



Effects of prenatal diesel exhaust inhalation on pulmonary inflammation and development of specific immune responses

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ARTICLE INFO

Article history:

Received 15 January 2010

Received in revised form 18 March 2010

Accepted 24 March 2010

Available online 1 April 2010

Keywords:

Diesel

In utero exposure

Toxicity

Immunity

Allergy

ABSTRACT

There is increasing evidence that exposure to air pollutants during pregnancy can result in a number of deleterious effects including low birth weight and the incidence of allergic asthma. To investigate the *in utero* effects of DE exposure, timed pregnant BALB/c mice were exposed to 0, 0.8 or 3.1 mg/m³ of DE during gestation days (GD) 9 to GD 18. The number of successful pregnancies was 15/20 in the air controls and 10/20 in each of the diesel exposures. Immune function in the 6-week-old offspring as determined by development of delayed type hypersensitivity (DTH) reactions to bovine serum albumin (BSA), antibody titers to injected sheep red blood cells (SRBC), splenic T cells expressing CD45⁺CD3⁺CD8⁺ and CD3⁺CD25⁺, and mRNA expression of TNF- α , TLR2, SP-A, TGF- β and Foxp3 in the lung were not affected by prenatal DE exposure. On the other hand, lung TLR4 mRNA expression, the number of neutrophils in the bronchoalveolar lavage fluid (BALF) and splenic T cells expressing CD45⁺CD3⁺CD4⁺ and CD4⁺CD25⁺ were differentially affected depending on the DE concentration and gender. When additional groups of mice were sensitized and challenged via the respiratory tract with ovalbumin to induce allergic airway inflammation, female mice had higher protein levels in the BALF compared to males and this was reduced by prenatal exposure to either concentration of DE. No other changes in allergen-induced immunity, lung function or severity of inflammation were noted. Collectively, the results show that *in utero* exposure to DE altered some baseline inflammatory indices in the lung in a gender-specific manner, but had no effect on development of specific immune responses to experimental antigens, or the severity of allergic lung inflammation.

Published by Elsevier Ireland Ltd.

1. Introduction

Epidemiology studies have reported that exposures to various air pollutants including street traffic exhaust (Ising et al., 2003) and cigarette smoke (Li et al., 2005) during pre- and post-natal stages of life are risk factors for the development of allergic airway inflammation. In addition experimental studies have shown that the offspring of pregnant mice that were exposed to residual oil fly ash by oropharyngeal aspiration exhibited increased numbers of lung eosinophils, and more pronounced airway hyperreactivity and Th2-biased immune responses following immunization and challenge with antigen (Hamada et al., 2007). More recently the

same group reported that *in utero* exposure to diesel particles as well as less reactive materials including titanium dioxide and carbon black by oropharyngeal aspiration also resulted in stronger allergic immune responses and airway inflammation in the offspring of exposed dams (Fedulov et al., 2008). Prenatal exposure to environmental contaminants therefore may significantly affect the potential to develop allergic asthma (Kurukulaaratchy et al., 2004).

Diesel exhaust (DE) consists of various hazardous compounds in both particle and gaseous forms, and induces toxicity and inflammation through oxidative stress pathways (Bonvallot et al., 2002; Bleck et al., 2006). Studies indicate that direct exposure to DE can alter the expression of many pro-inflammatory cytokines, chemokines, receptors and proteins (Imrich et al., 2007; Ito et al., 2006a,b) depending upon the particle size, chemistry, concentration, and route of administration (Rao et al., 2005;

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Hougaard et al., 2008). Alteration in the expression and production of many of these biomolecules can interfere with the normal development of innate and adaptive immune processes. During non-inflammatory immune stimulation, antigen presenting cells promote naïve T cells to differentiate into T regulatory cells in the influence of transforming growth factor (TGF)- β and/or interleukin (IL)-10 resulting in development of immune tolerance (Sun et al., 2010). Under pro-inflammatory circumstances, dendritic cells and macrophages recognize pathogens through pattern recognition receptors including toll-like receptors (TLR), and process and present antigens in association with the major histocompatibility complex (MHC) class I or II as a peptide (Areschoug and Gordon, 2008). This signaling generates co-stimulatory molecules, chemokines and cytokines required for T cell activation and proliferation, and subsequent secretion of immunoglobulins (Ig) from B cells in adaptive immunity (Iwasaki and Medzhitov, 2010). DE or its individual components, including the particle elemental carbon core and condensed organic carbon species, can promote B and T cell proliferation in the lymph nodes and cytokine secretion and allergen-specific IgE and IgG1 responses in both experimental animals and humans (Rao et al., 2005; Riedl and Diaz-Sanchez, 2005). This adjuvant effect of DE has been associated with decreased Th1 cell function by interfering with dendritic cell maturation and activating a signaling pathway for suppression of IL-12 (Chan et al., 2006). Pregnant mice exposed to diesel exhaust have increased placental expression of immunomodulatory cytokines including IL-2, IL-5, IL-12 and granulocyte monocyte colony stimulating factor, GM-CSF (Fujimoto et al., 2005).

We have previously reported that exposure to a number of air pollutants including DE promotes the development of allergic airway inflammation through the expansion of Th2 cytokine responses (Stevens et al., 2009). In light of the increased incidence of allergic asthma in near-road environments (Ising et al., 2003; Venn et al., 2005), we postulated that inhalation exposure of DE during pregnancy would alter immune signaling resulting in increased allergy in the offspring of exposed dams. We first evaluated the effect of DE on pregnancy outcome, litter size and body weight of the offspring, and then examined whether prenatal exposure to DE affected baseline immune function, a variety of different signalling molecules associated with innate and adaptive immune function as well as pulmonary homeostasis in untreated male and female offspring. Secondly, humoral and cell mediated immune responses to novel antigens were evaluated in additional groups of offspring to assess effects of prenatal diesel exposure on systemic immune function. Finally, additional groups of offspring were sensitized and challenged with ovalbumin antigen via the airways to determine whether the prenatal exposure affected development of allergic lung disease.

2. Materials and methods

2.1. Animals

Timed pregnant (plug positive) BALB/c mice aged 10–12 weeks were purchased from Charles River Laboratories, Raleigh, NC, and delivered to the animal facility on gestational day (GD) 7. Mice were housed individually in polycarbonate cages with hardwood chip bedding (Warrensburg, NY) maintained at $22.3 \pm 1^\circ\text{C}$ and $50 \pm 10\%$ humidity in filtered animal housing racks and provided access to mouse chow (SPOO Prolab RMH3000, PMI Nutrition International, Richmond, IN) and water *ad libitum*. All procedures employed in this study were approved in advance by the Institutional Animal Care and Use Committee of the National Health and Environmental Effects Research Laboratory, US EPA.

