Toxicology Reports 12 (2024) 135–147

Contents lists available at ScienceDirect

Toxicology Reports

journal homepage: www.elsevier.com/locate/toxrep

Effects of inhaled tier-2 diesel engine exhaust on immunotoxicity in a rat model: A hazard identification study. Part II. Immunotoxicology

Lisa M. Weatherly *, Hillary L. Shane, Rachel Baur, Ewa Lukomska, Walter McKinney, Jenny R. Roberts, Jeffrey S. Fedan, Stacey E. Anderson

Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV 26505, USA

ARTICLE INFO

Handling editor: Prof. L.H. Lash

Keywords:
Diesel exhaust
Immunotoxicity
Immunomodulation
Immune phenotyping
Whole-body inhalation

ABSTRACT

Diesel exhaust (DE) is an air pollutant containing gaseous compounds and particulate matter. Diesel engines are common on gas extraction and oil sites, leading to complex DE exposure to a broad range of compounds through occupational settings. The US EPA concluded that short-term exposure to DE leads to allergic inflammatory disorders of the airways. To further evaluate the immunotoxicity of DE, the effects of whole-body inhalation of 0.2 and 1 mg/m^3 DE (total carbon; 6 h/d for 4 days) were investigated 1-, 7-, and 27-days post exposure in Sprague-Dawley rats using an occupationally relevant exposure system. DE exposure of 1 mg/m^3 increased total cellularity, number of CD4+ and CD8+ T-cells, and B-cells at 1 d post-exposure in the lung lymph nodes. At 7 d post-exposure to 1 mg/m^3, cellularity and the number of CD4+ and CD8+ T-cells decreased in the LLNs. In the bronchoalveolar lavage, B-cell number and frequency increased at 1 d post-exposure, Natural Killer cell number and frequency decreased at 7 d post-exposure, and at 27 d post-exposure CD8+ T-cell and CD11b+ cell number and frequency decreased with 0.2 mg/m^3 exposure. In the spleen, 0.2 mg/m^3 increased CD4+ T-cell frequency at 1 and 7 d post-exposure and at 27 d post-exposure increased CD4+ and CD8+ T-cell number and CD8+ T-cell frequency. B-cells were the only immune cell subset altered in the three tissues (spleen, LLNs, and BALF), suggesting the induction of the adaptive immune response. The increase in lymphocytes in several different organ types also suggests an induction of a systemic inflammatory response occurring following DE exposure. These results show that DE exposure induced modifications of cellularity of phenotypic subsets that may impair immune function and contribute to airway inflammation induced by DE exposure in rats.

1. Introduction

Diesel exhaust (DE) is a chemical mixture that contains hundreds of different gaseous compounds and particulate matter, both organic and inorganic, many designated as toxic air pollutants [31]. The particulate fraction of DE can contain hydrocarbons, sulfur compounds, and oxides of nitrogen. The gaseous fraction of DE can include carbon dioxide, carbon monoxide, nitrogen, and aldehydes such as formaldehyde and acetaldehyde ([19,55]). Diesel engine use has increased due to their superior energy efficiency and durability leading to an increase in DE exposure in everyday life. Diesel engines are common on gas extraction and oil sites, also leading to a high potential for DE exposure through occupational settings. According to a study published in 2016, air pollution is in the top 10 risk factors for mortality in men and women [23]. Also, in the same study, diesel exhaust occupational exposure had an increase in its summary exposure value (measure of a population's exposure to a risk factor) [23]. Therefore, it is critical to fully understand the health effects of DE exposure.

In the US, as of the beginning of 2023, there were 148,610 people employed in the Oil Drilling and Gas Extraction Industry (IBIS World 2023) [27]. Workers in these industries that are exposed to DE can be exposed to higher levels than that found in ambient air [48,53,58]. Although new tier-4 diesel engines have been developed [18], the older design tier-2 engines are still being used, specifically at hydraulic fracturing ("fracking") sites [44]. Older diesel engines that are already in operation are "grandfathered in", allowing the continued use of the tier-2 engines [18]. At fracking sites, pumping fracking fluid requires very large diesel engines, where off-road tier-2 diesel engines are still being used. Some hydraulic fracturing sites have 20 or more diesel-powered pumps that could contain tier-2 diesel engines for on-site

* Correspondence to: Allergy and Clinical Immunology Branch National Institute for Occupational Safety and Health (NIOSH), 1000 Frederick Lane, Morgantown, WV 26508, USA
E-mail address: nux6@cdc.gov (L.M. Weatherly).

https://doi.org/10.1016/j.toxrep.2024.01.004
Received 18 December 2023; Received in revised form 8 January 2024; Accepted 9 January 2024
Available online 15 January 2024

water transportation [19].

The International Agency for Research on Cancer (IARC) classified DE as carcinogenic to humans (Group 1) due to associations between inhaled DE exposure and an increased risk of lung cancer [26]. A strong association between occupations where diesel engines are used and an increase in lung cancer has been observed in humans [55]. Additionally, numerous non-carcinogenic changes are also associated with DE exposure including cardiovascular health effects, immunological and allergic effects, and reproductive and developmental effects [24]. Acute short-term DE exposure effects include eye and throat irritation, lightheadedness, nausea, and exacerbation of asthma and allergy-like symptoms [55].

In a study done by the National Institute for Occupational Safety and Health (NIOSH) researchers collected 104 air samples and measured diesel particulate matter (expressed as elemental carbon) at sites that were representative of typical oil and gas extraction. The arithmetic mean of the time weighted average (TWA) was 17 μg/m^3, with 10% of the samples measuring over 20 μg/m^3 [19]. No federal agency has established an occupational exposure limit for DE; however, the State of California has a TWA for elemental carbon exposure of 20 μg/m^3 [49]. In an epidemiology study on workers that tested diesel engines, there was a significant alteration in blood serum cytokine expression (IL-1β, IL-6, IL-8) in DE exposed workers compared to controls, suggesting inflammation and potential immunotoxicity [15]. The US EPA concluded in 2002 that short-term exposure scenarios lead to allergic inflammatory disorders of the airways after DE exposure [55]. In addition, there is also data from animal studies suggesting that DE exposure affects the immune response to infection [24]. In Brown Norway rats, a single high dose DE particle exposure decreased early innate immune response to infection followed by an increase in late cell-mediated response (increases in CD4+, CD8+ T-cells and increase in production of IL-2, IL-6, and IFN-γ) [63,64]. With lower DE particle concentrations and repeated exposure with infection, both innate and T-cell mediated immune responses were decreased [61,62].

The present study was undertaken to evaluate immunotoxicity following inhalation exposure to DE in a rat animal model as a surrogate for potential effects in humans. To evaluate immunotoxicity, the endpoints investigated were natural killer (NK) cell-mediated cytotoxicity, antibody response to sheep red blood cells (SRBC), and immune cell phenotyping. This is a hazard identification study designed to deliver fresh DE immediately to the animal to improve our understanding of DE effects on immune function.

2. Methods

2.1. Animals

All studies were conducted in facilities accredited by AAALAC International, were approved by the CDC-Morgantown Institutional Animal Care and Use Committee (protocols 16-JF-R-018 and 19–015) and were in compliance with the Public Health Service Policy on Humane Care and Use of Laboratory Animals and the NIH Guide for the Care and Use of Laboratory Animals. Male Sprague-Dawley rats [H1a: (SD) CVF], approximate body weight of 200 – 275 g at arrival, were obtained from Hilltop Lab Animals, Inc. (Scottdale, PA). All animals were free of viral pathogens, parasites, mycoplasm, *Heliobacter* and cilia-associated respiratory bacillus. Animals were acclimated for one week and housed in ventilated micro-isolator units supplied with HEPA-filtered laminar flow air (Lab Products OneCage; Seaford, DE), with Teklad Sanichip and Teklad Diamond Dry cellulose bedding or Shepherd Specialty Paper's Alpha-Dri cellulose (Shepherd Specialty Papers; Watertown, TN) bedding instead of Diamond Dry. They were provided tap water and autoclaved HaCXan Teklad Global 18% protein rodent diet (Harlan Teklad; Madison, WI) ad libitum. Rats were housed in pairs under controlled light cycle (12 h light/12 h dark) and temperature (22 – 25 °C) conditions.

2.2. Diesel exhaust exposures

2.2.1. Inhalation exposure system

An eight-kilowatt (KW) diesel generator (Onan QD 8000, part number 8HDKAK11451J, Cummins Inc., Columbus IN) was used to produce diesel exhaust in real time during inhalation exposures. The US EPA has set emission standards for diesel engines. The first federal standards (Tier 1) for new nonroad diesel engines was adopted in 1994. More stringent Tier 2 standards were adopted in 1998 and phased-in between 2000 and 2008. This diesel engine was tier 2 EPA compliant [57]. A load bank was connected to the generator and set to 4 KW (50% generator load) for all inhalation exposures. Mobil 1 Delvac 1300 Super Motor Oil 15w40 was used as the engine oil. The fuel used in the generator was ultra-low sulfur (15 ppm or less sulfur), No. 2 dyed winter blend from Jacobs petroleum products in Waynsburg PA.

Inhalation exposure system was based on [40] with several adaptations. A custom exposure system with software written in LabVIEW automatically controlled chamber air flows, particle concentration, and exposure duration. The software monitored exposure chamber temperature, relative humidity, NO, O_2, SO_2. and CO_2 levels. Diesel engine parameters were monitored and recorded, including fuel level, oil pressure, coolant temperature, and battery voltage. A portion of the diesel exhaust was directed into the laboratory, and a computer-controlled ball valve determined the amount. Clean dry dilution air (80 L/min) from a mass flow controller was added to the diesel exhaust delivery line before it entered the top of the exposure chamber.

The aerosol mass concentration inside the exposure chamber was continuously monitored with a Data RAM (DR-40000 Thermo Electron Co.) and gravimetric determinations (37 mm cassettes with 0.45 μm pore-size Teflon filters, (2 l/min. sample flow) were used to calibrate and verify the Data RAM readings during each exposure run. Particle size distribution inside the exposure chamber (count based) was collected (SMPS Model 3081, TSI Inc., Shoreview, MN). The count median electric mobility diameter was 120 nm. Mass-based aerodynamic particle size distribution was determined in the exposure chamber by using a Micro-Orifice Uniform Deposit Impactor (MOUDI, MSP Model 110, MSP Corporation, Shoreview, MN). The mass median aerodynamic diameter was found to be 170 nm.

Rats (8 per group) were placed in the custom-made, whole-body exposure chamber. Exposures were conducted under controlled conditions with a target diesel particulate (DP) concentration of 0.2 or 1 mg/m^3 (or filtered air) for 6 h/d for 4 days. Experimental endpoints were examined at 1-, 7-, and 27-days post exposure for each concentration of DE. For the first exposure study (A), NK cell activity, immune cell phenotyping, and complete blood counts were evaluated. The data presented for the second study (B) was restricted to the IgM response to sheep red blood cells (SRBC) due to immunization of rats.

2.2.2. Exposure dose

.

Table 1
Measured exposure chamber conditions for all exposures used in this study. Gravimetric filter data was used for the particle concentration data.

