Methods:

Animals and Diet

Male Sprague-Dawley (SD) and Brown Norway (BN) rats were selected as the strains of interest for this study due to the strains' propensities to exhibit unique immune, metabolic, and lung responses (28, 29). SD rats are an outbred strain commonly used in obesity models and pulmonary toxicity studies. Comparatively, BN rats are an inbred strain predisposed to developing Type 2 immune responses, IgE-mediated allergic reactivity, and autoimmunity. Accordingly, specific pathogen-free male SD (Hla: SD CVF; Hilltop Lab Animals, Scottdale, Pennsylvania) and BN (BN/RijHsd; Harlan Laboratories, Inc, Indianapolis, Indiana) rats (120 of each strain) were obtained at 5 weeks of age. All rats were co-housed in ventilated polycarbonate cages with HEPA-filtered air and maintained in a controlled humidity/temperature environment with a 12h light/dark cycle in the AAALAC-approved National Institute for Occupational Safety and Health (NIOSH) Animal Facility.

Upon arrival, rats were provided tap water and irradiated Teklad 2918 regular diet (Reg; 18.6% protein, 44.2% carbohydrate, 6.2% fat; Envigo Teklad Diets, Madison, Wisconsin) ad libitum. After one week of acclimation, a set of animals from each strain (n = 60 rats/strain) was continued on the REG Teklad 2918 diet or transitioned onto a Teklad Custom 45% Fat Kcal high-fat diet (HF; 14.8% protein, 40.6% carbohydrate, 44.6% fat; Envigo Teklad). The 45% Fat Kcal diet was designed with similarities to the western diet, and included the addition of 21% anhydrous milk fat, 34% sucrose, and 2% soybean for essential fatty acid supplementation. Animals were maintained on the REG or HF diets until humanely euthanized with an intraperitoneal injection of sodium pentobarbital (> 100 mg/kg body weight; Fatal-Plus Solution, Vortech Pharmaceutical, Inc, Dearborn, Michigan) and subsequent exsanguination via the abdominal aorta at the selected time points. All procedures in the studies comply with the ethical standards set forth by Animal Welfare Act and the Office of Laboratory Animal Welfare (OLAW). The studies were approved by the NIOSH Health Effects Laboratory Division (HELD) Institutional Animal Care and Use Committee within the Center for Disease Control and Prevention in accordance with an approved animal protocol (protocol number 18-004).

Experimental Design and Welding Fume Exposure

Beginning at 6 weeks of age, SD and BN rats were maintained on HF or Reg diets for 7 weeks (figure 1). After the 7 weeks of diet maintenance, a set of rats from each strain was euthanized for collection of baseline parameters prior to WF exposure (7 wk time point). At the same time point, the remaining groups of rats were exposed by inhalation to stainless steel WF (target concentration of 20 mg/m3 × 3 h/day × 4 days/week × 5 weeks) or filtered air (control) until week 12, at which time, half of the remaining animals from each strain was euthanized (12 wk time point). Finally, the last set of SD and BN rats was allowed to recover from welding fume exposure for 12 weeks, and then euthanized (24 wk time point). Following euthanasia, whole blood and serum were collected from each rat, bronchoalveolar lavage (BAL) was performed, and lymphoid tissues were collected for subsequent evaluation of different immune markers. Body weight was also recorded throughout the 24-week regimen. The 7, 12, and 24 wk time points were selected in order to characterize immune effects directly following WF exposure (12 wk) and after a recovery period (24 wk)—exposure scenarios designed to mimic worker responses during a period of active exposure, as well as after a latency period.

The design and construction of the welding fume aerosol generator and the characterization of the fume were previously described (30, 31). Briefly, welding fume composition was determined by inductively coupled plasma-atomic emission spectroscopy according to NIOSH method 7300 and was composed of the following metals (weight %): Fe (53%), Cr (17%), Mn (24%), Ni (6%), and Cu (0.4%) (32). Particle size distribution was determined in the exposure chamber within the breathing zone of the rats by using a Micro-Orifice Uniform Deposit Impactor (MOUDI, MSP Model 110, MSP Corporation, Shoreview, Minnesota) for general purpose aerosol sampling, and a Nano-MOUDI (MSP Model 115) that is specifically designed for sampling aerosols in the size range down to 0.010 µm. The mass median aerodynamic diameter was determined from a series of randomly-collected samples, measuring 0.26 µm with a geometric standard deviation of 1.4. The actual animal chamber concentrations (mean ± standard deviation) achieved during the exposures were 20.3 mg/m^3 ± 6.4 for the SD strain and 19.4 mg/m^3 ± 7.1 for the BN strain.