2.2. Diesel exhaust generation, exposure, and monitoring

Diesel exhaust for exposure experiments was generated using a 4.8 kW (6.4 hp) direct injection single-cylinder 0.320 L displacement Yanmar L70 V diesel generator operated at a constant 3600 rpm. Resistance heating elements provided a constant 3 kW load. Low sulfur diesel fuel (32 ppm), purchased from a local distributor was available from a large storage tank. Engine lubrication oil (Shell Rotella, 15W-40)

was changed before each set of exposure tests. From the engine, approximately 85 L/min of the exhaust was directed to a cone diluter and mixed with approximately 595 L/min (7:1 dilution) of high efficiency particulate air (HEPA) filtered room air. The diluted exhaust then traveled approximately 12 m through 7.6 cm diameter stainless steel tubing to two Hazelton 1000 (984 L) exposure chambers housed in an isolated animal exposure room. Target DE concentrations in the two chambers were 0.5 mg of particulate matter (PM)/m³ (low) and 2 mg of PM/m³ (high). The chamber concentrations were controlled by periodic adjustments of dilution air based on continuous mass concentrations determined by tapered element oscillating microbalance (TEOM, Rupprecht and Patashnick Co., series 1400, Albany, NY) instruments. These instruments include a heated (50°C) chamber that could theoretically vaporize low temperature volatiles. The actual retrospective concentrations as determined by gravimetric mass (at 22°C) were approximately 0.8 and 3.1 mg/m³. Control animals were placed in a third chamber supplied with the same HEPA filtered room air. DE concentrations in the low (0.8 mg/m³) 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 (283 L/min) which resulted in approximately 17 air exchanges per hour. Integrated 4 h filter samples (14.1 L/min) were collected daily from each chamber and analyzed gravimetrically to determine particle concentrations. In addition, triplicate 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) by the NIOSH 5040 method (<http://www.cdc.gov/niosh/nmam/pdfs/5040f3.pdf>) to determine organic carbon and elemental carbon (OC–EC) partitioning of the collected DE. Continuous emission monitors (CEMs) were used to measure chamber concentrations of PM by TEOM, oxygen (O₂, Beckman Corp., model 755, La Habra, CA), carbon monoxide (CO, Thermo Electron Corp., model 48, Franklin, MA), nitrogen oxides (NO and NO₂, Teledyne Technology Co., model 200A4, San Diego, CA), and sulfur dioxide (SO₂, Thermo Electron Corp., model 43C, Franklin, 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 of air was adjusted periodically to maintain target PM concentrations as measured by the TEOM. Particle size distributions were characterized during each exposure 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, St. Paul, MN). Chamber temperatures, relative humidity, and noise were also monitored, and maintained within acceptable ranges.

2.3. Experimental design and evaluation of reproductive status

Timed pregnant mice were randomly divided into three groups of 20. Pregnant animals were placed in individual stainless steel wire exposure cages and were exposed to HEPA filtered room air or diesel emissions diluted to yield 0.8 or 3.1 mg/m³ of diesel emission particulate for 4 h per day for 10 consecutive days, from GD 9 to GD 18. The ramp up to target exposure concentration occurred within 5 min of onset of exposure. After 4 h, the engine was switched off and the chambers were evacuated of residual exhaust within 10 min post-exposure. Mice gave birth between GD 20 and 21, and the litters were kept separate until weaning at postnatal day (PND) 21. Offspring were then separated into subgroups of non-littermates for evaluation of specific endpoints. To detect the pregnancy loss, the uteri of females that had not given birth were visually inspected for size, thickness, blood supply, resorption sites and presence of dead embryos. The pregnancy loss was evaluated in total dams per group. The percentage of pregnancy was calculated by dividing the number of the dams giving birth by the total number of the dams per group. Litter size was expressed by the average numbers of live offspring per treatment group.

2.4. Toxicity assessment and allergen sensitization

A group of male and female mice ($n=6$ of each gender, total 12 per exposure concentration) was euthanized on PND 42 for assessment of immune and inflammatory parameters including protein and inflammatory cells ($n=6$ of each gender, total 12 mice per exposure concentration), and mRNA expression and T cell populations ($n=3$ of each gender, total 6 mice per exposure concentration). Additionally two separate groups of male and female mice ($n=6$ of each gender, total 12 per exposure concentration) were identified for immunotoxicity testing. Further groups of male and female mice ($n=6$ of each gender, total 12 per exposure concentration) were lightly anesthetized with isoflurane and intranasally (i.n.) instilled with 25 μL per nostril containing 50 μg of chicken ovalbumin (OVA, Fraction V, Sigma) in normal saline (Hospira Inc., Lake forest, IL) or normal saline alone on PND 42 and 43 (Fig. 1). This procedure was then repeated at challenge with 5 μg of OVA in 20 μL normal saline or normal saline only per nostril on PND 54, 55 and 56. One day after the final OVA challenge, immune responses, lung function and inflammation were assessed.

2.5. Delayed type hypersensitivity (DTH) assessment

The DTH response was assessed as described by Rooney et al. (2003). Briefly, purified bovine serum albumin (BSA) (Fraction V, Sigma) at a concentration of 2 mg/ml in sterile normal saline was emulsified in Freund's complete adjuvant (CFA;

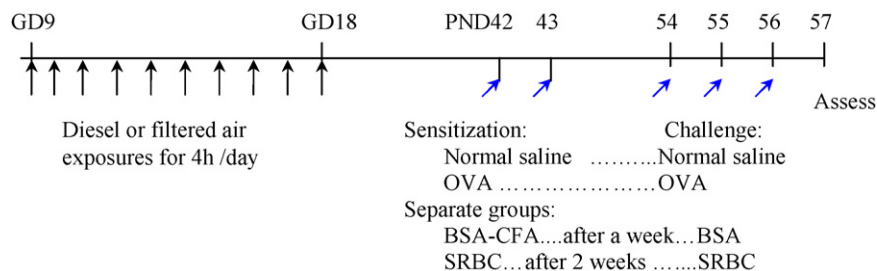


Fig. 1. Experimental regimen. Timed pregnant mice were exposed to either DE or air, and the offspring were assessed for inflammation and immune status at PND 42. Different separate groups of offspring were sensitized and challenged to OVA and control group was treated with normal saline. Separate groups of offspring were immunized and challenged with BSA or SRBC.

Difco, Detroit, MI) at a 1:1 ratio. Offspring were anesthetized with isoflurane and sensitized with 0.05 ml BSA-CFA by subcutaneous injection into the caudal tail fold after PND 42. Seven days later, animals were re-anesthetized and challenged by injecting 0.05 ml of heat-aggregated BSA into the right rear footpad. The left rear footpad was injected with the same volume of normal saline as a control. 24 h after challenge, footpad thickness was determined by triplicate measurements in anesthetized animals with an electronic caliper. The degree of edema, calculated by difference between saline- and BSA-injected footpad thickness, was then used to assess the degree of DTH response.