	Target DEP of 0.2 mg/m3		Target DEP of 1.0 mg/m3	
	Mean	STD	Mean	STD
DP Concentration (mg/m3)	0.20	0.01	0.94	0.08
CO_2 (PPM)	3550	580	3611	1100
NO (PPM)	8	2	8	4
SO_2 (PPM)	0	0	1	1
CO (PPM)	4	2	18	2
O_2 (%)	21	0.8	20	0.1
Temperature (F)	75	1	74	1
Relative Humidity (%)	30	6	45	8

2.3. Tissue processing (A)

Tissue processing was conducted as previously described [59]. On days 1, 7, and 27, following the last DE or filtered air exposure, rats were given an i.p. injection of sodium pentobarbital (100 – 300 mg/kg; Fatal Plus; Vortech Pharmaceuticals; Dearborn, MI). No significant difference in final body weights were seen between control and DE exposure groups at any time point; there was a significant increase in body weights over time (linear trend test), but this occurred in both the control and DE groups (data not shown). Following exsanguination, the trachea was cannulated, the chest cavity was opened, the right bronchus was clamped off, and bronchoalveolar lavage (BAL) fluid was collected from the left lung. The first lavage sample was obtained by filling the left lung with 3 ml of phosphate-buffered saline (PBS), massaging for 30 s, withdrawing, and repeating the process one more time. This concentrated aliquot was withdrawn, retained, kept separately, and was designated as the first fraction of BAL fluid. Subsequent aliquots of PBS in 5 ml volumes were instilled once with light massaging, withdrawn, and combined until a 30 ml total volume was obtained. For each animal, both lavage fractions were centrifuged (10 min, 598 x *g*), and the cell pellets were combined and resuspended in 1 ml of PBS.

The lung lymph nodes (LLNs) and spleen were excised from each animal, cleaned of connective tissue, and placed in sterile PBS or complete medium (CM)[RPMI-1640, 10% fetal bovine serum (FBS; HyClone, Waltham, MA) and 100 IU penicillin/100 μg streptomycin (Sigma-Aldrich; St. Louis, MO) per ml of medium], respectively. LLN single cell suspensions were prepared by mechanical disruption of tissues between frosted microscope slides in PBS. Spleens were prepared using a 30 ml syringe plunger and subsequently passing the homogenate through a cell strainer to obtain a single cell suspension. Cells were washed with PBS and resuspended in complete media. To enumerate the total number of cells, 20 μl of cells was added to 10 ml of Isoton II diluent (1:500; Beckman Coulter; Brea, CA) and two drops of ZAP-OGLOBIN (Beckman Coulter) were added to lyse red blood cells. Cells were then counted using a Coulter counter.

2.3.1. Flow cytometry

Flow cytometry was conducted as previously described [59]. For phenotypic analysis, single cell suspensions obtained from the BAL, spleen, and LLN ($1 - 2 \times 10^6$) were dispensed into a 96-well, round-bottom plate and washed in staining buffer (PBS + 1% bovine serum albumin + 0.1% sodium azide). Erythrocytes were lysed via RBC lysis buffer before staining of the spleen. For blocking of Fc receptors, cells were resuspended in staining buffer containing α-rat CD32 antibody. Cells were then incubated with a staining cocktail of fluorochrome-conjugated antibodies specific for rat cell surface epitopes: CD3-FITC (clone G4.18), CD4-APC-Cy7 (OX-35), CD8-PerCP (OX-8), CD11b-V450 (WT.5), CD45RA-PE (OX-33), CD45-PE-Cy7 (OX-1), CD161a-AF647 (10/78). Following an incubation, cells were washed twice in staining buffer and fixed in Cytofix buffer according to the manufacturer's instructions (BD Biosciences, San Jose, CA). Within 24 h, cells were resuspended in staining buffer and analyzed on a LSR II flow cytometer (BD Biosciences). Data analysis was performed with FlowJo 7.6.5 software (TreeStar Inc.; Fenton, MI). Cells were first gated on single cells using SSC-A×SSC-H doublet discrimination, followed by cellular discrimination using FSC-A and SSC-A parameters, then cells were identified as CD4+ T cells (CD4+ CD3+), CD8+ T cells (CD8+ CD3+), B cells (CD45RA+ CD3-), NK cells ($CD161a^{hi}$ CD3-), and CD11b+ myeloid cells.

2.3.2. Natural killer (NK) cell-mediated cytotoxicity

NK cell cyotoxicity was conducted as previously described [2,59]. NK cell activity was evaluated using Yac-1, a murine T-cell lymphoma cell line (ATCC; Manassas, VA) as the target cell. Target cells were cultured in complete media (CM), maintained at 37 ℃ in a humidified, 5% CO_2 atmosphere, and monitored daily. Cells were harvested in the log phase of growth, washed with Dulbecco's PBS, and counted using 0.4% trypan blue solution. Only the cultures with greater than 95% cell viability were selected for use in the assay. Target cells were labeled with carboxyfluorescein succinmidyl ester (CellTrace™ CFSE Proliferation Kit; Life Technologies; Waltham, MA) according to the manufacturer's directions. Cells were pelleted, washed twice in CM to wash off the unincorporated dye, counted in 0.4% trypan blue solution, and diluted to the desired concentration in CM. Splenocytes collected and processed as described above were used as the effector cells in this assay. The cytotoxicity assay was performed in 96-well, round bottom tissue culture-treated microtiter plates. Effector cells were seeded in a 100 μl volume/well with a fixed number of CFSE-stained target cells (20, 000/well) with effector to target ratios ranging from 50:1 to 150:1. Control wells for spontaneous death of target contained CFSE-stained target with no effectors (0:1, E:T ratio). Recombinant rat interleukin (IL)− 2 (final concentration 0.05 ng/μl; R&D Systems; Minneapolis, MN) was added to well containing effector cells for enhancement of baseline cytotoxic activity. The contents of each well were mixed gently by pipetting, centrifuged briefly at 526 x *g*, and incubated at 37 °C in a humidified 5% CO_2 atmosphere for 4 h. Killing was stopped by placing the plate on ice for 5 min. All wells were subsequently stained with Live/Dead Fixable Violet Dead Cell Stain (Invitrogen; Waltham, MA) and fixed in Cytofix fixation buffer (BD Bioscience) according to the manufacturer's directions. Effectors only, unstained target, and Live/-Dead Fixable Violet Dead Cell -stained targets were also included as assay controls. Stained samples were analyzed on a LSR II flow cytometer (BD Biosciences). The results were expressed as percentage of dead targets on a cell-to-cell basis.

2.3.3. Hematology

Following administration of an overdose of sodium pentobarbital and exsanguination, whole blood was collected from the abdominal aorta of the rats. A 150 μl aliquot was used for hematological evaluations (Procyte, IDEXX). Endpoints analyzed included peripheral erythrocyte and leukocyte counts, leukocyte differentials (lymphocytes, neutrophils, monocytes, basophils, and eosinophils), reticulocytes, platelet counts, hematocrit, hemoglobin levels, mean corpuscular hemoglobin (MCH) and hemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean platelet volume (MPV), platelet hematocrit (PCT), and platelet distribution width (PDW). Corrected reticulocyte percentage was calculated as [reticulocyte %]* [(animal hematocrit/normal animal hematocrit)] [25]. Normal animal hematocrit number was obtained [12], and a hematocrit value of 43.93 was used for the calculations as the male rats had an average age of 8–12 weeks during exposure. Reticulocyte production index was calculated as (corrected reticulocyte percentage/reticulocyte lifespan). Reticulocyte lifespan was determined following the chart at (Cornell University College of Veterinary Medicine 2023)[14] and a lifespan of 1.5 was used for the calculation.

2.4. Spleen IgM response to SRBC (B)

For study B, the primary IgM response to SRBC was enumerated using a modified hemolytic plaque assay [2,29,59]. Four days prior to euthanasia, the rats were immunized with 2×10^8 SRBC (in 1 ml volume) by i.v. injection. All SRBC for these studies were drawn from a single donor animal (Lampire Laboratories; Pipersville, PA). On days 1, 7, and 27 following the last exposure to DE or air, rats were euthanized by CO_2 asphyxiation, body and spleen weights were recorded, and spleens were collected in 10 ml of Hank's balanced salt solution (HBSS).

Single cell suspensions of the spleens from individual animals were prepared in HBSS by disruption using a 30 ml syringe plunger and subsequent passage through a cell strainer to filter cellular debris. To quantify the total number of spleen cells, 20 μl of cells was added to 10 ml of Isoton II diluent (1:500; Beckman Coulter) and two drops of ZAP-OGLOBIN (Beckman Coulter) were added to lyse red blood cells. Cells were then counted using a Coulter counter.

Dilutions (1:60 and 1:120) of spleen cells were then prepared and 100 μl of each dilution were added to test tubes containing a 0.5 ml warm agar/dextran mixture (0.5% Bacto-Agar; Thermo Scientific; Waltham, MA) and 0.05% DEAE dextran (Sigma-Aldrich), 25 μl of 1:1 ratio of SRBC suspension, and 25 μl of 1:4 dilution (1 ml lyophilized) guinea pig complement (Cedarlane Laboratories; Burlington, Ontario). Each sample was vortexed, poured into a petri dish, covered with a microscope coverslip, and incubated for 3 h at 37 °C. The plaques (representing antibody-forming B-cells) were then counted. Results were expressed in terms of both specific activity (IgM plaque forming cells (PFC) per 10^6 spleen cells) and total activity (IgM PFC per spleen) based on the average of the two dilutions.

2.5. Statistical analyses

To determine statistically significant differences in immunological endpoints, a 2-tailed unpaired *t*-test was used to compare rats exposed to filtered air or diesel exhaust at the specified time point. Presented data are based on the means ± SEM of 6–8 rats for each treatment group at each time point, except for the NK cell assay, in which 4 rats per exposure group were used [2,59]. Differences between control and experimental groups were accepted as significant when *P < 0.05, * *P < 0.01, * **P < 0.001. Statistical analysis was performed using Graph Pad Prism version 5.0 (San Diego, CA).

3. Results

3.1. Immunophenotyping

Flow cytometry was used to investigate immune cell subsets present in LLNs, BAL, and spleen, along with total cellularity, at each of the post-exposure time points. Following exposure to 0.2 mg/m^3 DE, the only significant change in the LLNs was observed at 27 d post-exposure with an increase in the frequency of CD4+ T-cells cells (Supplemental Table 1). However, exposure to 1 mg/m^3 increased total cellularity, the number of CD4+ T-cells, CD8+ T-cells, B-cells at 1 d post-exposure (Fig. 1A, C, E, G). Total cellularity, CD4+ T-cells, CD8+ T-cells, and B-cells increased by 69%, 66%, 81%, and 84%, respectively. At 7 d post-exposure to 1 mg/m^3, cellularity decreased (32%) along with the number of CD4+ T-cells (33%) and CD8+ T cells (43%) in the LLNs (Fig. 1B, D, F). No changes were observed with 1 mg/m^3 DE at 27 d post-exposure in the LLN (Supplemental Table 2). All changes in the LLN were in cell number and no changes were seen in cell frequency with any cell subtype. This suggests that DE is altering the overall cell number without specifically targeting certain cell sub-groups. While no changes in total cellularity were observed in the BAL with 0.2 mg/m^3 exposure (Supplemental Table 3), B-cell number and frequency increased at 1 d post-exposure (Fig. 2A, B), NK cell number and frequency decreased at 7 d post-exposure (Fig. 2C, D), and at 27 d post-exposure NK frequency decreased, CD8+ T-cell number and frequency decreased, and CD11b+ cell number and frequency decreased (Fig. 2F-J). B-cells had large changes in both number and frequency with increases of 188% and 173%, respectively. With 1 mg/m^3 diesel exhaust, CD4+ T-cell frequency decreased at 7 d post-exposure and B-cell frequency and number increased at 27 d post-exposure (Supplemental Table 4). B-cells also had a large increase with 1 mg/m^3, with cell number increasing by 156% and frequency by 100%. Also, in the absence of changes in cellularity (Supplemental Table 5), 0.2 mg/m^3 increased CD4+ T-cells frequency in the spleen at 1 and 7 d post-exposure (Fig. 3B, D) and at 27 d post-exposure increased CD4+ and CD8+ T-cell number and CD8+ T-cell frequency (Fig. 3E, G, H). CD4+ T-cell had the largest increase at 27 d post-exposure, increasing by 26%. CD8+ T cell number increased by 38% and frequency by 18% at 27 d post-exposure. No changes were observed after 1 mg/m^3 at 1 d post-exposure in the spleen. However, B-cell number increased at 7 d post-exposure (20% increase), and CD4 T-cell number and frequency increased at 27 d post-exposure (29% and 17%, respectively) (Supplemental Table 6).

