

Methods

1.1. Characterization and preparation of MWCNTs and C60F

The MWCNTs used in this study were obtained from Mitsui & Company (Mitsui-7, XNRI 1, lot # - 0507 2001K28, Tokyo, Japan). C60F was purchased from Sigma Aldrich (St. Louis, MO, USA). Characterization of MWCNTs and C60F was performed using transmission electron microscopy (TEM). A sample of MWCNTs and C60F were suspended in isopropanol, sonicated, and dispersed onto a TEM grid with a carbon film. For MWCNTs, length measurements were taken from the longest straight distance between two points. The width measurement was the distance perpendicular to the structural walls of the CNTs. To determine C60F diameter, two perpendicular measurements were collected on each particle. Morphology of C60F was further examined using field emission scanning electron microscopy (FESEM).

A dispersion medium (DM; 0.9% saline supplemented with 5.5 mM D-glucose, 0.6 mg/ml mouse serum albumin, and 0.01 mg/ml 1,2-dipalmitoyl-sn-glycero-3-phosphocholine) was modified from one previously developed and validated by our laboratory as a vehicle for nanotoxicology studies, and was used to prepare suspensions of MWCNTs and C60F following a two-step dispersion procedure.

1.2. Animals and treatment

Six-week old male B6C3F1 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Mice were maintained in an accredited, specific pathogen-free and environmentally controlled facility at the National Institute for Occupational Safety and Health. All animals received humane care and all experiments involving animals were approved by the Institutional Animal Care and Use Committee. 7 to 10 mice per treatment at each timepoint were treated with a single dose of 50 μ l of DM, MWCNTs (40 μ g/mouse), or C60F (640 or 1,280 μ g/mouse) in suspensions by pharyngeal aspiration as described elsewhere. A saline control was used to establish baseline levels of the measurements. At day 1, 7, or 28 post-exposure, the mice were euthanized with an intraperitoneal injection of a sodium pentobarbital euthanasia solution (100-300 mg/kg body weight; Zoetis, Florham Park, NJ, USA), followed by exsanguination once the mouse was unresponsive to a toe pinch, for molecular, immunologic, and pathological examinations.

Given the apparent paucity of data concerning human exposure to ENMs, the MWCNT and C60F doses chosen for the mouse study were estimated to approximate human occupational exposure. A recent study reported peak MWCNT-containing airborne dust levels of approximately 10.6 μ g MWCNTs/m³ among workers exposed to MWCNTs in eight U.S. manufacturing facilities. Airborne particle concentrations of carbonaceous nanomaterials, including fullerenes, was measured in a commercial nanotechnology facility. Fine particle mass concentrations were measured in three different areas, ranged from 50 to 125 μ g/m³. Assuming a worker performs light work in an environment with MWCNT aerosol of 10.6 μ g/m³ or C60F aerosol of 50 μ g/m³, and has a minute ventilation of 20 L/min with a deposition fraction of 30% and an alveolar epithelium surface area of 102 m², the estimated human exposure per year would be 7.3 mg/m² alveolar epithelium for MWCNTs and 34.6 mg/m² alveolar epithelium for C60F. Therefore, a 40 μ g MWCNT exposure in mouse approximates human deposition for a person performing light work for 11 years and a 1,280 μ g C60F exposure in mouse for 9 months.

1.3. Macrophage culture, polarization, and treatment

The J774A.1 murine monocyte/macrophage cell line was purchased from American Type Culture Collection (TIB-67, ATCC, Manassas, VA, USA). The cells were grown in the Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) (both from Thermo Fisher Scientific, Waltham, MA, USA). For differential polarization of M1 and M2 macrophages, cells at a density of 5×10^5 cells/ml were seeded in DMEM with 3% FBS for 1 day. M1 polarization was then induced by incubation with interferon γ (IFN- γ , Sigma Aldrich) at 20 ng/ml plus lipopolysaccharide (LPS, Sigma Aldrich) at 100 ng/ml for indicated time (typically three days). M2 polarization was induced by incubation of the cells with interleukin (IL) 4 (Sigma Aldrich) at 20 ng/ml for indicated time (typically three days). Polarized M1 or M2-macrophages were treated with DM or MWCNTs at 2.5 $\mu\text{g/ml}$ for indicated time (i.e., 1, 2, or 3 days post-polarization), before the cells were examined for protein expression and production of LMs.