2.6. Sheep red blood cell (SRBC)-specific antibody responses

Offspring were intravenously injected with 2×10^7 SRBC in 0.2 ml of sterile normal saline on PND 42 and bled 5 days later from the facial vein for assessment of SRBC-specific IgM in the serum. An intravenous booster immunization with the same number of SRBC was given to all mice 2 weeks after primary immunization and blood was collected 5 days after the secondary immunization for determination of IgG. The relative serum titers of IgM and IgG antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as described by Rooney et al. (2003). Absorbance was read on a SpectraMax 250-plate reader at 410 nm and processed using SOFTmax PRO software as $\log_2$ antibody titers from the log-log curve of optical density versus dilution, as described by Temple et al. (1995). All ELISA procedures were optimized for differences in SRBC membrane preparations and standards were assessed as described by DeWitt et al. (2005).

2.7. Lung lavage cytology

Following euthanasia and cardiac puncture for blood collection, the chest wall was opened and the left main stem bronchus was isolated, clamped and the trachea cannulated. The lung left lobe was excised and frozen at -70°C for PCR. Bronchoalveolar lavage fluid (BALF) from the right lung lobes was collected using 3×0.6 ml volumes of warmed Hanks balanced salt solution (HBSS) and centrifuged at 1200 rpm for 5 min. Total cells were counted using a coulter counter (Coulter Corp., Miami, FL). Differential cell counts were performed on 200 stained (Wright-Giemsa, Fisher Scientific) cells prepared on glass slides using a Cytospin centrifuge (Shandon Inc., Pittsburgh, PA) and the total numbers of each cell type was calculated in the total cells obtained by the coulter counter.

2.8. Biochemical assays

BALF was analyzed for lactate dehydrogenase (LDH) and protein to detect lung injury and edema, respectively, using a commercially prepared kit and controls and Pierce Coomassie Plus Protein Assay Reagent (Pierce) from Sigma Chemical Co. (St. Louis, MO). The assays were modified for use on the KONELAB 30 clinical chemistry Spectrophotometer analyzer (Thermo Clinical Labsystems, Espoo, Finland).

2.9. Quantitation of mRNA by RT-PCR

RNA was isolated and purified with RNeasy mini-columns according to the manufacturer's protocol (QIAGEN, Valencia, CA) and the quality of RNA was assessed with a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Samples were stored at -80°C until RT-PCR analysis. One-step RT-PCR was carried out on 100 ng of total RNA using SuperScript III Platinum reagent (Invitrogen, Carlsbad, CA) as per manufacturers' recommendations. Real-time PCR was conducted on a Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). RT-PCR conditions consisted of reverse transcription for 20 min at 53°C and inactivation of reverse transcriptase activity for 2 min at 95°C . There were 40 amplification cycles at 95°C for 15 s followed by 60°C for 45 s. Genbank mRNA primers included NM.023134.4 for surfactant protein (SP)-A, NM.013693.2 for tumor necrosis factor (TNF)- α , NM.011905.3 for TLR2, NM.021297.2 for TLR4, NM.011577.1 for TGF- β 1 and NM.054039.1 for forkhead box P3 (Foxp3) (Applied Biosystems, CA). Data were analyzed using ABI Sequence Detection System software version 2.2. The comparative cycle threshold (Ct) method was used to calculate the relative concentrations.

Target gene Ct was normalized to reference 18S and expression of each exposure group was quantified relative to increase over air exposure group.

2.10. Flow cytometry analysis

Single cell suspensions of the spleen were prepared by pressing the tissue through a $70\text{ }\mu\text{m}$ cell strainer in 1% FCS-HBSS buffer. Red blood cells (RBC) were lysed with 2 ml of RBC lysis solution and cells were incubated on ice with labeled antibodies to CD45 PE-cy7, CD25 PE, CD4 APC, CD3 FITC and CD8 PE and appropriate isotype controls (BD Pharmingen, San-Diego, CA) in PBS containing 1% BSA/0.1% sodium azide for 30 min. A total of 10,000 events were collected using a Becton Dickinson model LSR-II flow cytometer and the data were analyzed using CellQuest (Becton Dickinson) and FlowJo software (Tree Star Inc., CA). CD45 $^+$ cells were further gated for CD3 $^+$ CD4 $^+$ or CD3 $^+$ CD8 $^+$ subpopulations. The populations of CD3 $^+$ CD25 $^+$ and CD4 $^+$ CD25 $^+$ in the spleen were also quantified.

2.11. Immediate airway response (IAR) to allergen

Antigen-specific IAR was measured immediately after each challenge to OVA in unrestrained mice using a whole body plethysmograph (Buxco Electronics, Sharon, CT) to derive the calculated parameter enhanced pause (Penh). Penh is a parameter that correlates with airway obstruction. Baseline measurements for each previously OVA- or saline-administered animal were recorded for 10 min. The animals were then lightly anesthetized and challenged to OVA or saline intranasally. Mice were placed back into the chambers within 5 min of dosing and Penh readings were then monitored for 1 h. Average maximum responses over a 1 h for the separate 3 challenges were reported as percentage increase over baseline.

2.12. Airway hyperreactivity (AHR) to methacholine (MCh) aerosol

One day after the last OVA challenge, Penh to aerosolized MCh (Sigma-Aldrich Chemical Co., St. Louis, MO) in unanesthetized and unrestrained mice was measured in a 12-chamber plethysmography system. After measuring baseline parameters for 10 min, an aerosol of saline or MCh in increasing concentrations was nebulized through an inlet of the chamber. Airflow was 6 L/min. For each animal, the data were expressed as percentage of Penh over baseline values.

2.13. Pathological changes

The whole lungs were inflated after lavaging and fixed in 10% buffered formalin (Fisher Scientific, Fair Lawn, NJ). The slides were stained with H&E for eosinophils, and periodic acid-Schiff (PAS) for mucus-containing epithelial cells. The slides of the whole lungs were screened and examined under the light microscope (100 $\times$ of magnification), and then the number of cells were enumerated by averaging the score of the fields (5) under high magnification (400 $\times$) contained the bronchioles and alveolar regions.

2.14. Antigen-specific antibodies

Microtiter wells were coated with 10 $\mu\text{g}/\text{ml}$ OVA for IgG1 or 1 mg/ml for IgE detection (Pharmingen, CA) in carbonate buffer, pH 9.6. Nonspecific binding was blocked with 1% PBS buffer containing 0.05% Tween 20 and 2% bovine serum albumin. Sera were diluted 1:100,000 for IgG1 and undiluted for IgE antibody. Mouse standard IgE (Serotec, Kidlington, Oxford) or IgG1 (Sigma Chemical Co., St. Louis) antibodies were used. The antigen-antibody complex was detected with biotinylated antibodies (PharMingen, San-Diego, CA) to IgE or IgG1. The binding was visualized using Streptavidin-HRP (Zymed, San-Francisco, CA) and TMB substrate (Dako Co., Carpinteria, CA). The plates were read on a Thermomax $^{\text{®}}$ Plate Reader (Molecular Devices Co., Menlo Park, CA) after 20 min at a wavelength of 650 nm and analyzed using Softmax Pro $^{\text{®}}$ version 5.2 (Molecular Devices Co., Sunnyvale, CA).