3.2. Natural killer cell assay

Diesel exhaust exposure of 0.2 mg/m^3 suppressed NK cell function but only at 27 d post-exposure (Fig. 4E). A significant decrease in the percent killing was observed at one effector to target ratio, with 1:150 inducing a decrease of 12.6% at 27 d post-exposure. No changes were observed with 0.2 mg/m^3 at 1 or 7 d post-exposure at any effector to target ratios. With 1 mg/m^3 diesel exhaust exposure no changes were observed at any effector to target ratios at 7 and 27 d post-exposure. However, at 1 d post-exposure 1 mg/m^3 diesel exhaust increased the percent killing at 1:100 by 10.9% (Fig. 4B).

3.3. Hematology

No significant hematological changes were observed following exposure to 0.2 mg/m^3 DE exposure at the time points evaluated (Table 2). However, with 1 mg/m^3 exposure, erythrocytes, hemoglobin, and hematocrit significantly increase at 1 d post-exposure (5%, 6%, and 7% increase, respectively) (Table 3). At 7 d post-exposure (1 mg/m^3) MPV decreased and at 27 d post-exposure platelet count decreased and MCH increased (Table 3). Platelet count decreased by 10% (27 d post-exposure) and MPV decreased by 4% (7 d post-exposure). Platelet count showed a significant decrease over time (linear trend test) with 1.0 mg/m^3 DE exposure while control groups showed no change over time, further supporting a DE effect on platelets. Corrected reticulocyte (%) and reticulocyte product index (RPI) showed no change with 0.2 or 1 mg/m^3 DE at any timepoint compared to control. There was a significant decrease over time (linear trend test) in both these parameters, but this occurred in both the control and DE exposure groups, showing the decreases over time were not due to DE exposure.

3.4. IgM response to SRBC

Immunosuppression was evaluated following inhalation exposure to DE via IgM response to SRBC. No significant reductions in the specific (PFC/10^6 cells) or PFC/spleen IgM antibody activity against SRBC were observed after exposure to 0.2 mg/m^3 (Figs. 5) or 1 mg/m^3 (Fig. 6) DE at any of the post-exposure time points.

4. Discussion

Acute immunotoxicologic and hematologic toxic effects of DE were investigated in these studies due to the potential for worker exposure to a chemical mixture. To examine these effects, rats were exposed to 0.2 or 1 mg/m^3 DE and endpoints were measured 1-, 7-, and 27-days post exposure. Following whole-body inhalation exposure to DE, phenotypic changes were observed at multiple time points with most changes occurring in the LLNs. In the LLNs, the majority of changes were observed at the 1-day post exposure timepoint to 1 mg/m^3, resolving by 27 days, suggesting an acute inflammatory response. This is in part supported by the findings in the BALF. While increases in the frequency and number of B-cells in the BALF were observed at 1 day following 0.2 mg/m^3 DE exposure, this increase was only observed at 27 days post exposure for the high dose. It is worth noting that the control B-cell number and frequency at 27 d post-exposure are much lower than the control values at 1 and 7 d post-exposure, therefore, this increase in B-cells after 27 d post-exposure could be due to variation in BAL collection and/or processing. Similar findings were observed in the spleen with more changes occurring following exposure to the 0.2 mg/m^3 low dose. Fujimaki *et al.* also saw an increased number of lymphocytes in the BAL fluid of mice after 4 weeks of DE exposure to low dose (1 mg/m^3) DE but not in the high dose (3 mg/m^3) DE exposure [20]. B-cells were the only immune cell subset altered in the spleen, LLNs, and BALF. This increase suggests the induction of the adaptive immune response; however, these

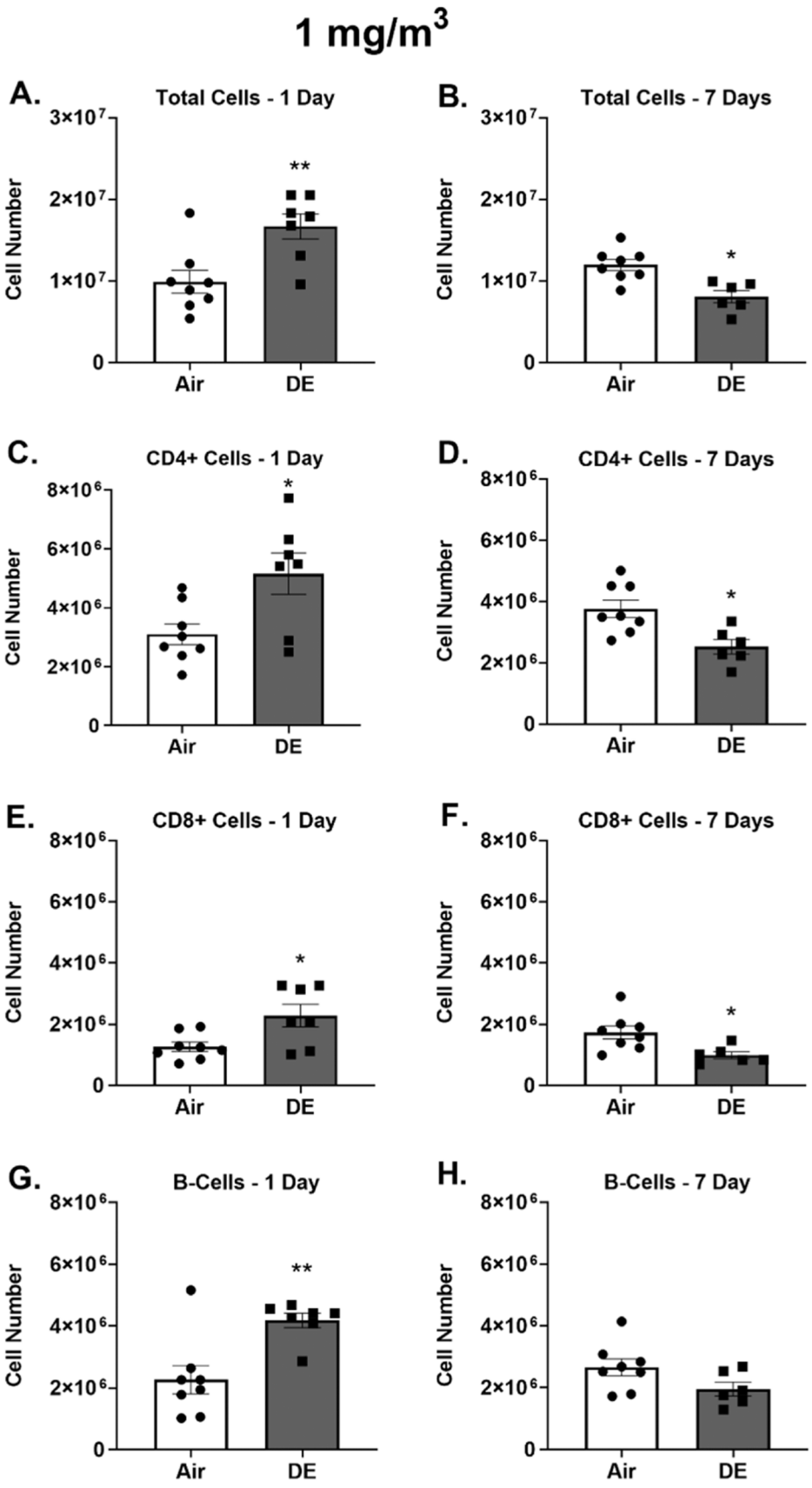

Fig. 1. Inhalation exposure to DE alters immune cell subsets in the LLN. Phenotyping was evaluated in LLN after in vivo exposure to 1 mg/m^3 DE. Total cellularity (A, B), CD4 + cell number (C, D), CD8 + cell number (E, F), and B-cell number (G, H) were quantified via flow cytometry. Statistical analysis was determined using a one-way ANOVA with a Dunnett's post-test. Data shown as the means ± SEM of 6–8 rats per group. P values are represented by *s where *p < 0.05 and * *p < 0.01.

