

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. (Scottsdale, PA). All animals were free of viral pathogens, parasites, mycoplasma, *Helicobacter* 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.

Diesel exhaust exposures

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. This diesel engine was tier 2 EPA compliant. 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 (15ppm or less sulfur), No. 2 dyed winter blend from Jacobs petroleum products in Waynsburg PA.

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, O₂, SO₂, and CO₂ 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 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/m³ (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 gives the measured exposure chamber conditions for all exposures used in this study. Gravimetric filter data was used for the particle concentration data.

Table 1

	Target DP of 0.2 mg/m ³		Target DP of 1.0 mg/m ³	
	Mean	STD	Mean	STD
DP Concentration (mg/m³)	0.20	0.01	0.94	0.08
CO₂ (PPM)	3550	580	3611	1100
NO (PPM)	8	2	8	4
SO₂ (PPM)	0	0	1	1
CO (PPM)	4	2	18	2
O₂ (%)	21	0.8	20	0.1
Temperature (F)	75	1	74	1
Relative Humidity (%)	30	6	45	8

Tissue processing

On days 1, 7, and 27, following the last diesel exhaust or filtered air exposure, rats were given an i.p. injection of sodium pentobarbital (100 – 300 mg/kg; Fatal Plus; Vortech Pharmaceuticals; Dearborn, MI). 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 sec, 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 g), and the cell pellets were combined and resuspended in 1 ml of PBS.

The lung lymph nodes (LLNs) and spleen were removed 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

Flow Cytometry

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 an 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 \times 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.

Natural killer (NK) cell assay

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 °C in a humidified, 5% CO₂ 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 succinimidyl 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 all wells for enhancement of baseline cytotoxic activity. The contents of each well were mixed gently by pipetting, centrifuged briefly at 526 g, and incubated at 37 °C in a humidified 5% CO₂ atmosphere for 4 h. Killing was stopped by placing the plate on ice for 5 min. Appropriate wells were subsequently stained with Live/Dead Fixable Violet Dead Cell Stain and fixed in Cytifix fixation buffer (BD Bioscience) according to the manufacturer's directions. Effectors only, unstained target, and Live/Dead Fixable Violet Dead Cell (Invitrogen; Waltham, MA)-stained targets were also included as assay controls. Stained samples were analyzed on LSR II flow cytometer (BD Biosciences). The results were expressed as percentage of dead targets on a cell-to-cell basis.

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. 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).

Spleen IgM response to SRBC

For study B, the primary IgM response to SRBC was enumerated using a modified hemolytic plaque assay (Anderson et al. 2020; Jerne and Nordin 1963; Weatherly et al. 2022). 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 diesel exhaust or air, rats were euthanized by CO₂ 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.

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