Table 1Summary of concentrations and characteristics of the diesel exhaust particles and gases within the animal exposure chambers.^a

Constituent	Units	Low exposure	High exposure
Particle mass concentration (TEOM)	μg/m ³	535 ± 28	2065 ± 118
Particle mass concentration (filter) ^b	μg/m ³	782 ± 36	3091 ± 151
Particle number concentration ^c	/cm ³	5.7 × 10 ⁷ ± 4.0 × 10 ⁷	1.8 × 10 ⁸ ± 1.7 × 10 ⁸
Oxygen (O ₂)	%	20.7 ± 0.2	20.4 ± 0.1
Carbon monoxide (CO)	ppm	5.7 ± 0.9	17.7 ± 1.7
Nitrogen oxide (NO)	ppm	6.3 ± 0.7	21.2 ± 1.4
Nitrogen dioxide (NO ₂)	ppm	0.43 ± 0.11	1.22 ± 0.22
Sulfur dioxide (SO ₂)	ppm	<1	<1
Number median D _p ^d	nm	66 ± 2	74 ± 2
Volume median D _p	nm	162 ± 2	199 ± 2
OC/EC ^e	wt ratio	0.6 ± 0.06	0.6 ± 0.07

^a Tapered element oscillating microbalance (TEOM), O₂, CO, NO, NO₂, and SO₂ data represent mean values from continuous measurements taken over the exposure periods ± standard error of mean (SEM).

^b Filter data represent mean values from one measurement per day taken over the exposure periods ± SEM.

^c Particle number concentration data represent mean values from seven (high exposure) and five (low exposure) measurements.

^d D_p indicates particle number and volume median diameters for exposure day particle size distributions ± geometric standard deviation. Note that volume information is calculated from number based mobility diameters and assume spherical particles.

^e OC/EC (organic carbon to elemental carbon ratio) data represent mean values from one measurement per day taken over the exposures ± SEM.

2.15. Cytokine quantitation

A total of 2 × 10⁷ cells/ml from the peribronchial lymph nodes (PBLN) were pooled from 3 mice and stimulated in quadruplicate with 1 mg/ml OVA, and cultured in 200 μl complete culture medium RPMI (Invitrogen, Grand Ireland, NY) per well of 96-well plates at 37 °C for 72 h. The concentration of IL-4, IL-13, and IFN-γ in the cell-free supernatants was measured with ELISA using cytoset kits (Biosource, Carlsbad, CA). Briefly, 96-well plates were coated with appropriate antibodies and then blocked with blocking buffer. Standard antibodies and samples were added and washed. Plates were incubated with biotinylated antibodies and visualized with HPR-streptavidin and TMB substrate. The result was read at 650 nm. The sensitivity of detection was 7.8 pg/ml for IL-13, and 15.6 pg/ml for IL-4 and IFN-γ.

2.16. Statistics

The percentages of pregnancy and pregnancy loss were analyzed for differences among the exposure groups, using a Fisher's exact test. Single measurement responses were analyzed using a two-way analysis of variance (ANOVA) model. The two factors were gender and exposure. Pair wise comparisons were performed as subtests of the overall ANOVA. If the ANOVA requirements of normality and homoskedasticity were not satisfied, the data were transformed. Subsequent to the transformation, the data were checked for requirement compliance and if acceptable, the analysis proceeded. If, however, the transformed data also failed to meet the requirements, a distribution free method of analysis was employed. Data from repeated measurements, of the same statistical unit, were analyzed in two steps. First, a multivariate repeated measures analysis was performed to establish the existence of differences among the various treatment and gender combinations. Secondly, and subsequent to a significant finding, pair wise comparisons were made as subtests of the overall multivariate analysis of variance for repeated measures. The level of significance was set at 0.05. No adjustments were made for multiple testing, as this controls the Type II error rate. We recognize that this will tend to increase the Type I error rate. The statistics were generated using SAS statistical software (SAS V9.2, SAS Institute, Cary, NC).

3. Results

3.1. The characteristics of DE exposure in the chambers

Table 1 presents a summary of the average exposure data for the low (0.5 mg/m³) and high (2.0 mg/m³) DE concentrations. These target chamber PM concentrations, determined and adjusted based

on continuous TEOM measurements, were achieved with relatively low variability either within a particular 4 h exposure or between different days. Chamber particle concentrations determined gravimetrically from integrated filter samples (one 4 h sample per exposure day) however, showed that the TEOMs underestimated the mass by approximately 50%, and that the actual mean concentrations were 0.782 and 3.091 mg/m³ for the low and high chambers respectively. These differences were quite possibly due to the fact that the TEOM stage has a heating component that may promote sublimation of organic components that remained on the filter based system. CO, NO, and NO₂ concentrations were all maintained below thresholds of concern. SO₂ concentrations were below detection levels for all exposures. Particle number concentrations were relatively high, and correspond to particle size distributions (PSDs) with well-established accumulation modes and little evidence of notable nuclei or coarse modes. Median number and volume (assuming spherical particles) diameters of approximately 75 and 200 nm, respectively, were measured in the chambers. It should be noted, that the SMPS system (with long column) limited measurements to particles greater than approximately 15 nm. OC/EC wt ratios of 0.6 indicate that 38% of the DE was comprised of organic carbon.

3.2. The effects of DE inhalation on reproductive health

The percentage of pregnancy was 75% in the air exposure group and 50% for both diesel concentrations which were not statistically significant (Table 2). Retained dead fetuses in early and late stage of development, and evidence of resorption sites were found in non-productive dams exposed to the high concentration of DE. This high exposure group also had the lowest litter size although this was not statistically significant. The whole litters of each dam were weighed together at PND 1, 3, 7, 14 and 21, and individually at PND 42. No differences in the bodyweight of the offspring were observed in either of the DE exposure groups compared with air controls (data not shown).

Table 2

Reproductive outcomes.