1.4. Whole lung lavage (WLL) preparation

After 1, 7, or 28 days post-exposure, mice were euthanized by intraperitoneal injection of a sodium pentobarbital euthanasia solution, followed by exsanguination, as described under Animals and treatment. A tracheal cannula was inserted and WLL was performed through the cannula using ice cold Ca^{2+} - and Mg^{2+} -free phosphate buffered saline (PBS), pH 7.4. The first lavage (0.6 ml) was kept separated from the rest of the lavage fluid. Subsequent lavages, each with 1 ml of PBS, were performed until a total of 3 ml of lavage fluid was collected. WLL cells were isolated by centrifugation (650 \times g, 5 min, 4°C). An aliquot of the acellular supernatant from the first WLL was decanted, transferred to tubes, and stored at -80°C for later analyses. The acellular supernatants from the remaining lavage samples were decanted and discarded. WLL cells isolated from the first and subsequent lavages from the same mouse were pooled after resuspension in PBS, centrifuged a second time (650 \times g, 5 min, 4°C), and the supernatant decanted and discarded. The WLL cell pellet was then resuspended in PBS and placed on ice. Total WLL cell counts were obtained using a Coulter Multisizer 3 (Coulter Electronics, Hialeah, FL, USA) and cell differentials were determined by flow cytometry.

1.5. Enhanced darkfield microscopy

ENMs, such as CNTs, have dimensions less than the wavelength of light and closely packed atoms and, hence, a refractive index that is significantly different from those of the environment, such as biologic tissues and the mounting medium. These characteristics of ENMs produce significantly greater scattering of light than by the surrounding tissues. The enhanced-darkfield optical system images light scattered in tissue sections and ENMs in tissues would stand out from the surrounding tissues with a high contrast.

To visualize ENMs and their deposition in lung tissue, formalin-fixed lung sections were examined under an enhanced darkfield microscope as described previously. Using this method of imaging, it is practical to scan whole lung sections at a relatively low magnification (40-60 \times objectives) to identify ENMs that would not be detected by other means. The optical system consists of high signal-to-noise, darkfield-based illumination optics adapted to an Olympus BX-41 microscope (CytoViva, Auburn, AL, USA). Sections for dark-field examination were specifically cut from paraffin blocks and collected on ultrasonically cleaned, laser cut slides (Schott North America Inc, Elmsford, NY, USA) to avoid nanoparticle contamination from the ground edges of traditional glass slides. After staining with Picrosirius red-hematoxylin, the sections were cover-slipped with Permount (Vector Laboratories, Inc., Burlingame, CA, USA). After alignment of the substage oil immersion optics with a 10 \times objective, sections were examined with 60 \times air or 100 \times oil immersion objectives. Enhanced darkfield images were taken at 2400 \times 4800 pixels with an Olympus DP-73 digital camera (Olympus America Inc., Center Valley, PA, USA).

1.6. Histopathology

For histopathological examination, 3 mice per treatment group, separate from those used for WLL studies, were euthanized by intraperitoneal injection of sodium pentobarbital euthanasia solution, followed by exsanguination, as described under Animals and Treatment. The lung was removed, fixed with 10 % neutral buffered formalin by intratracheal perfusion, and embedded in paraffin. Sections of 5 μ m thickness were subjected to hematoxylin and eosin (H&E) staining or Picrosirius red staining following standard procedures.

1.7. Immune cell profiling by flow cytometry

Cells were recovered from WLL fluids by centrifugation (400xg, 5 min at 4°C). Cells were resuspended in 1x PBS with normal rat sera (Sigma Aldrich) and stained using rat anti-mouse CD16/32 (clone 2.4G2, BD Biosciences, San Jose, CA, USA) for 5 min at room temperature. Cells were then stained with the following fluorescent antibody cocktail in a fluorescence-activated cell sorting (FACS) buffer (1x PBS, 5% BSA, 2 mM EDTA) for 25 min at 4°C, : anti-CD103 (clone M290), anti-CD19 (clone 1D3), anti-Siglec F (clone E50-2440) (BD Biosciences), and anti-CD11b (clone M1/70), anti-CD11c (clone N418), anti-CD3 (clone 17A2), anti-CD49b (clone DX5), anti-Ly6C (HK1.4), anti-Ly6G (1A8) (Biolegend, San Diego, CA, USA). After washing with 2 ml of the FACS buffer, cells were centrifuged (400xg, 5 min at 4°C). Cell were fixed in the BD Cytofix Fixation Buffer (BD Biosciences) for 10 min at room temperature, then washed with 2 ml of FACS buffer and centrifuged. Cells were resuspended in 250 μ l of FACS buffer and more than 10,000 events were acquired using LSR II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (FlowJo LLC, Ashland, Oregon, USA). Live cells were discriminated from dead cells, doublets, and debris first using forward scatter (FSC)-A vs side scatter (SSC)-A, then gating upon singlet populations in FSC-H vs FSC-W view then SSC-H vs SSC-W view. Innate immune cells and lymphocytes were subsequently identified based on the cell surface markers indicated on the X and Y axes of the flow cytometry pictographs. Cell populations were gated sequentially on populations negative for the previous populations as follows: alveolar macrophages, eosinophils, neutrophils, lymphocytes, dendritic cells (DCs), and monocytes.