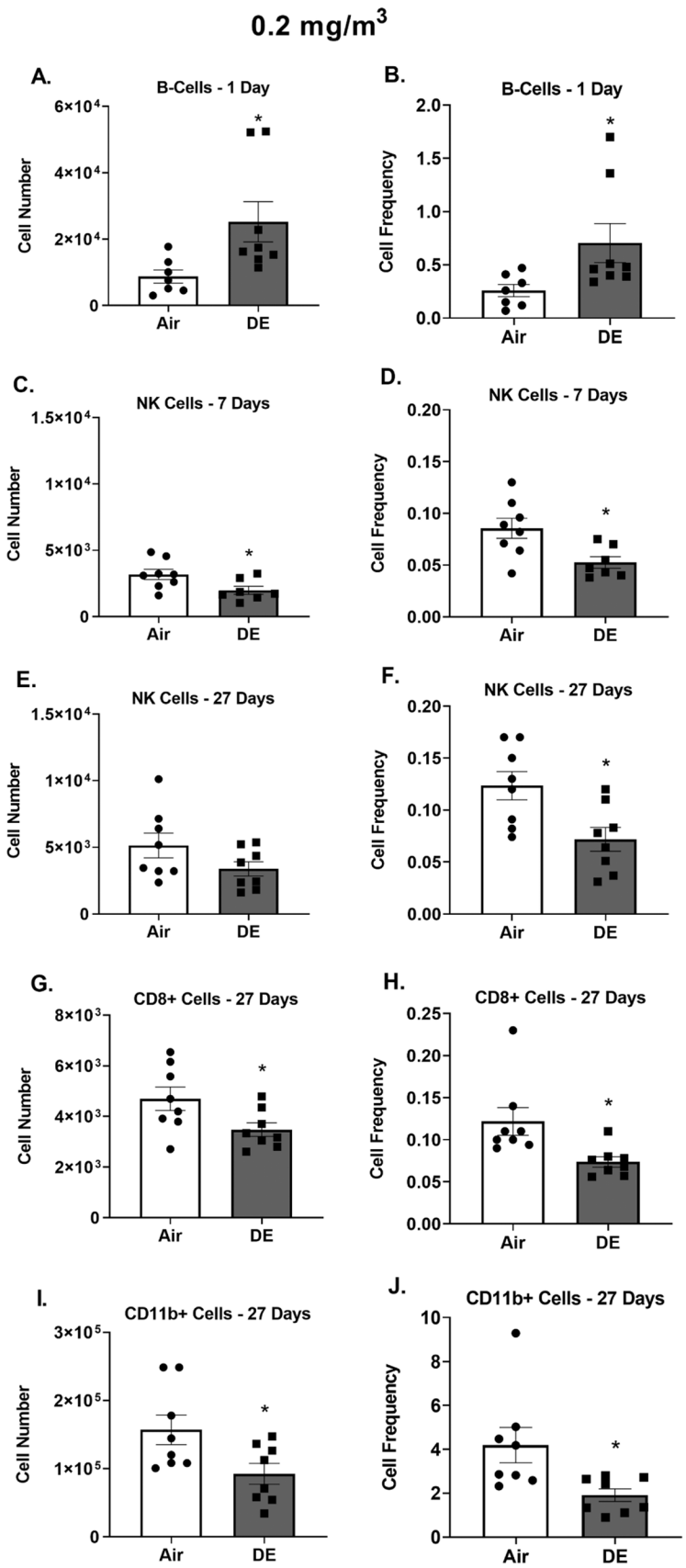

Fig. 2. Inhalation exposure to DE alters immune cell subsets in the BAL fluid. Phenotyping was evaluated in BALF after in vivo exposure to 0.2 mg/m^3 DE. B-cell number and frequency (A, B), NK number and frequency (C-F), CD8 + cell number and frequency (G, H), and CD11b+ cell number and frequency (I, J) were quantified via flow cytometry. Statistical analysis was determined using a one-way ANOVA with a Dunnett's post-test. Data shown as the means ± SEM of 6–8 rats per group. P values are represented by *s where *$p < 0.05$.

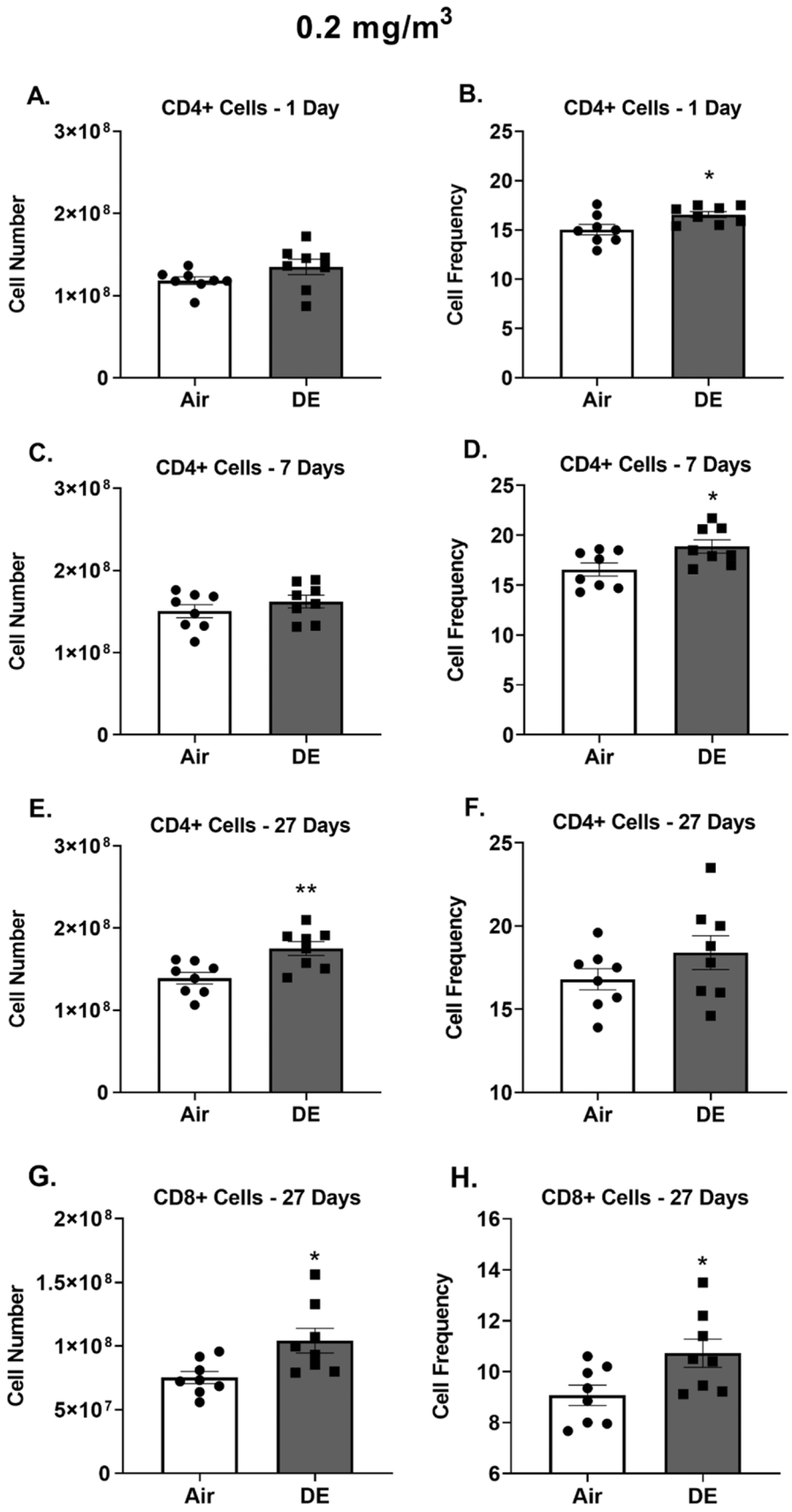

Fig. 3. Inhalation exposure to DE alters immune cell subsets in the spleen. Phenotyping was evaluated in the spleen after in vivo exposure to 0.2 mg/m^3 DE. CD4 + cell number and frequency (A-F), and CD8 + cell number and frequency (G, H) were quantified via flow cytometry. Statistical analysis was determined using a one-way ANOVA with a Dunnett's post-test. Data shown as the means ± SEM of 6–8 rats per group. P values are represented by *s where *p < 0.05 and * *p < 0.01.

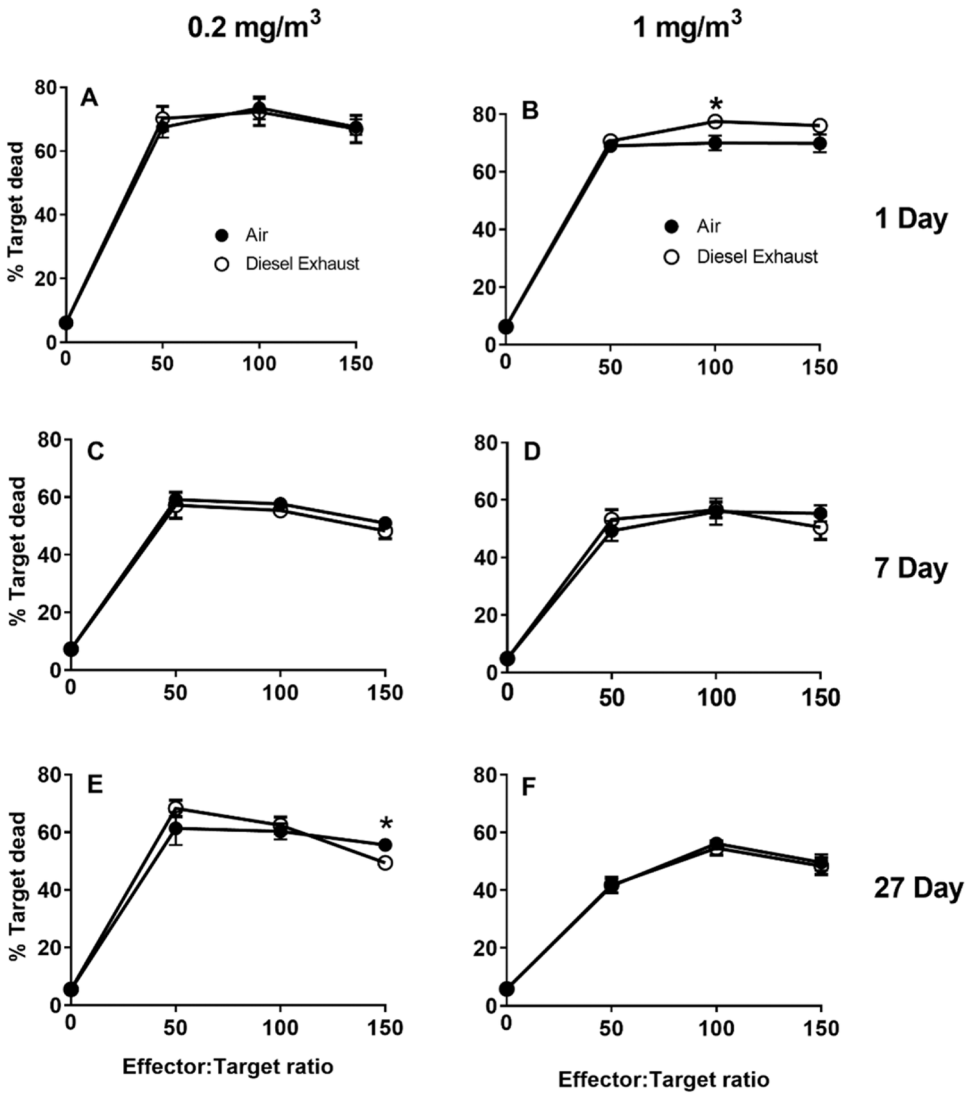

Fig. 4. Inhalation exposure to DE alters spleen NK cell activity. NK cell activity was evaluated in splenocytes after in vivo exposure to 0.2 (A, C, E) and 1 mg/m^3 (B, D, F). NK cell percent killing is shown at 1 d (A, B), 7 d (C, D), and 27 d (E, F) post-exposure. Data shown as the means ± SEM of 4 rats per group. *P < 0.05 vs air control at indicated effector: target ratio.

changes were not observed over multiple time points, although the changes were observed in multiple tissue types. The increase in lymphocytes in several different organ types also suggests an induction of a systemic inflammatory response occurring following DE exposure. Data from several research studies support the findings presented in this manuscript. In a human study, particulates [similar to that of DE Particulate (DEP)] were shown to accumulate in the LLNs over time [56]. Additionally, an epidemiology study investigating occupational exposure to DE found an association between exposure and an increase in CD4 + T cells, CD8 + T cells, and B-cells in peripheral blood [35]. Another study in healthy human volunteers, showed an increase in CD4 + and CD8 + T cells in bronchial biopsies and an increase in B-cells in the BAL after acute DE exposure [50]. These results support our own findings and suggest that the increase in T cells may contribute to airway inflammation induced by DE exposure, similar to what has been observed by other investigators.