	Percent pregnancy (dams given birth/total dams per group)	Percent detectable pregnancy loss (dams lost litters/total dams)	Average litter size per dam (total live pups/total pregnant dams)	Number and gender of offspring	
				Male	Female
Air	75% (15/20)	0% (0/20)	6.4 ± 0.3 (96/15)	36	60
0.8 mg/m ³	50% (10/20)	0% (0/20)	6.7 ± 0.4 (67/10)	24	43
3.1 mg/m ³	50% (10/20)	20% (4/20)	5.6 ± 0.6 (56/10)	27	29

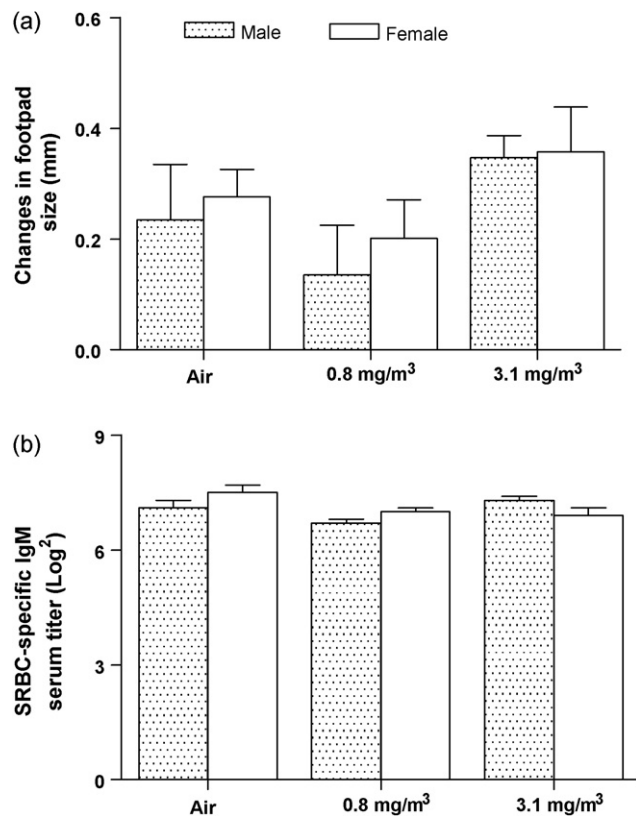


Fig. 2. Developmental immunotoxicity in offspring exposed to DE *in utero*. The offspring were immunized with BSA or SRBC at PND 42 as described in Section 2. (a) DTH was determined by difference of thickness between normal saline and BSA-injected footpad. (b) The levels of SRBC-specific antibodies in the sera were determined by ELISA. Data in each assay are the mean $\pm$ SEM of 6 offspring in each gender.

3.3. Prenatal DE inhalation on immunotoxicity of the offspring

In order to determine the effects of prenatal DE inhalation exposure on developmental immunotoxicity, we assessed DTH to BSA, and development of SRBC-specific antibodies after a primary and secondary immunizations. There were no statistical differences in the degree of footpad swelling between male or female mice, nor between any of the exposure groups (Fig. 2a). All mice produced similar titers of IgM (Fig. 2b) and IgG antibodies following a booster injection of SRBC (data not shown), regardless of gender or prenatal treatment.

3.4. Pulmonary inflammatory responses in non-allergic offspring

Offspring from dams exposed to either DE or air were assessed for pulmonary inflammation at PND 42. No difference was observed

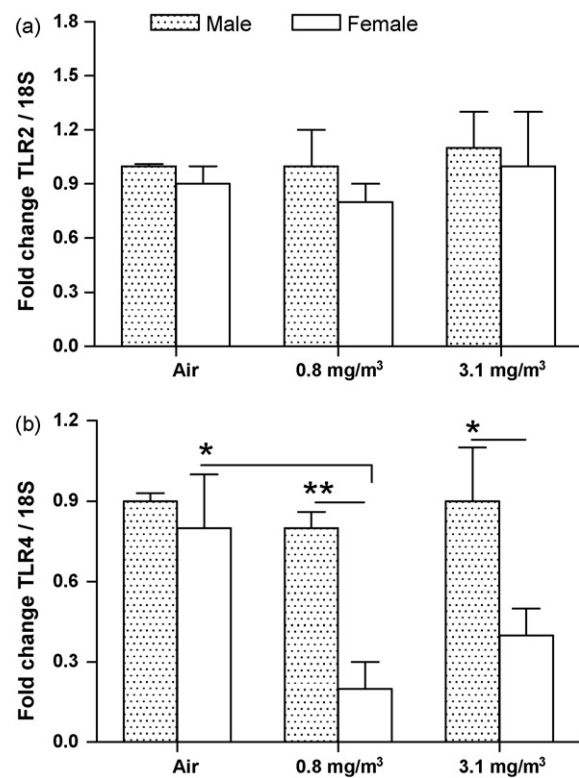


Fig. 3. A representative data of mRNA expression of immune related molecules in lung tissue. mRNA expression of (a) TLR2 and (b) TLR4 in the lung were quantified by real-time RT-PCR. The value of each gene was normalized to control gene 18S, and mRNA expression of treatment groups were expressed as fold induction. Data are reported as mean $\pm$ SEM of 3 offspring in each gender. * $P < 0.05$ and ** $P < 0.01$, significantly different from other groups.

between groups in the number of BALF macrophages and lymphocytes. The number of neutrophils was significantly reduced in female offspring of the 0.8 mg/m³ DE compared to air controls, but increased in the males prenatally exposed to the 3.1 mg/m³ DE concentration compared to air controls. No differences were seen in LDH levels across exposure groups (data not shown), however, BALF protein levels in female offspring to the 3.1 mg/m³ DE concentration were increased compared to air controls (Table 3).

No statistical difference was observed in expression of TNF- α , TLR2, SP-A, TGF- β and Foxp3 mRNA level by real-time PCR. A significant reduction was however observed in TLR4 mRNA level of female offspring in the 0.8 mg/m³ DE exposure group compared to the air exposure group. A reduction at the higher exposure level was also evident, but was not statistically different. At both DE concentrations, the reduction of TLR4 mRNA in females was statistically significant compared to male offspring (Fig. 3b).

Table 3
Inflammatory cell population and total protein in BALF of non-allergic offspring.

Treatment group	Gender	Number of cells ($\times 10^4$) per ml				Total protein (μ g/ml)
		Macrophages	Lymphocytes	Neutrophils	Eosinophils	
Air	♂	9.2 $\pm$ 1.7	0.1 $\pm$ 0.04	0.3 $\pm$ 0.09		65 $\pm$ 12
	♀	6.7 $\pm$ 0.9	0.04 $\pm$ 0.02	0.36 $\pm$ 0.04		63 $\pm$ 5
0.8 mg/m³	♂	6.5 $\pm$ 1.2	0.5 $\pm$ 0.3	0.5 $\pm$ 0.07		97 $\pm$ 15
	♀	6.3 $\pm$ 0.1	0.8 $\pm$ 0.5	0.18 $\pm$ 0.06*		75 $\pm$ 8
3.1 mg/m³	♂	6.5 $\pm$ 1.3	0.7 $\pm$ 0.3	1 $\pm$ 0.2*	0.2 $\pm$ 0.2	82 $\pm$ 6
	♀	7.2 $\pm$ 1	0.2 $\pm$ 0.08	0.4 $\pm$ 0.04		98 $\pm$ 10*

Note: Data in the table are presented as mean $\pm$ SEM of 6 offspring in each gender.