Phenotypic Differentiation of Circulating Immune Cell Populations

Blood was drawn from the abdominal aorta directly following euthanasia by sodium pentobarbital overdose. A 500 µL aliquot of whole blood was separated from the total volume and collected into EDTA-coated vacutainers to prevent clotting and agitated continuously until analysis. Total leukocyte number was determined using an IDEXX Procyte Dx Hematology Analyzer (IDEXX Laboratories; Westbrook, ME). White blood cell populations were also

differentiated to determine absolute number and percentage of circulating monocytes, neutrophils, eosinophils, lymphocytes, and basophils.

Bronchoalveolar Lavage

BAL was performed on rats to assess lung inflammation and retrieve immune cells present in the airway lumen. The lungs were lavaged with calcium- and magnesium-free PBS (pH 7.4) to yield 30ml of fluid, which was then centrifuged at 500 × g for 10 min. Cell pellets from each BAL sample were washed and resuspended in 1 ml of PBS buffer. Cell suspensions were diluted into isotonic buffer and used for enumeration of total BAL cell burden, as well as size-specific population differentiation using a Coulter Multisizer II and AccuComp software (Coulter Electronics, Hialeah, Florida). Two separate aliquots, each containing 5.0 x 10^5 BAL cells, was retained for each sample for subsequent analysis by flow cytometry.

Collection and Processing of Spleens and Lymph Nodes

After the lungs of each animal were lavaged, the lung-associated lymph nodes were harvested and collected into sterile PBS for phenotypic analysis. The spleen was also collected from each rat and sectioned into two halves—one half was placed directly into fixative solution for future histopathological analysis and the other half was retained for flow cytometric analysis. Both lymphoid tissues were processed between frosted microscope slides to yield single cell suspensions in sterile PBS. Cell concentrations were then evaluated in the lymph node samples using a Coulter Multisizer II (Coulter electronics; Hialeah, FL) to calculate total cell number for each animal.

Differentiation of Immune Cells in the Lymph Node, Spleen, and BAL by Flow Cytometry

For phenotypic differentiation of immune cell subsets in the BAL, spleen, and lymph nodes, 5.0 x 10^5 cells form each sample were plated and suspended in FACS buffer (PBS + 1% bovine serum albumin + 0.1% sodium azide) containing F_c receptor-blocking anti-rat CD32 (BD Biosciences). Cells were incubated for 5 minutes at 4ºC, washed, and resuspended in a staining solution containing a cocktail of fluorophore-conjugated antibodies. Two different staining panels were designed in order to differentiate immune cell subsets of lymphoid and myeloid origin. For each panel, a set of compensation controls was prepared using the respective cell type stained with a single fluorophore.

All three sample types were stained with the lymphocyte-differentiating panel. Lymph node, spleen, and BAL cells were stained with CD3-APC, CD4-BV421, CD8-BV605, CD44-FITC, CD45-PE-Cy7, CD45R (B220)-APC-Cy7, CD86-PE, and CD161-BV605 (BD Biosciences) to allow for identification of T-cells (CD45+/CD3+), CD4+ T-cells (CD45+/CD4+/CD8-), CD8+ T-cells (CD45+/CD4-/CD8+), B-cells (CD45+/CD45R+), and NK cells (CD45+/CD161+). Additionally, T-cell activation status was able to be evaluated in accordance with CD44 expression and B-cell activation was determined by CD86 expression levels. The gating strategy used to analyze cells in this staining panel in shown in figure S1.

The second aliquot of BAL cells was stained with the panel of antibodies to differentiate immune cells of myeloid origin. The panel contained CD11b-APC, CD43-AF647, CD45-PE-Cy7, CD68-PE, His48-FITC, and MHCII-PerCP (BD Biosciences), which allowed for differentiation of macrophages (CD45+/CD11b+/CD68+, high autofluorescence), neutrophils (CD45+/CD43+ high granularity), monocytes (CD45+/CD11b+/CD43+/His48+), and lymphocytes (CD45+/CD11b-/CD43-/His48-). In addition, MHC II expression level was used to determine macrophage activation status.

Cells were incubated in the corresponding staining cocktails for 30 minutes at 4ºC, washed, and resuspended in 100 µL Cytofix Buffer (BD Biosciences). After 20 minutes of incubation, the samples were washed and resuspended in FACS buffer, covered in foil, and stored at 4ºC until analysis. For each sample, 100,000 events were recorded on an LSR II (BD Biosciences; San Diego, CA). In all analyses, doublet exclusion was performed (SSC-A vs SSC-H) and cell populations were gated using the FSC-A x SSC-A parameters for subsequent analysis. All data analyses were performed using FlowJo 7.6.5 Software (Treestar Inc.; Ashland, OR).