While the phenotypic findings suggest the potential for alterations in the humoral immune response, functional evaluation of the adaptive immune response was not consistent with the phenotypic data. The primary murine assay to evaluate humoral immune function is the SRBC PFC assay; this is considered the gold standard for assessing immunomodulation. This assay requires the cooperation of B and T-lymphocytes and macrophages. Although a previous study in $B_6C_3F_1$ mice showed a decrease in both the specific activity and total activity of spleen IgM response to SRBC following exposure to DEP [60], no changes in immune suppression were identified in the current study. [60] also observed a decrease in CD4+ and CD8+ T cells in the absence of B-cell changes. This is contradictory to our phenotyping results where we saw an increase in CD4+ (0.2 and 1 mg/m^3) and CD8+ (0.2 mg/m^3) T cells along with an increase in B cells (1 mg/m^3). These differences in findings could be due to Yang et al. using DEP for exposure via intratracheal aspiration while the current study utilized whole DE exposure via whole-body inhalation. Yang et al. also exposed over 2 or 4 weeks while the current study exposed for 4 days. Similar to our findings, an earlier study with a much longer exposure (6–24 months) found no effect on IgM response with DEP inhalation exposure in both rats and mice [4]. The lack of effect on humoral immune function in this study further suggests that the immune changes observed are acute and inflammatory in nature.

Support for innate immune effects was demonstrated by the findings of the NK assay. The present study did observe a suppression of NK cell function after DE exposure, but only at one timepoint (27 days post exposure) with 0.2 mg/m^3. This could possibly be the start of a subchronic response; however, this study did not allow further

Table 2
Hematology parameters of rats exposed to 0.2 mg/m^3 diesel exhaust by whole body inhalation.

	Days Post-Exposure					
	1 d		7 d		27 d	
Parameter[a]	*Air*	*Diesel Exhaust*	*Air*	*Diesel Exhaust*	*Air*	*Diesel Exhaust*
Erythrocytes (M/μl)	6.71 ± 0.31	6.81 ± 0.32	7.04 ± 0.52	7.25 ± 0.28	8.18 ± 0.39	8.24 ± 0.22
Hemoglobin (g/dl)	13.25 ± 0.47	13.58 ± 0.43	13.43 ± 0.54	13.76 ± 0.56	14.98 ± 0.51	15.07 ± 0.58
Hematocrit (%)	39.89 ± 1.68	40.46 ± 1.26	39.41 ± 1.96	40.12 ± 2.02	43.85 ± 1.98	44.20 ± 2.04
MCV (fl)	59.52 ± 1.47	59.32 ± 2.68	56.11 ± 1.48	55.55 ± 1.34	53.62 ± 1.42	53.6 ± 1.59
MCH (pg)	19.78 ± 0.50	19.90 ± 0.88	19.11 ± 0.68	18.99 ± 0.33	18.34 ± 0.43	18.27 ± 0.41
MCHC (g/dl)	33.24 ± 0.44	33.55 ± 0.23	34.09 ± 0.39	34.15 ± 0.48	34.18 ± 0.46	34.11 ± 0.53
Reticulocytes (K/μl)	383.11 ± 31.59	365.91 ± 54.98	288.64 ± 29.21	269.99 ± 33.54	193.57 ± 12.80	194.32 ± 15.61
Reticulocytes (%)	5.74 ± 0.72	5.38 ± 0.92	4.11 ± 0.41	3.73 ± 0.50	2.37 ± 0.12	2.35 ± 0.15
Corrected Reticulocyte (%)	5.20 ± 0.20	4.95 ± 0.32	3.68 ± 0.14	3.41 ± 0.18	2.35 ± 0.05	2.35 ± 0.09
Reticulocyte Product Index (RPI)	3.47 ± 0.13	3.30 ± 0.21	2.46 ± 0.09	2.28 ± 0.12	1.67 ± 0.13	1.92 ± 0.21
Leukocytes (K/μl)	6.65 ± 1.07	6.53 ± 1.15	6.12 ± 1.79	5.33 ± 1.49	6.86 ± 1.45	7.18 ± 1.64
% Neutrophils	8.70 ± 2.16	9.24 ± 1.68	8.4 ± 1.89	8.45 ± 1.49	10.3 ± 1.63	10.04 ± 1.72
% Lymphocytes	85.86 ± 3.24	87.25 ± 2.84	86.86 ± 3.02	87.11 ± 2.24	85.11 ± 1.85	84.25 ± 1.12
% Monocytes	4.01 ± 1.68	2.49 ± 1.18	3.37 ± 1.35	3.00 ± 1.36	2.88 ± 1.98	4.12 ± 1.30
% Eosinophils	1.26 ± 0.35	0.80 ± 0.30	1.13 ± 0.56	1.25 ± 0.29	1.58 ± 0.47	1.45 ± 0.22
% Basophils	0.39 ± 0.62	0.24 ± 0.11	0.11 ± 0.11	0.16 ± 0.11	0.11 ± 0.11	0.11 ± 0.06
Platelets (K/μl)	907.5 ± 60.89	902.25 ± 103.06	854.2 ± 71.91	781.14 ± 68.56	740.71 ± 95.33	780.85 ± 70.28
PDW (FL)	7.96 ± 0.47	7.89 ± 0.42	8.43 ± 0.95	7.69 ± 0.32	7.88 ± 0.22	8.08 ± 0.28
MPV (FL)	6.76 ± 0.19	6.85 ± 0.28	6.98 ± 0.26	6.59 ± 0.21	6.71 ± 0.14	6.88 ± 0.11
PCT (%)	0.62 ± 0.05	0.62 ± 0.08	0.52 ± 0.15	0.52 ± 0.05	0.49 ± 0.05	0.54 ± 0.05

[a]MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; PDW, platelet distribution width; MCHC, mean corpuscular hemoglobin concentration, MPV, mean platelet volume; and PCT, platelet hematocrit. Values are expressed as the means (± SD) for each group (n = 7-8 rats/group).

Table 3
Hematology parameters of rats exposed to 1 mg/m^3 diesel exhaust by whole body inhalation.

	Days Post-Exposure					
	1 d		7 d		27 d	
Parameter[a]	*Air*	*Diesel*	*Air*	*Diesel*	*Air*	*Diesel*
Erythrocytes (M/μl)	6.63 ± 0.13	**6.99 ± 0.09** *	7.19 ± 0.10	7.01 ± 0.14	7.93 ± 0.07	7.81 ± 0.04
Hemoglobin (g/dl)	13.11 ± 0.24	**13.85 ± 0.18** *	13.89 ± 0.27	13.44 ± 0.18	14.19 ± 0.11	14.25 ± 0.12
Hematocrit (%)	38.63 ± 0.78	**41.31 ± 0.74** *	41.78 ± 1.01	39.99 ± 0.61	41.29 ± 0.46	41.20 ± 0.25
MCV (fl)	58.30 ± 0.45	59.16 ± 1.06	58.11 ± 0.95	57.11 ± 0.52	52.03 ± 0.31	52.78 ± 0.22
MCH (pg)	19.81 ± 0.18	19.85 ± 0.26	19.34 ± 0.24	19.21 ± 0.20	17.90 ± 0.06	**18.25 ± 0.10** *
MCHC (g/dl)	33.96 ± 0.13	33.54 ± 0.20	33.28 ± 0.22	33.63 ± 0.18	34.37 ± 0.28	34.58 ± 0.17
Reticulocytes (K/μl)	354.9 ± 19.53	390.5 ± 14.03	302.3 ± 8.17	278.7 ± 14.57	245.6 ± 35.60	206.3 ± 5.96
Reticulocytes (%)	5.37 ± 0.31	5.59 ± 0.21	4.21 ± 0.12	3.98 ± 0.18	3.09 ± 0.43	2.64 ± 0.08
Corrected Reticulocyte (%)	4.71 ± 0.27	5.27 ± 0.24	4.01 ± 0.18	3.62 ± 0.19	2.92 ± 0.43	2.48 ± 0.06
Reticulocyte Product Index (RPI)	3.14 ± 0.18	3.51 ± 0.16	2.88 ± 0.31	2.41 ± 0.12	1.94 ± 0.29	1.65 ± 0.04
Leukocytes (K/μl)	6.08 ± 0.66	6.81 ± 0.42	7.31 ± 0.83	6.26 ± 0.42	6.17 ± 0.25	6.39 ± 0.60
% Neutrophils	10.86 ± 0.81	10.16 ± 0.67	10.00 ± 1.03	10.50 ± 0.66	10.37 ± 0.77	9.48 ± 0.89
% Lymphocytes	83.33 ± 1.28	84.28 ± 1.55	84.40 ± 1.43	83.74 ± 1.31	84.70 ± 1.122	85.56 ± 0.75
% Monocytes	4.44 ± 0.49	4.51 ± 1.03	4.15 ± 0.66	3.94 ± 0.63	3.10 ± 0.57	3.20 ± 0.48
% Eosinophils	1.10 ± 0.24	0.98 ± 0.13	1.38 ± 0.20	1.60 ± 0.56	1.60 ± 0.19	1.59 ± 0.08
% Basophils	0.34 ± 0.10	0.15 ± 0.03	0.20 ± 0.01	0.23 ± 0.04	0.23 ± 0.06	0.18 ± 0.05
Platelets (K/μl)	977.4 ± 69.46	946.9 ± 53.69	921.5 ± 36.46	895.5 ± 49.89	846.6 ± 27.22	**758.4 ± 28.66** *
PDW (FL)	8.31 ± 0.21	7.98 ± 0.10	8.33 ± 0.17	7.98 ± 0.10	8.39 ± 0.15	8.23 ± 0.17
MPV (FL)	7.48 ± 0.41	6.83 ± 0.05	7.10 ± 0.08	**6.79 ± 0.08** *	7.00 ± 0.09	6.86 ± 0.09
PCT (%)	0.74 ± 0.09	0.65 ± 0.04	0.66 ± 0.03	0.61 ± 0.04	0.59 ± 0.03	0.52 ± 0.02

[a]MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; PDW, platelet distribution width; MCHC, mean corpuscular hemoglobin concentration, MPV, mean platelet volume; and PCT, platelet hematocrit. Values are expressed as the means (± SE) for each group (n = 7-8 rats/group). Several samples were omitted from % Basophils calculations as they were below the accurate limits of detection. *$P < 0.05$

examination of this since acute effects were examined (less than 28 days) after initial exposure. NK cells kill diseased or stressed cells via cytolytic granules. NK cell activity is an innate immune function that plays a critical role in defense against infectious diseases as well as tumor surveillance. NK cells are vital in the innate immune response to infection and act as a bridge between innate and adaptive immune response. A study using isolated peripheral blood from human volunteers observed a decrease in NK cell cytotoxicity with DEP stimulation [41]. Another study found a decrease in cytotoxic activity of NK cells from peripheral blood of inhabitants living near oil industries compared to control group [6]. In the current study, no changes in NK cell number or frequency in the spleen were observed after DE exposure, suggesting changes in NK cell function were not due to an effect on phenotypic changes in NK cells but rather a functional immune defect occurring. NK cell frequency decreased at multiple time points (7- and 27-days post exposure) but only in the BALF. However, due to these changes being relatively minor and not consistent between time points, the biological relevance is questionable.