* $P < 0.05$, significantly different from the same gender in air-exposed group.

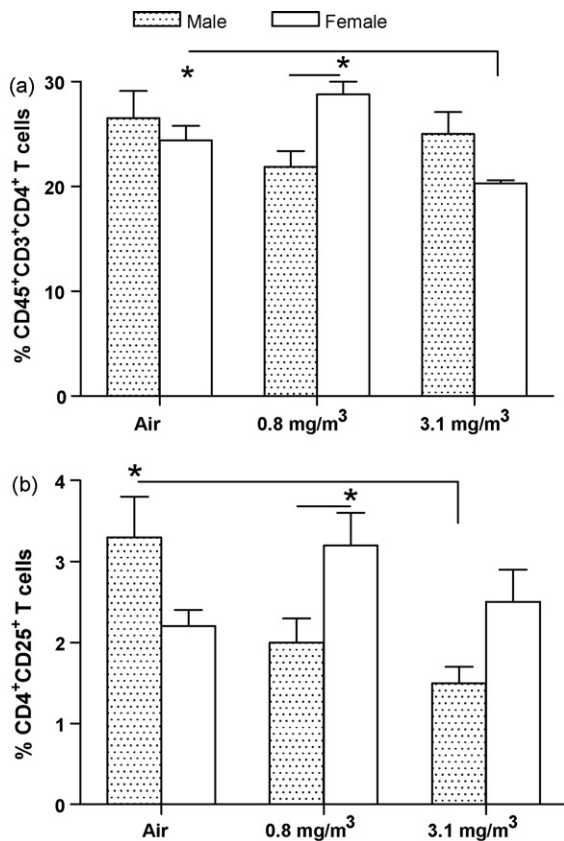


Fig. 4. A representative data of T cell populations in the spleen in non-allergic offspring. A total of 10,000 events of the spleen cells were collected for analysis and gated for CD45⁺ marker, which was further gated for (a) CD3⁺CD4⁺. The total events were also gated for the population of (b) CD4⁺CD25⁺. Data are reported as mean $\pm$ SEM of 3 offspring for each gender.

3.5. T cell phenotypes in non-allergic offspring to prenatal DE inhalation

Flow cytometric analysis was performed on spleen cells from both genders and each exposure group. There were no significant differences in the percentage of CD45⁺CD3⁺CD8⁺ and CD3⁺CD25⁺ markers across gender or exposure groups (data not shown). The number of CD45⁺CD3⁺CD4⁺ T cells in female (Fig. 4a) and CD4⁺CD25⁺ T cell populations in male offspring of the 3.1 mg/m³ DE exposure group were significantly decreased compared to controls (Fig. 4b). At the 0.8 mg/m³ DE concentration, increases in CD4⁺ and

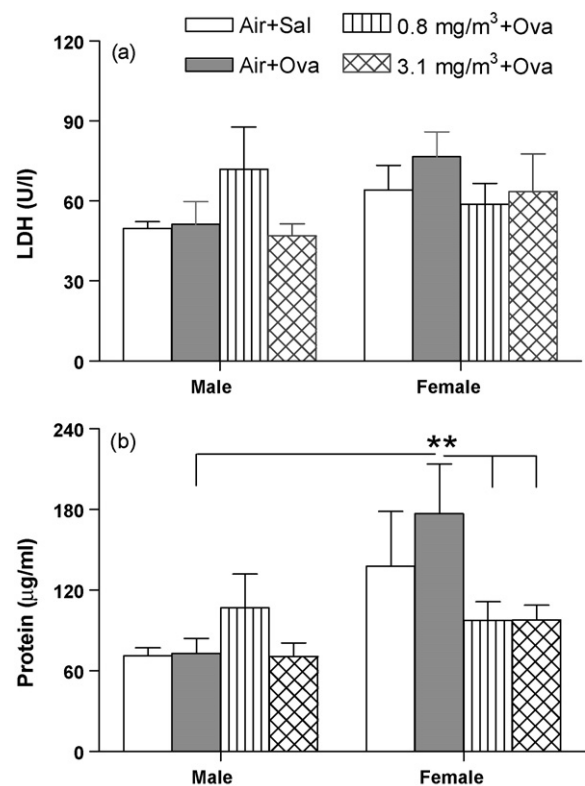


Fig. 5. Lung injury and edema in allergic offspring. One day after the last OVA challenge, BALF was assessed for (a) LDH and (b) total protein. Data are reported as mean $\pm$ SEM of 6 offspring in each gender. ** $P < 0.01$, significantly different from other groups.

CD4⁺CD25⁺ T cells in female spleen were statistically significant compared to male offspring.

3.6. Pulmonary inflammation in allergic offspring

No differences were observed in LDH levels in BALF of any of the treatment groups (Fig. 5a). Total protein levels in BALF were significantly higher in the female air group compared to the male air group. The level of protein was significantly decreased in female offspring exposed to both DE concentrations *in utero* (Fig. 5b). There were no exposure-related effects in either gender on BALF inflammatory cells of any type (Fig. 6a). Eosinophil numbers in lung tissue were only significantly increased in the air-exposed allergic female

Table 4
Lung function in allergic offspring.

Penh in IAR (% increase over baseline)		Penh (% increase over baseline) to methacholine in AHR			
		Normal saline	6.25 mg/ml	12.5 mg/ml	25 mg/ml
Air + Sal					
♂	56 ± 11	10 ± 4	68 ± 12	199 ± 28	380 ± 48
♀	123 ± 25	23 ± 7	63 ± 10	204 ± 39	380 ± 84
Air + OVA					
♂	84 ± 23	21 ± 7	72 ± 7	255 ± 25	377 ± 92
♀	143 ± 15	16 ± 6	59 ± 12	179 ± 36	343 ± 53
0.8 mg/m ³ + OVA					
♂	68 ± 13	10 ± 4	66 ± 5	275 ± 28	354 ± 35
♀	89 ± 6	13 ± 6	54 ± 9	216 ± 40	343 ± 53
3.1 mg/m ³ + OVA					
♂	97 ± 16	14 ± 6	73 ± 11	229 ± 25	423 ± 58
♀	131 ± 25	23 ± 5	82 ± 13	205 ± 18	388 ± 51

Note: Data in the table are presented as mean $\pm$ SEM of 6 offspring in each gender.

