Materials and Methods

Animals

Male A/J mice (age 4–6 week) were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in an AAALAC International - specific pathogen-free, environmentally-controlled facility. All mice were free of endogenous pathogens including viruses, bacteria, mycoplasmas, and parasites. Mice were housed in groups of two in ventilated cages and provided high-efficiency particulate filtered air under a controlled light cycle (12 h light/12 h dark) at a standard temperature (22-24^{o}C) and 30-70% relative humidity. Animals were acclimated to the animal facility for at least one week before beginning the experimental protocol and allowed access to a conventional diet (6% irradiated NIH-31 Diet, Envigo RMS, Inc.; Madison, WI) and tap water *ad libitum.* All procedures were performed using protocols approved by the National Institute for Occupational Safety and Health (NIOSH) Institutional Animal Care and Use Committee.

Welding fume inhalation exposure system

The design and construction of the welding fume aerosol generator were previously described (Antonini et al., 2006). This automated robotic welder continuously generated welding fumes by welding beads onto ¼ inch thick plates of mild steel. The welding wire used was 0.045 inch diameter Lincoln Electric Techalloy 413 MIG (Lincoln Electric; Cleveland, OH) and the welding parameters were set to 25 volts DC, 300 inch per minute wire feed, 30 L/min of 75% argon – 25% helium shielding gas, and a typical welding current of 200 amps. The resulting fume was carried into a whole-body exposure chamber through a ¾ inch flexible tube by maintaining the chamber at a negative pressure (0.70 inch H_2O). Particle concentrations within the exposure chamber were continuously monitored with a Data RAM (DR-40000 Thermo Electron Co; Franklin, MA), and gravimetric determinations (37 mm cassettes with 0.45 µm pore-size Teflon filters) were used to calibrate and verify the Data RAM readings each day. Gas generation, including carbon monoxide (CO), carbon dioxide (CO_2), oxygen (O_2), and ozone (O_3), was continuously monitored. During the welding exposure, O_2 levels were maintained above the OSHA minimal acceptable level. O_3, CO, CO_2 were below OSHA permissible exposure limits and NIOSH recommended exposure limits (REL) during the entire exposure duration. In the exposure chamber, CO and O_3 levels were not significantly higher than background. The exposure system was modified slightly from that described previously to reduce the travel time of the particulate fume from the welding torch to the exposure chamber.

Welding fume characterization

For elemental analysis of Cu-Ni fume, generated particles were collected inside the exposure chamber onto 5.0 µm polyvinyl chloride membrane filters in 37-mm cassettes during three 30 minute collection periods. The particle samples were digested and the metals determined by Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) according to the NIOSH method 7303 for hot block/HCL/HNO_3 digestion (NIOSH, 1994).[15] The welding fume was analyzed for aluminum (Al), barium (Ba), calcium (Ca), Cr, cobalt (Co), copper (Cu), Fe, potassium (K), lithium (Li), Mn, magnesium (Mg), Ni, phosphorus (P), lead (Pb), strontium (Sr), titanium (Ti), vanadium (V), zinc (Zn), and zirconium (Zr). Metal content of blank filters was also analyzed for control purposes.

A small amount of welding fume was collected gravimetrically onto 47-mm Nucleopore polycarbonate filters (Whatman; Clinton, PA) for field emission scanning electron microscopy (FESEM) to assess particle size and morphology. The particles were imaged using a Hitachi S4800 Field Emission Scanning Electron Microscope (Hitachi; Tokyo, Japan). To determine particle mass size distribution, a Micro-Orifice Uniform Deposit Impactor (MOUDI, model 110; MSP corp., Shoreview, MN) with additional Nano-MOUDI stages (MSP model 115) was used. Elemental profiles of collected welding fume samples were determined using energy dispersive X-ray analysis (SEM-EDS; Princeton Gamma-Tech, Rocky Hill, NJ) at 20 keV to map specific metal components.

Whole lung metal analysis

Weight-matched A/J mice were exposed by whole-body inhalation in individual steel mesh cages to Cu-Ni welding aerosols (mean concentration 43 mg/m^3) for 4 hours (n = 10) or filtered air (n = 6). Immediately following exposure (time zero), mice were euthanized with sodium pentobarbital [100-300 mg/kg IP] (Vortech Pharmaceuticals; Dearborn, MI) followed by exsanguination via the vena cava. Whole lungs were excised, trimmed, and lyophilized. The freeze-dried tissue was weighed then acid digested. ICP-AES was used to determine the amount of Al, Ba, Ca, Co, Cr, Cu, Fe, K, Li, Mg, Mn, Ni, P, Pb, Sr, Ti, V, Zn and Zr present in the lung according to the draft NIOSH method 8200 used for bulk tissue samples (NIOSH 2003).

Experimental protocol for assessment of lung toxicity

Mice were exposed by whole-body inhalation to Cu-Ni welding fume aerosols or filtered air for 2 [low deposition (LD)] or 4 [high deposition (HD)] hours/day for 10 days at a target concentration of 40 mg/m^3 (actual mean concentrations were 23 and 27.3 mg/m^3; respectively). Before the start of the exposure mice were weight-matched then weighed biweekly and again at the terminal sacrifice of 1,7,28, and 84 days after the 10 day exposure. Mice were euthanized as described above. Body weights were determined by placing the conscious animal into a weight bucket on a calibrated digital scale. Mice were euthanized as described above.into a weight container on a calibrated digital scale.

For whole lung bronchoalveolar lavage, a blunted cannula was placed in the trachea through a small incision and the thorax was massaged as 0.6 mL of cold Ca^{2+} and Mg^{2+} -free phosphate buffered saline (PBS) was instilled into the lungs. After 10 seconds, the BAL fluid was withdrawn and placed in a 15 ml conical tube. This consisted of the first lavage fraction. This process was then repeated 3 times using 1 ml of PBS per instillate and this second fraction was collected in a separate 15 ml conical tube. The BAL fluid was kept on ice and then centrifuged (500 x g, 10 minutes, 4°C).

Lung toxicity parameters

The first fraction acellular BAL supernatant was used to measure LDH activity, indicative of lung cytotoxicity. LDH activity was analyzed using a COBAS MIRA Plus auto-analyzer (Roche Diagnostic Systems; Montclair, NJ) which measured the oxidation of lactate to pyruvate coupled with the formation of NADH at 340 nm.

For analysis of the BAL cells, the supernatant from the second lavage fraction was discarded and the cell pellets of both fractions were combined. The final cell pellet suspended in 800 µl of PBS was used for cell counts and differential staining. Total cell numbers were determined using a hemocytometer. For cell differentials, cells were plated onto glass slides using a Cytospin 3 centrifuge (Shandon Life Sciences

International; Cheshire, England) set at 800 rpm for 5 minutes. Slides were stained with Hema 3 Fixative and Solutions (Fisher Scientific; Pittsburgh, PA) then coverslipped. A minimum of 300 cells/slide, consisting of macrophages, lymphocytes, and polymorphonuclear leukocytes were identified using light microscopy.

Macrophage functional assay

The macrophage functional assay was done to examine the effect of the LD Cu-Ni fume exposure on innate immune function. Lung macrophages harvested by BAL at 1, 7, and 28 days post-exposure were challenged with *Escherichia coli* (*E. coli*) GFP for 2 hours at 1:25 MOI (multiplicity of infection) as previously described (Kodali et al., 2017). The uptake of *E.coli* by macrophages was quantified by flow cytometry.