The data on DE effects on hematological parameters are minimal or inconsistent and considered to be due to biological variability. The decrease in platelet count observed in this study (with no changes in corrected reticulocyte count or RPI) suggest DE effects acting on platelet production. Platelets can regulate the maturation and activation of certain innate immune cells (macrophages, neutrophils, and DCs) and have been shown to have additional roles in modulating the adaptive immune response [1]. Platelets can affect leukocyte recruitment to

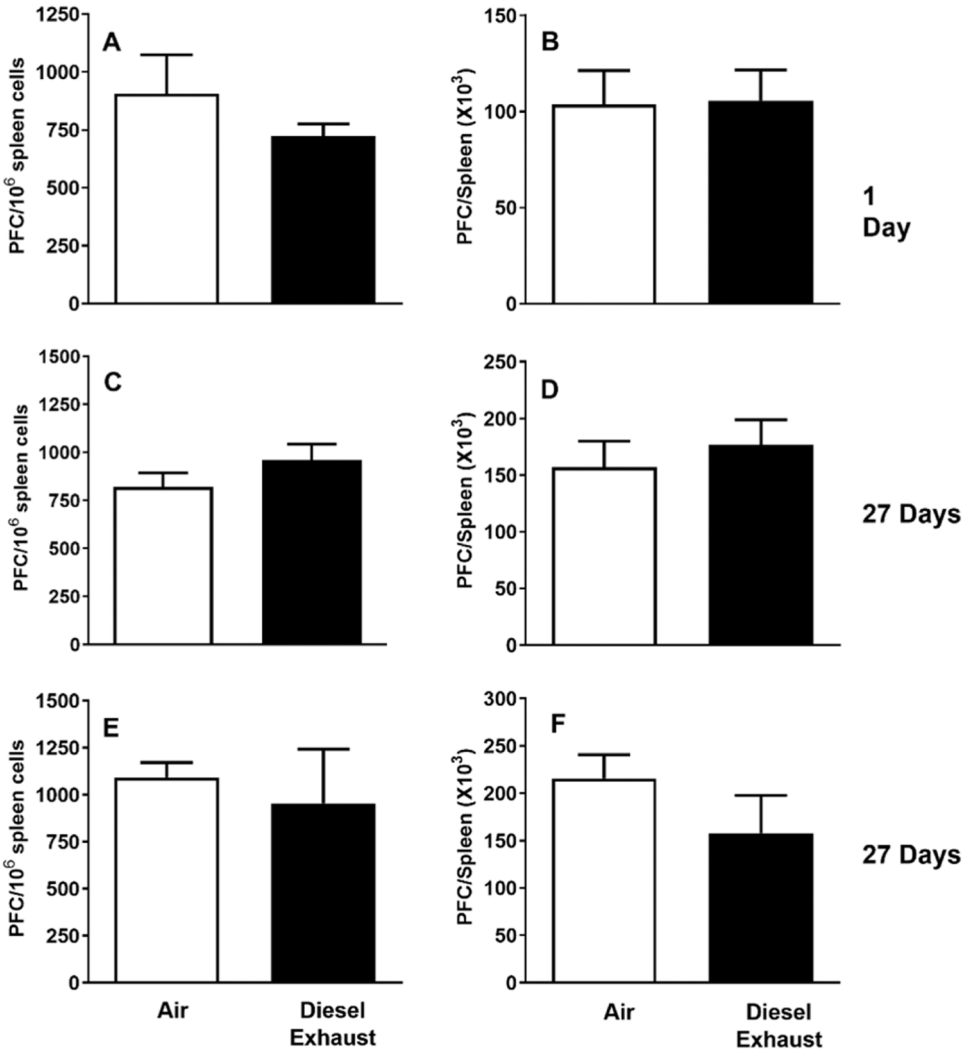

Fig. 5. Inhalation exposure to 0.2 mg/m^3 DE does not suppress the spleen IgM response to sheep red blood cells (SRBC). The spleen IgM response to SRBC was evaluated after exposure to 0.2 mg/m^3 of DE. The IgM response to SRBC/10^6 splenocytes and IgM response to SRBC/spleen is illustrated at 1 d (A, B), 7 d (C, D), and 27 d (E, F) post-exposure. Data shown as the means ± SEM of 8 rats per group.

tissues undergoing inflammation [1] and can activate T and B cells [21]. In the current study, phenotyping analysis showed alterations in lymphocytes, however, activation status was not investigated. Decreases in platelet count along with the effects on immune phenotyping markers may suggest a more complex interaction across these two systems in response to DE. A significant decrease over time (linear trend test) in corrected reticulocyte count and RPI was observed in both the control and DE exposure groups and no differences were observed between control and DE exposure at any timepoint; suggesting the decrease over time was not due to DE exposure. The decrease in reticulocytes over time could be due to overall stress of the animal or another external factor. In a human study with inhaled DE exposure, a significant increase in hematocrit was observed 7 h after DE [34] and following 4 weeks of DEP exposure, a decrease in platelet count was observed in mice exposed to 15 mg/kg DEP [60], both similar to that in the current study. Opposite to our findings, another human study found no effect on platelet number after DE exposure, compared to filtered air, 3, 6, and 22 h after exposure [8]. In a mouse study, no effect on any hematological parameters (measured 1 day after a 4-week exposure) after DE exposure was observed [10]. DEP induced a reduction in red blood cell number and hemoglobin in rats [42], however, this study used intravenous administration of the DEP as opposed to inhalation. A possible reason for the differences in hematology results could be the species dependent degrees of internalization of DEP and resistance to effects in the erythrocytes after DEP exposure [43].

Although not evaluated in the studies presented in this manuscript, many studies report DE and DEP as adjuvants rather than inducing direct immunogenic effects. Mediators of inflammation (RANTES, TNF-α, MIP-1α, MCP-3) increased in production after exposure to DEP with allergen and led to an increase in lymphocyte, monocyte/macrophage and neutrophil cell number ([17,28,45, 55]). These findings are supported by the acute inflammation observed in the present study, further supporting immunotoxic effects of DE.

As mentioned previously, DE is composed of multiple chemicals, some of which have been shown to individually alter immune function. One group of compounds usually found in DE are polycyclic aromatic hydrocarbons (PAHs). PAHs show effects on both immunosuppression and immune enhancement [7]. PAHs can penetrate the epithelial barrier and activate the aryl hydrocarbon receptor (AhR). AhR activation is used by both innate and adaptive immune cells leading to effects on macrophages, mast cells, B cells and T cells [65]. PAHs can also interfere or activate signaling pathways in both murine and human B- and T- cells [7,16]. While the current study did not observe suppression of the humoral immune response, alterations in T and B-cell number and frequency were observed. Nitric oxide is another component of DE. Following nitric oxide exposure in 15 healthy volunteers, a decrease in

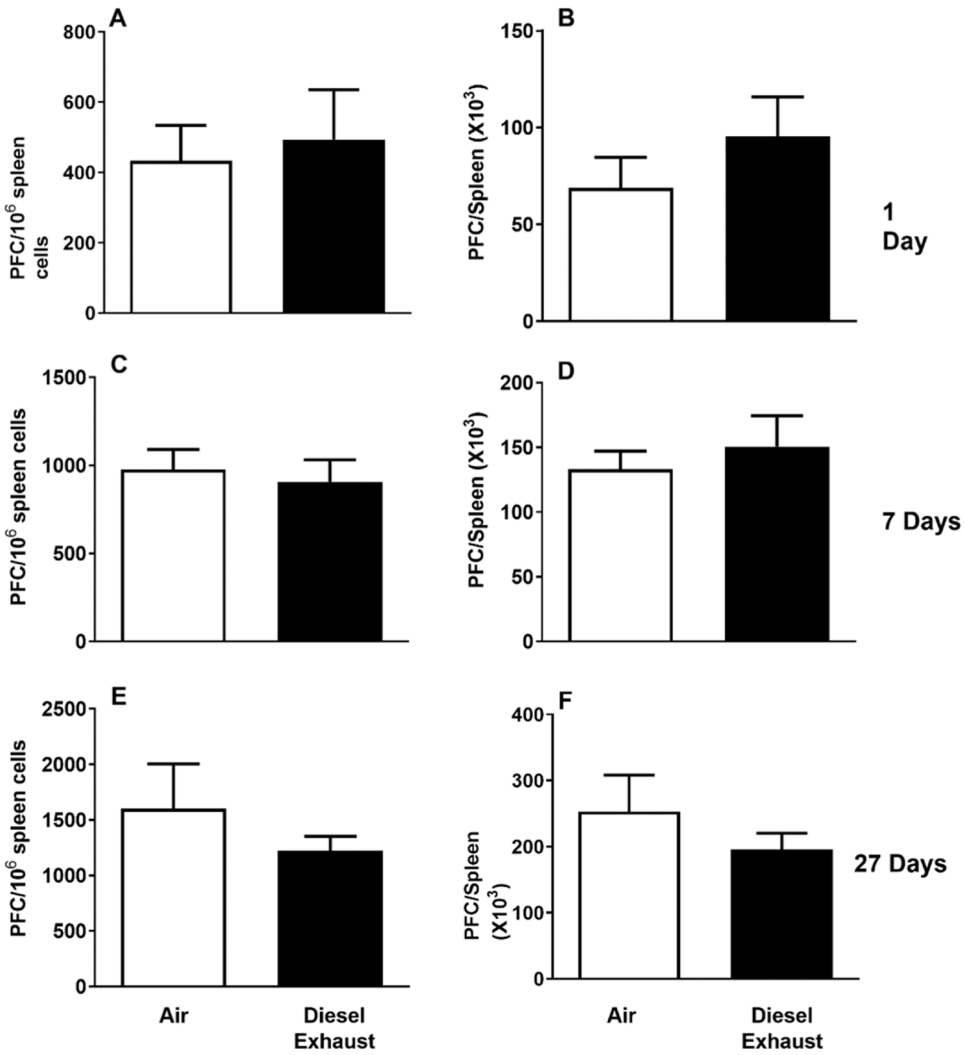

Fig. 6. Inhalation exposure to 1 mg/m^3 DE does not suppress the spleen IgM response to sheep red blood cells (SRBC). The spleen IgM response to SRBC was evaluated after exposure to 1 mg/m^3 of DE. The IgM response to SRBC/10^6 splenocytes and IgM response to SRBC/spleen is illustrated at 1 d (A, B), 7 d (C, D), and 27 d (E, F) post-exposure. Data shown as the means ± SEM of 8 rats per group.

T-cells in the BAL was observed (compared to filtered air exposure) [52] which is consistent with the finding in our study. Higher outdoor nitric oxide levels have also been associated with childhood asthma [22]. After nitric oxide exposure, all subjects (18 healthy non-smokers) showed an inflammatory response with altered numbers of lymphocytes in the BAL fluid [51], and another study showed an increase in B-cells in the BAL fluid after exposure in healthy subjects [5]. Composition of DE (including particle diameter, surface area, and PAH concentration) have been shown to differ between diesel fuel types and the DE created [3]. DEs are variable mixtures that have been shown to induce differing biological results depending on the components [54]. Careful interpretation of exposures occurring with mixtures need to be conducted as each mixture is not exactly the same and may contain varying concentrations of immunomodulating substances. Only male rats were used in this study, in order to compare results with previous and future studies. While interpreting the results, it is important to note that previous studies have found differences between males and females in a variety of endpoints after DE exposure [13,30,33,36] and the immune response is known to differ between males and females [32].