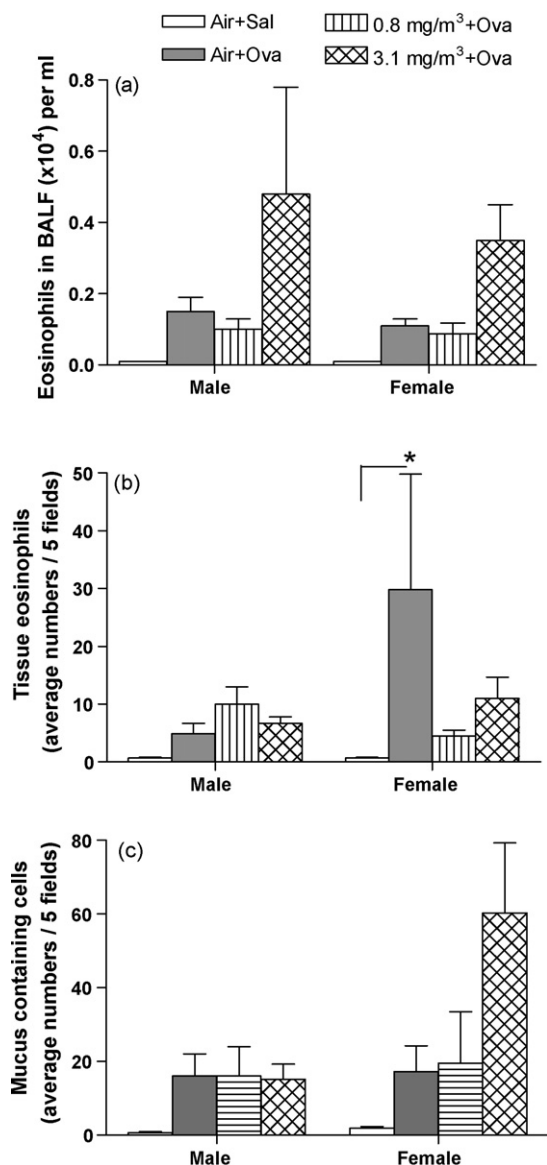


Fig. 6. BALF and tissue eosinophils in allergic offspring. One day after the last OVA challenge, (a) BALF eosinophils were counted on the cytospin slides and lung sections were stained with H&E for (b) eosinophils or PAS for (c) mucus-containing epithelial cells. Data are reported as mean $\pm$ SEM of 3 offspring in each gender. ** $P < 0.01$, significantly different from other groups.

offspring over saline control (Fig. 6b). There were no differences between this value and those of the animals that had been exposed to diesel *in utero*. The immunization and challenge regimen resulted in a large increase in mucus-containing cells in the airways that was not seen in saline group (Fig. 6c). No significant differences were observed between DE exposure and air allergic groups in either gender.

3.7. Pulmonary function testing in allergic offspring

No significant differences were observed in IAR data across gender and treatment groups. All animals responded to methacholine aerosol challenge with a steady increase in Penh values to approximately 400% of the original baseline values. There were no significant differences between male and female offspring for any of the exposure concentrations of DE and air (Table 4).

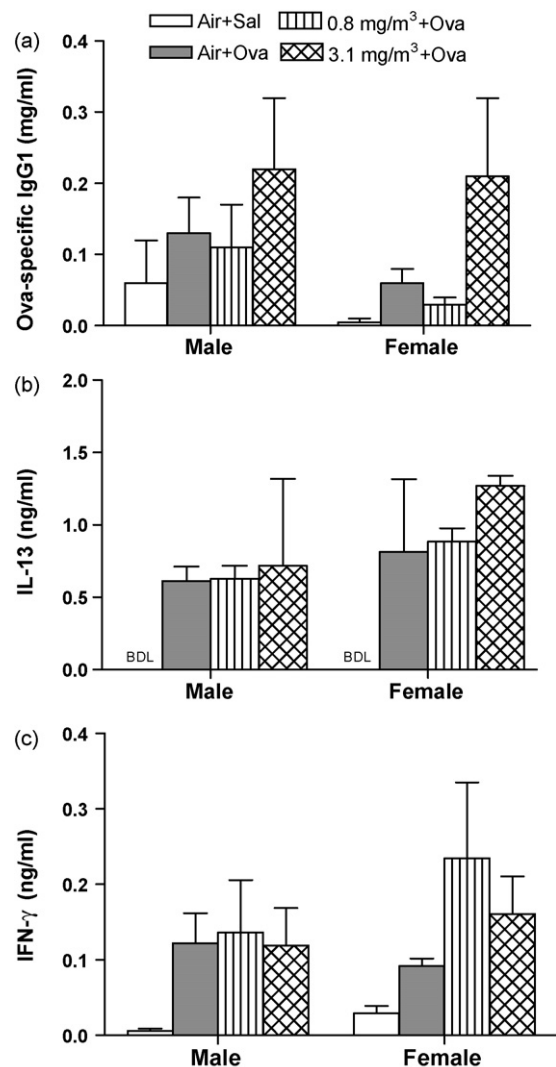


Fig. 7. Antigen-specific antibody and cytokine responses in allergic offspring. One day after the last OVA challenge, the sera were collected for OVA-specific antibodies and the PBLN were pooled from 3 mice per group and cultured and re-stimulated with OVA. (a) OVA-specific IgG1 was measured in the serum and data are the mean $\pm$ SEM of total $n = 6$. (b) IL-13 and (c) IFN- γ were detected in culture supernatants by ELISA. Data are the mean $\pm$ SEM of 2 sets of pooled PBLN per group.

3.8. OVA-specific antibody and cytokine responses in allergic offspring

The OVA sensitization and challenge regimen resulted in modest increases in OVA-specific IgE and IgG1 antibody levels, which were not altered by *in utero* exposure to diesel (Fig. 7a). The cytokines IL-4 and IL-13 were below detection limit in lymph node supernatant of the saline group and were elevated in the samples from sensitized animals regardless of diesel exposure. Levels of IFN- γ were also similarly lower in the non-sensitized animals compared to sensitized animals and again no differences were detected between exposure scenarios (Fig. 7b and c).

4. Discussion

In utero exposure to environmental agents during fetal development can dramatically influence the magnitude and type of immune responses after birth (Wang et al., 2008), and in later life (Hamada et al., 2007). Reported effects include immunosuppression (Burns-Naas et al., 2008; Dietert and Zelikoff, 2008) as

well as enhanced sensitization to allergens (Fedulov et al., 2008). Because there is emerging evidence to suggest that traffic-related pollutants increase the incidence of allergic asthma, this study was designed to investigate whether an analogous phenomenon occurred in murine offspring exposed prenatally to different levels of freshly generated and diluted diesel exhaust. Both concentrations of DE reduced the numerical pregnancy outcome of the dams from 75% to 50%, but did not affect the body weight of the surviving offspring. Despite the reduced birth rate with prenatal exposure to DE, subsequent standard immunotoxicity endpoints in the surviving offspring immunized with experimental antigens as well as markers of allergic lung disease following mucosal allergen sensitization and challenge were not affected. When non-immunized pups were assessed for baseline markers of inflammation and host defense however, significant gender-specific alterations in lung protein and inflammatory cells, and splenic T cell subsets were observed in the diesel-exposed groups.