1.8. Cytokine measurement

Measurement of cytokines in WLL fluids and lung tissue extract was performed by multiplex immunoassay using ProcartaPlex mouse cytokine/chemokine 36-plex kit (Thermo Fisher Scientific) on a Luminex 200 instrument system equipped with xPONENT software (Thermo Fisher Scientific) following the manufacturer's instructions. 25 μ L of WLL fluids or lung tissue homogenates in 1x PBS containing 1x protease inhibitor cocktail (Thermo Fisher) was analyzed to determine the concentration of each cytokine. For lung tissue, concentrations of cytokines were calculated and expressed as pg/mg protein.

1.9. Enzyme-linked immunosorbent assay (ELISA)

Proinflammatory cytokines (LTB₄, PGE₂) or SPMs (RvD1, LXA₄, RvE1) were detected in WLL fluids collected from mouse lungs exposed to saline, DM, MWCNTs, or C60F for the indicated dose and days using ELISA. All ELISA kits were purchased from MyBioSource (San Diego, CA, USA) and measurement was performed by following the manufacturer's protocol using a microplate reader equipped with SOFTmax PRO 4.0 (Molecular Devices, Sunnyvale, CA). To detect and quantify SPMs released from polarized M1 or M2-macrophages *in vitro*, cell-free media from cultures of M1 or M2 macrophages were collected and used for quantification of LTB₄, PGE₂, and RvD1 using ELISA.

1.10. Immunofluorescent staining and confocal microscopy

Formalin-fixed, paraffin-embedded lung tissue sections (left lung lobe, 5 μm) were deparaffinized, antigen-unmasked by heating tissue sections in universal antigen retrieval reagent (Cell Signaling Technology, Danvers, MA), and used to perform immunofluorescent staining. The primary antibodies used were rabbit anti-F4/80 (1:100, Thermo Fisher Scientific) or mouse anti-F4/80 (1:100, Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-CD68 (1:100, Novus Biologicals, Centennial, CA), mouse anti-CD206 (1:100, Abcam, Cambridge, MA, USA), rabbit anti-ALOX5AP (1:100, Abcam), and mouse anti-ALOX15 (1:200, Abcam) antibodies. Images were taken in three to five randomly selected fields per lung slice, three lung slices per mouse, using a Zeiss LSM 780 confocal microscope with a 63x magnification lens (Carl Zeiss Microscopy, Jena, Germany). More than 500 cells from captured microscopic images per each treatment were counted using the ImageJ software (NIH, Bethesda, USA) and the number of cells with double positive staining per every 100 cells were presented as mean \pm SEM (n=3).

1.11. Immunoblotting

J774A.1 cells (a mouse macrophage cell line) were treated as indicated and the cells were lysed in a lysis buffer (10 mM Tris, pH 7.4, 1% SDS) with 1x proteinase inhibitor cocktail (Thermo Fisher). Cell lysates were collected and sonicated for 10 seconds. The supernatant was collected and lysate proteins (20 μg each sample) were analyzed on 8, 10, or 12% SDS-PAGE gel and transferred onto nitrocellulose membrane. The membrane was incubated with 5% nonfat dry milk in tris-buffered saline with 0.05% Tween 20 for 1 hour at room temperature to block non-specific binding, before incubation with primary antibodies. The primary antibodies used were mouse anti-CD68 (1:200, Novus Biologicals), mouse anti-CD86 (1:200, Novus Biologicals), rabbit anti-CD163 (1:250, Abcam), mouse anti-CD206 (1:250, Abcam), rabbit anti-ALOX5 (1:1,000, Abcam), mouse anti-ALOX15 (1:500, Abcam), rabbit anti-ALOX5AP (1:500, Abcam), or mouse anti- β -actin (1:4,000, Sigma Aldrich) antibodies. After incubation with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (both from Jackson ImmunoResearch laboratories, West Grove, PA; 1:5,000), immunoreactive bands were developed with Enhanced chemiluminescence (Thermo Fisher Scientific) on X-ray films. Film images were scanned using HP scanjet (Hewlett-Packard, Palo Alto, CA, USA) and were used to quantify each band with ImageJ software (NIH) with normalization to β -actin level.

1.12. Statistical analysis

For statistical analysis of multiple samples, such as days post-exposure, one-way analysis of variance (ANOVA) was performed, followed by Tukey's test for comparison between two samples, using the GraphPad Prism 8 software (GraphPad, San Diego, CA, USA). Statistical analyses of differences between two groups were also performed using two-tailed Student's *t* test. Data were presented as means \pm SEM. A *p* value of less than 0.05 was considered statistically significant as indicated for individual experiment. *, *p* < 0.05; **, *p* < 0.01; and ***, *p* < 0.001.