Diesel particulate mass has historically been the measure of exposure to simulate whole DE exposure [55]. The majority of articles published on DEP toxicity use standard reference particles that are administered intranasally or intratracheally. The exposure method utilized in the current study overcome some of the limitations of reference particle exposure as the particles are freshly generated with the volatile organics and gases still present and exposure is occurring via inhalation which is the main route of exposure for humans. As mentioned above, it is important when interpreting DE findings to consider the type of DE/DEP being utilized, the exposure method, the concentration of the DE/DEP, and the animal model. The doses utilized in the current study were chosen to bracket potential occupational exposure levels using both a low and high dose [48]. Similar concentrations to what was used in this study have been published previously both in human exposure (0.3 mg/m^3 [11,37,47,9] and 0.11 mg/m^3, [46]) and in rodents (0.1 to 0.996 mg/m^3 [38], and 0.2 mg/m^3 [39]). In summary, the present study observed quantitative changes in cellularity in the BAL, LLNs, and spleen, alterations in NK function, and alterations in hematology parameters in rats after DE whole-body inhalation exposure. The suspected health effects of DE exposure with the high risk of occupational exposure demonstrates the need for additional studies to help evaluate the adverse effects of DE to human health.

5. Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National

Institute for Occupational Safety and Health, Centers for Disease Control and Prevention. Mention of brand name does not constitute product endorsement.

CRediT authorship contribution statement

Lukomska Ewa: Writing – review & editing, Data curation. **Baur Rachel:** Writing – review & editing, Data curation. **Shane Hillary L.:** Writing – review & editing, Formal analysis, Data curation. **Weatherly Lisa M.:** Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Anderson Stacey E.:** Writing – review & editing, Supervision, Data curation. **Fedan Jeffrey S.:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Roberts Jenny R.:** Writing – review & editing, Data curation. **McKinney Walter:** Writing – review & editing, Methodology, Data curation.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. The authors alone are responsible for the content of this manuscript. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention. All study data will be made available on the NIOSH Data and Statistics Gateway.

Data availability

All study data will be made available on the NIOSH Data and Statistics Gateway.

Acknowledgements

Funding was provided by the National Institute for Occupational Safety and Health, Project Number 6927ZLDC.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2024.01.004.

References

[1] R.A. Ali, L.M. Wuescher, R.G. Worth, Platelets: essential components of the immune system, Curr. Trends Immunol. 16 (2015) 65–78.
[2] S.E. Anderson, H. Shane, C. Long, A. Marrocco, E. Lukomska, J.R. Roberts, N. Marshall, J.S. Fedan, Biological effects of inhaled hydraulic fracturing sand dust. Viii. Immunotoxicity, Toxicol. Appl. Pharm. 408 (2020) 115256, https://doi.org/10.1016/j.taap.2020.115256.
[3] K.M. Bendtsen, L. Gren, V.B. Malmborg, P.C. Shukla, M. Tunér, Y.J. Essig, A. M. Krais, P.A. Clausen, T. Berthing, K. Loeschner, et al., Particle characterization and toxicity in c57bl/6 mice following instillation of five different diesel exhaust particles designed to differ in physicochemical properties, Part Fibre Toxicol. 17 (1) (2020) 38, https://doi.org/10.1186/s12989-020-00369-9.
[4] D.E. Bice, J.L. Mauderly, R.K. Jones, R.O. McClellan, Effects of inhaled diesel exhaust on immune responses after lung immunization. Fundam. Appl. Toxicol. 5 (6 Pt 1) (1985) 1075–1086, https://doi.org/10.1016/0272-0590(85)90143-5.
[5] A. Blomberg, M.T. Krishna, V. Bocchino, G.L. Biscione, J.K. Shute, F.J. Kelly, A. J. Frew, S.T. Holgate, T. Sandström, The inflammatory effects of 2 ppm no2 on the airways of healthy subjects, Am. J. Respir. Crit. Care Med 156 (2 Pt 1) (1997) 418–424, https://doi.org/10.1164/ajrccm.156.2.9612042.
[6] A. Bulog, I.B. Karaconji, I. Sutić, V. Mićović, Immunomodulation of cell-mediated cytotoxicity after chronic exposure to vapors, Coll. Antropol. 35 (Suppl 2) (2011) 61–64.
[7] S.W. Burchiel, M.I. Luster, Signaling by environmental polycyclic aromatic hydrocarbons in human lymphocytes, Clin. Immunol. 98 (1) (2001) 2–10, https://doi.org/10.1006/clim.2000.4934.
[8] C. Carlsten, J.D. Kaufman, A. Peretz, C.A. Trenga, L. Sheppard, J.H. Sullivan, Coagulation markers in healthy human subjects exposed to diesel exhaust, Thromb. Res. 120 (6) (2007) 849–855, https://doi.org/10.1016/j.thromres.2007.01.005.
[9] C. Carlsten, M.J. MacNutt, Z. Zhang, F. Sava, M.M. Pui, Anti-oxidant n-acetylcysteine diminishes diesel exhaust-induced increased airway responsiveness in person with airway hyper-reactivity, Toxicol. Sci. 139 (2) (2014) 479–487, https://doi.org/10.1093/toxsci/kfu040.
[10] F.R. Cassee, A. Campbell, A.J. Boere, S.G. McLean, R. Duffin, P. Krystek, I. Gosens, M.R. Miller, The biological effects of subacute inhalation of diesel exhaust following addition of cerium oxide nanoparticles in atherosclerosis-prone mice, Environ. Res 115 (1) (2012) 1–10, https://doi.org/10.1016/j.envres.2012.03.004.
[11] R. Cliff, J. Curran, J.A. Hirota, M. Brauer, H. Feldman, C. Carlsten, Effect of diesel exhaust inhalation on blood markers of inflammation and neurotoxicity: a controlled, blinded crossover study, Inhal. Toxicol. 28 (3) (2016) 145–153, https://doi.org/10.3109/08958378.2016.1145770.
[12] Clifford MGaC. 2006. Clinical laboratory parameters for crl:Cd(sd) rats. In: Laboratories CR, editor.
[13] T.B. Cole, J. Coburn, K. Dao, P. Roqué, Y.-C. Chang, V. Kalia, T.R. Guilarte, J. Dziedzic, L.G. Costa, Sex and genetic differences in the effects of acute diesel exhaust exposure on inflammation and oxidative stress in mouse brain, Toxicology 374 (2016) 1–9, https://doi.org/10.1016/j.tox.2016.11.010.
[14] Cornell University College of Veterinary Medicine.Absolute retic count. 2023. [accessed 2023]. https://eclinpath.com/hematology/tests/absolute-reticulocyte-count/.
[15] Y. Dai, X. Zhang, R. Zhang, X. Zhao, H. Duan, Y. Niu, C. Huang, T. Meng, M. Ye, P. Bin, et al., Long-term exposure to diesel engine exhaust affects cytokine expression among occupational population, Toxicol. Res. 5 (2) (2016) 674–681, https://doi.org/10.1039/c5tx00462d.
[16] D.R. Davila, D.P. Davis, K. Campbell, J.C. Cambier, L.A. Zigmond, S.W. Burchiel, Role of alterations in ca(2+)-associated signaling pathways in the immunotoxicity of polycyclic aromatic hydrocarbons, J. Toxicol. Environ. Health 45 (2) (1995) 101–126, https://doi.org/10.1080/15287399509531985.
[17] D. Diaz-Sanchez, M. Jyrala, D. Ng, A. Nel, A. Saxon, In vivo nasal challenge with diesel exhaust particles enhances expression of the cc chemokines rantes, mip-1alpha, and mcp-3 in humans, Clin. Immunol. 97 (2) (2000) 140–145, https://doi.org/10.1006/clim.2000.4921.
[18] EPA EPA. 2004. Control of emissions of air pollution from nonroad diesel engines and fuel; final rule. https://www.govinfo.gov/content/pkg/FR-2004–06-29/pdf/FR-2004–06-29.pdf.
[19] E.J. Esswein, M. Alexander-Scott, J. Snawder, M. Breitenstein, Measurement of area and personal breathing zone concentrations of diesel particulate matter (dpm) during oil and gas extraction operations, including hydraulic fracturing, J. Occup. Environ. Hyg. 15 (1) (2018) 63–70, https://doi.org/10.1080/15459624.2017.1388512.
[20] H. Fujimaki, Y. Kurokawa, S. Yamamoto, M. Satoh, Distinct requirements for interleukin-6 in airway inflammation induced by diesel exhaust in mice, Immunopharmacol. Immunotoxicol. 28 (4) (2006) 703–714, https://doi.org/10.1080/08923970601067433.
[21] O. Garraud, F. Cognasse, Are platelets cells? And if yes, are they immune cells? Front. Immunol. 6 (2015) 70, https://doi.org/10.3389/fimmu.2015.00070.
[22] W.J. Gauderman, E. Avol, F. Lurmann, N. Kuenzli, F. Gilliland, J. Peters, R. McConnell, Childhood asthma and exposure to traffic and nitrogen dioxide, Epidemiology 16 (6) (2005) 737–743, https://doi.org/10.1097/01.ede.0000181308.51440.75.
[23] GBD 2017 Risk Factor Collaborators. 2017. Global, regional, and national comparative risk assessment of 84 behavioural, environmental and occupational, and metabolic risks or clusters of risks, 1990–2016: A systematic analysis for the global burden of disease study 2016. Lancet. 390(10100):1345–1422. 10.1016/s0140–6736(17)32366–8.
[24] T.W. Hesterberg, C.M. Long, W.B. Bunn, S.N. Sax, C.A. Lapin, P.A. Valberg, Non-cancer health effects of diesel exhaust: a critical assessment of recent human and animal toxicological literature, Crit. Rev. Toxicol. 39 (3) (2009) 195–227, https://doi.org/10.1080/10408440802220603.
[25] R.S. Hillman, Characteristics of marrow production and reticulocyte maturation in normal man in response to anemia, J. Clin. Invest 48 (3) (1969) 443–453, https://doi.org/10.1172/jci106001.
[26] IARC, Diesel and gasoline engine exhausts and some nitroarenes, IARC Monogr. Eval. Carcinog. Risks Hum. IARC Monogr. Eval. Carcinog. Risks Hum. 105 (2014) 9–699.
[27] IBIS World. Oil drilling and gas extraction in the us - employment statistics 2023. [accessed 2023]. https://www.ibisworld.com/industry-statistics/employment/oil-drilling-gas-extraction-united-states/#:~:text=There%20are%20148%2C610%20people%20employed,the%20US%20as%20of%202023.
[28] K. Inoue, H. Takano, R. Yanagisawa, S. Hirano, T. Kobayashi, Y. Fujitani, A. Shimada, T. Yoshikawa, Effects of inhaled nanoparticles on acute lung injury induced by lipopolysaccharide in mice, Toxicology 238 (2-3) (2007) 99–110, https://doi.org/10.1016/j.tox.2007.05.022.
[29] N.K. Jerne, A.A. Nordin, Plaque formation in agar by single antibody-producing cells, Science 140 (3565) (1963) 405, https://doi.org/10.1126/science.140.3565.405.
[30] T.L. Jetton, O.T. Galbraith, M. Peshavaria, E.A. Bonney, B.A. Holmén, N. K. Fukagawa, Sex-specific metabolic adaptations from in utero exposure to particulate matter derived from combustion of petrodiesel and biodiesel fuels, Chemosphere 346 (2024) 140480, https://doi.org/10.1016/j.chemosphere.2023.140480.
[31] J. Kagawa, Health effects of diesel exhaust emissions–a mixture of air pollutants of worldwide concern. Toxicology 181-182 (2002) 349–353, https://doi.org/10.1016/s0300-483x(02)00461-4.