The decreased pregnancy outcome following DE exposure is consistent with a previous study that reported 25% abnormal pregnancies at an exposure concentration of 3 mg/m³ (Tsukue et al., 2002). In contrast to other reports, however, postnatal body weights of the surviving offspring were not affected by *in utero* exposure and may be explained either by the fact that the dams were not exposed prior to breeding and gestation (Tsukue et al., 2002), or that the other studies were conducted at higher exposure concentrations (e.g. 19 mg/m³; Hougaard et al., 2008). Lower litter size was recorded in the highest DE concentration with retained fetuses therefore, we suspected that some embryos in the uterus might not have survived until full-term of birth. Although the DE experimental concentrations used in this and other studies were at, or above occupational exposure levels, equating the short duration of gestational exposure in mice to that in humans would, from a concentration times time (C × T) perspective, justify a 10 fold to 15 fold increase in exposure concentration to achieve the same overall dose.

There is substantial evidence that *in utero* exposure to a variety of environmental toxicants including diethylstilbestrol (Luster et al., 1979), lead (Bunn et al., 2001) and tetrachlorodibenzo-p-dioxin (Vos and Moore, 1974), as well as drugs including diazepam (Schlumpf et al., 1992) result in decreased immune function and resistance to infection (Schlumpf et al., 1994) in the offspring. Recent critical reviews of hazard identification of immunotoxicants (Luebke et al., 2006; Dietert, 2009) concluded that testing in adult models may be inadequate to detect the effects of developmental exposure to a variety of chemicals, given the greater sensitivity of the developing immune system to chemical perturbation. Ito et al. (2006a,b) reported that organic chemicals on the surface of DE particles alter fetal thymocyte development via engagement of the aryl hydrocarbon receptor (AhR). In addition, the potent AhR agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin has been reported to cause persistent (essentially lifetime) suppression of immune function in the offspring of dams exposed to TCDD during gestation (Gehrs and Smialowicz, 1999). Given the potential immunotoxicity of AhR ligands for the developing immune system, we hypothesized that the developing immune system may be particularly susceptible to enriched diesel exhaust. However, using a standard immunotoxicity testing protocol, we did not identify any alterations in immune function resulting from exposure to either concentration of diesel exhaust.

In addition to classical immunotoxicity testing, we also assessed the effect of *in utero* DE exposure on baseline parameters of inflammation and immune signaling in the offspring. The decrease in effector T cell populations in the spleen and increased total protein and neutrophils in the BALF of the 3.1 mg/m³ DE exposure group suggested some alteration in immune homeostasis and immune signaling by this treatment. Gender-specific decreases in TLR4

mRNA expression and numbers of airway neutrophils were also observed with statistical significance at the low DE concentration. This effect has been previously reported *in vitro*, whereby diesel-enriched particles decreased LPS-induced TLR4 expression by dendritic cells (Porter et al., 2007). TLR4 protein has also been shown to be reduced following iron-associated oxidative stress as well as after hypoxic tubular injury in the kidney and seems to be associated with cellular injury (Zager et al., 2007). It is interesting to note that previous studies from our laboratory reported decreased expression of surfactant protein-A with an accompanying increased susceptibility to influenza infection in the low but not the high exposure DE concentration (Ciencewicki et al., 2007). Seeing these observations it is quite possible that, the effects of diesel exposure do not follow a linear dose response curve, but rather, certain thresholds may exist at exposure levels that differentially affect distinct homeostatic processes.

Previous studies have demonstrated increased potential of allergic lung disease following exposure to diesel exhaust or other particles. For example, DE exposure to rats *in utero* as well as exposure during the suckling period increased IgE levels following systemic immunization with pollen once the offspring reached maturity (Watanabe and Ohsawa, 2002). A more recent study in mice also reported that a single intranasal instillation of 50 µg of diesel particles or “inert” titanium dioxide particles at GD 14 led to increased systemic allergic sensitization in the two week old offspring (Fedulov et al., 2008). Although we utilized an experimental model that was designed to produce a moderate degree of allergic lung disease, the standard markers of immune function and subsequent pathophysiological readouts were not appreciably affected by *in utero* exposure to fresh diesel emissions. Differences that might explain the discrepancies between these reports might include the type and concentration of diesel exposure as well as a different sensitization regimen.

Male and female murine immune cells present antigens and proliferate to different degrees *in vitro*, presumably because of differences in hormonal profiles (Weinstein et al., 1984). In support of this, TLR4 mRNA in the lung of non-allergic females with exposure to the lower concentration of DE was reduced along with a decrease in BALF neutrophils, and effector T cells in the spleen of these mice were increased compared to males. On the other hand, the male mice had increased lung neutrophils in association with decreased T cells in the spleen with exposure to the high dose of DE. These observations suggest that the prenatal DE exposure differentially affected immune processes in the lung and spleen in a gender and concentration dependent manner. To support this apparent difference in gender we found that the OVA sensitization resulted in a greater degree of allergic inflammation for a number of markers including total protein as well as non-significant increases in LDH (~1.3 fold) in BALF, tissue eosinophils (~6 fold) and IAR (~1.7 fold) in the female offspring over the male counterparts. Consistent with this, Seymour et al. (2002) reported increased responses of Th2 cytokines, OVA-specific antibodies and blood eosinophils in female allergic mice exposed to environmental tobacco smoke from the neonatal stage until PND 42. In the female allergic offspring only, the high concentration of DE increased mucus-containing cells in the airway epithelium, BALF eosinophils and OVA-specific IgG1 by 2 fold. In contrast, the exposure to the low concentration reduced IAR (~1.5 fold) and tissue eosinophils (~5 fold), and increased in IFN-γ level (~2.5 fold).

In summary, our observations show that inhaled DE affected pregnancy outcome but did not alter bodyweight of the offspring, the levels of immune responses to experimental antigens or the degree of allergic sensitization following nasal administration and challenge with OVA. Additional work is needed to explore possible gender differences in relation to altered host defenses and allergic inflammation following *in utero* DE exposures as well as testing

how timing of allergen sensitization and challenges may affect the outcome.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

The project was funded by US EPA through ORISE, TN. We thank S.-H. Cho, W. Williams, W. Zhu, C. Copeland, E. Boykin, M. Daniels, K. Gowdy, G. McGee, J. Richards, J. Lehmann, D. Andrews, C. King and D. Janek for technical assistance and appreciate Drs. Janice Dye and David Kurtz for review of the manuscript. This paper has been reviewed by the US Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency, nor does the mention of trade names or commercial products constitute endorsement or recommendation for use.

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