[32] S.L. Klein, K.L. Flanagan, Sex differences in immune responses, Nat. Rev. Immunol. 16 (10) (2016) 626–638, https://doi.org/10.1038/nri.2016.90.
[33] C. Kochi, A. Salvi, F. Atrooz, S. Salim, Simulated vehicle exhaust exposure induces sex-dependent behavioral deficits in rats, Environ. Toxicol. Pharmacol. 86 (2021) 103660, https://doi.org/10.1016/j.etap.2021.103660.
[34] R.M. Krishnan, J.H. Sullivan, C. Carlsten, H.W. Wilkerson, R.P. Beyer, T. Bammler, F. Farin, A. Peretz, J.D. Kaufman, A randomized cross-over study of inhalation of diesel exhaust, hematological indices, and endothelial markers in humans, Part Fibre Toxicol. 10 (2013) 7, https://doi.org/10.1186/1743-8977-10-7.
[35] Q. Lan, R. Vermeulen, Y. Dai, D. Ren, W. Hu, H. Duan, Y. Niu, J. Xu, W. Fu, K. Meliefste, et al., Occupational exposure to diesel engine exhaust and alterations in lymphocyte subsets, Occup. Environ. Med 72 (5) (2015) 354–359, https://doi.org/10.1136/oemed-2014-102556.
[36] M. Liao, Z. Braunstein, X. Rao, Sex differences in particulate air pollution-related cardiovascular diseases: a review of human and animal evidence, Sci. Total Environ. 884 (2023) 163803, https://doi.org/10.1016/j.scitotenv.2023.163803.
[37] E. Long, C. Carlsten, Controlled human exposure to diesel exhaust: results illuminate health effects of traffic-related air pollution and inform future directions, Part Fibre Toxicol. 19 (1) (2022) 11, https://doi.org/10.1186/s12989-022-00450-5.
[38] J.L. Mauderly, S.K. Seilkop, The national environmental respiratory center (nerc) experiment in multi-pollutant air quality health research: Iii. Components of diesel and gasoline engine exhausts, hardwood smoke and simulated downwind coal emissions driving non-cancer biological responses in rodents, Inhal. Toxicol. 26 (11) (2014) 668–690, https://doi.org/10.3109/08958378.2014.920440.
[39] J.D. McDonald, K.S. Harrod, J. Seagrave, S.K. Seilkop, J.L. Mauderly, Effects of low sulfur fuel and a catalyzed particle trap on the composition and toxicity of diesel emissions, Environ. Health Perspect. 112 (13) (2004) 1307–1312, https://doi.org/10.1289/ehp.7059.
[40] W.ea McKinney, Automated crude oil vapor inhalation exposure system, Inhal. Toxicol. (2022).
[41] L. Müller, C.V. Chehrazi, M.W. Henderson, T.L. Noah, I. Jaspers, Diesel exhaust particles modify natural killer cell function and cytokine release, Part Fibre Toxicol. 10 (2013) 16, https://doi.org/10.1186/1743-8977-10-16.
[42] A. Nemmar, I.M. Inuwa, Diesel exhaust particles in blood trigger systemic and pulmonary morphological alterations, Toxicol. Lett. 176 (1) (2008) 20–30, https://doi.org/10.1016/j.toxlet.2007.09.006.
[43] A. Nemmar, S. Zia, D. Subramaniyan, I. Al-Amri, M.A. Al Kindi, B.H. Ali, Interaction of diesel exhaust particles with human, rat and mouse erythrocytes in vitro, Cell Physiol. Biochem 29 (1-2) (2012) 163–170, https://doi.org/10.1159/000337597.
[44] W. Nieuwenburg, A. Nix, D. Fu, T. Yeung, W. Zemlak, N. Wells, Analysis of emissions profiles of hydraulic fracturing engine technologies, Energy Power Eng. 15 (2023) 1–34, https://doi.org/10.4236/epe.2023.151001.
[45] R.J. Pandya, G. Solomon, A. Kinner, J.R. Balmes, Diesel exhaust and asthma: hypotheses and molecular mechanisms of action (Suppl 1), Environ. Health Perspect. 110 (Suppl 1) (2002) 103–112, https://doi.org/10.1289/ehp.02110s1103.
[46] E.A. Pawlak, T.L. Noah, H. Zhou, C. Chehrazi, C. Robinette, D. Diaz-Sanchez, L. Müller, I. Jaspers, Diesel exposure suppresses natural killer cell function and resolution of eosinophil inflammation: a randomized controlled trial of exposure in allergic rhinitics, Part Fibre Toxicol. 13 (1) (2016) 24, https://doi.org/10.1186/s12989-016-0135-7.
[47] A.P. Pettit, A. Brooks, R. Laumbach, N. Fiedler, Q. Wang, P.O. Strickland, K. Madura, J. Zhang, H.M. Kipen, Alteration of peripheral blood monocyte gene expression in humans following diesel exhaust inhalation, Inhal. Toxicol. 24 (3) (2012) 172–181, https://doi.org/10.3109/08958378.2012.654856.
[48] A. Pronk, J. Coble, P.A. Stewart, Occupational exposure to diesel engine exhaust: a literature review, J. Expo. Sci. Environ. Epidemiol. 19 (5) (2009) 443–457, https://doi.org/10.1038/jes.2009.21.
[49] Salmon L.G., Mayo P.R., Cass G.R. 2004. Determination of elemental carbon and organic carbon concentrations during the southern california children's health study, 1999–2001. https://ww2.arb.ca.gov/sites/default/files/classic/research/apr/past/01–309.pdf.
[50] S. Salvi, A. Blomberg, B. Rudell, F. Kelly, T. Sandström, S.T. Holgate, A. Frew, Acute inflammatory responses in the airways and peripheral blood after short-term exposure to diesel exhaust in healthy human volunteers, Am. J. Respir. Crit. Care Med 159 (3) (1999) 702–709, https://doi.org/10.1164/ajrccm.159.3.9709083.
[51] T. Sandström, N. Stjernberg, A. Eklund, M.C. Ledin, L. Bjermer, B. Kolmodin-Hedman, K. Lindström, L. Rosenhall, T. Angström, Inflammatory cell response in bronchoalveolar lavage fluid after nitrogen dioxide exposure of healthy subjects: A dose-response study, Eur. Respir. J. 4 (3) (1991) 332–339.
[52] C. Solomon, D.L. Christian, L.L. Chen, B.S. Welch, M.T. Kleinman, E. Dunham, D. J. Erle, J.R. Balmes, Effect of serial-day exposure to nitrogen dioxide on airway and blood leukocytes and lymphocyte subsets, Eur. Respir. J. 15 (5) (2000) 922–928, https://doi.org/10.1034/j.1399-3003.2000.15e19.x.
[53] P. Taxell, T. Santonen, Diesel engine exhaust: Basis for occupational exposure limit value, Toxicol. Sci. 158 (2) (2017) 243–251, https://doi.org/10.1093/toxsci/kfx110.
[54] A.I. Totlandsdal, J.I. Herseth, A.K. Bølling, A. Kubátová, A. Braun, R.E. Cochran, M. Refsnes, J. Ovrevik, M. Låg, Differential effects of the particle core and organic extract of diesel exhaust particles, Toxicol. Lett. 208 (3) (2012) 262–268, https://doi.org/10.1016/j.toxlet.2011.10.025.
[55] U.S. EPA USEPA. 2002. Health assessment document for diesel engine exhaust (final 2022).
[56] B.B. Ural, D.P. Caron, P. Dogra, S.B. Wells, P.A. Szabo, T. Granot, T. Senda, M.M. L. Poon, N. Lam, P. Thapa, et al., Inhaled particulate accumulation with age impairs immune function and architecture in human lung lymph nodes, Nat. Med 28 (12) (2022) 2622–2632, https://doi.org/10.1038/s41591-022-02073-x.
[57] US EPA USEPA. 1998. Final rule for control of emissions of air pollution from nonroad diesel engines. https://www.epa.gov/regulations-emissions-vehicles-and-engines/final-rule-control-emissions-air-pollution-nonroad-0.
[58] R. Vermeulen, L. Portengen, How serious are we about protecting workers health? The case of diesel engine exhaust, Occup. Environ. Med. 79 (8) (2022) 540–542, https://doi.org/10.1136/oemed-2021-107752.
[59] L.M. Weatherly, H.L. Shane, R. Baur, E. Lukomska, J.R. Roberts, J.S. Fedan, S. E. Anderson, Biological effects of inhaled crude oil. Vi. Immunotoxicity, Toxicol. Appl. Pharm. 449 (2022) 116100, https://doi.org/10.1016/j.taap.2022.116100.
[60] H.M. Yang, L. Butterworth, A.E. Munson, B.J. Meade, Respiratory exposure to diesel exhaust particles decreases the spleen igm response to a t cell-dependent antigen in female b6c3f1 mice, Toxicol. Sci. 71 (2) (2003) 207–216, https://doi.org/10.1093/toxsci/71.2.207.
[61] X.J. Yin, C.C. Dong, J.Y. Ma, J.M. Antonini, J.R. Roberts, M.W. Barger, J.K. Ma, Sustained effect of inhaled diesel exhaust particles on t-lymphocyte-mediated immune responses against listeria monocytogenes, Toxicol. Sci. 88 (1) (2005) 73–81, https://doi.org/10.1093/toxsci/kfi279.
[62] X.J. Yin, C.C. Dong, J.Y. Ma, J.M. Antonini, J.R. Roberts, C.F. Stanley, R. Schafer, J. K. Ma, Suppression of cell-mediated immune responses to listeria infection by repeated exposure to diesel exhaust particles in brown norway rats, Toxicol. Sci. 77 (2) (2004) 263–271, https://doi.org/10.1093/toxsci/kfh035.
[63] X.J. Yin, R. Schafer, J.Y. Ma, J.M. Antonini, J.R. Roberts, D.N. Weissman, P. D. Siegel, J.K. Ma, Alteration of pulmonary immunity to listeria monocytogenes by diesel exhaust particles (deps). Ii. Effects of deps on t-cell-mediated immune responses in rats, Environ. Health Perspect. 111 (4) (2003) 524–530, https://doi.org/10.1289/ehp.5709.
[64] X.J. Yin, R. Schafer, J.Y. Ma, J.M. Antonini, D.D. Weissman, P.D. Siegel, M. W. Barger, J.R. Roberts, J.K. Ma, Alteration of pulmonary immunity to listeria monocytogenes by diesel exhaust particles (deps). I. Effects of deps on early pulmonary responses, Environ. Health Perspect. 110 (11) (2002) 1105–1111, https://doi.org/10.1289/ehp.021101105.
[65] Y.Y. Yu, H. Jin, Q. Lu, Effect of polycyclic aromatic hydrocarbons on immunity, J. Transl. Autoimmun. 5 (2022) 100177, https://doi.org/10.1016/j.jtauto.2022.100177.