Methods

1.1. Characterization of nanoparticles

The MWCNTs used in this study were obtained from Mitsui & Company (Mitsui-7, XNRI 1, lot #-0507 2001K28, Tokyo, Japan). Some properties of the MWCNTs have been characterized previously. The MWCNTs are fiber-like in shape and are rigid with a distinctive multi-walled structure. Their length distribution is log normal with a mean of 4.46 mm (95% confidence interval of 4.08-4.88 mm) and the width distribution is normally distributed with a mean of 58.5 nm (95% confidence interval of 56.0-61.0 nm). The average surface area is 26 m^2/g as measured by nitrogen absorption-desorption. Trace elements were low with 0.78% for all metals and 0.32% for iron. The level of lipopolysaccharides (LPS) in the MWCNTs was determined to be <0.1 EU/ml (<0.01 mg/ml) using the Pierce LAL chromogenic endotoxin quantification kit (Thermo Scientific, Pittsburgh, PA).

Carbon black (CB) are amorphous, carbonaceous particulates and therefore is used as a non-fiber, carbon-based particle control for MWCNTs. The CB used was purchased commercially (Printex 90, Degussa Engineered Carbons, L.P., Parsippany, NJ, USA). The average size of CB particles is 15 nm in diameter.

For cell treatment, MWCNTs and CB were dispersed in the Dulbecco's Modified Eagle Medium (DMEM) with 1% fetal bovine serum (FBS) (both from Thermo Fisher Scientific, Waltham, MA, USA) at a concentration of 2 mg/ml by vortex and sonication as described previously. Stock solutions were further diluted with the culture media and sonicated immediately before use.

As comparison, MWCNTs and CB and their preparations in media were characterized in parallel. The morphology of the MWCNTs and CB was compared using images obtained from transmission electron microscopy shown in Supplemental Figure S1, which revealed consistent shapes and average diameters for MWCNTs and CB as described above. Zeta potentials of the particles in water and in the medium were measured using the Malvern spectrometer Zetasizer Nano Z (Malvern Panalytical Inc., Westborough, MA, USA) and were summarized in Supplemental Table 1. The MWCNTs have zeta potentials of -15.34 ± 0.29 mV in water and -12.03 ± 1.24 mV in the medium, respectively, whereas the CB particles have -10.58 ± 0.59 mV in water and -9.88 ± 1.62 mV in the medium, respectively. CB has slightly lower zeta potentials, mobility, and conductivity in both the medium and aqueous solution than MWCNTs. Their negative values may reflect the presence of carboxylic groups on the surface of MWCNTs and CB. The relatively small zeta potentials may be caused by formation of corona in the samples in the aqueous phase.

To ascertain the stability of MWCNTs and CB in culture media, the samples were incubated with the culture medium for 1-day or 3-day, and their particle numbers were analyzed using NanoSight NS300 (Malvern Panalytical Inc). Data was summarized in Supplemental Table 2. After incubation with the culture medium, the particle number of MWCNTs decreased only slightly, by 1.1% at 1 day ($7.90x10^9$/ml) and by 1.5% at 3 day ($7.87x10^9$), compared to preparations not incubated with the medium ($7.99x10^9$). The particle number of carbon black also showed only slight decrease upon incubation in the culture medium, showing $1.02x10^{10}$/ml at 1 day and $1.01x10^{10}$ at 3 day, compared to unincubated ($1.15x10^{10}$).

1.2. Cell culture, polarization, and treatment

The J774A.1 murine macrophage cell line was purchased from American Type Culture Collection (TIB-67, ATCC, Manassas, VA, USA). Upon culture, the cells adhere to plates like tissue macrophages and have been shown to polarize into M1 or M2 cells in response to

classical M1 and M2 inducers, respectively. Polarized J774A.1 cells respond to MWCNTs with increased M1 or M2 responses similarly to polarized human peripheral blood monocyte-derived macrophages in response to bacterial pathogens.

The cells were grown in DMEM with 10% FBS. For differential polarization of M1 and M2, cells at a density of 5 x 10^5 cells/ml were seeded in DMEM with 3% FBS for 1 day. Polarization of macrophages M1 cells was then induced by incubation with IFN-γ (Sigma Aldrich, St. Louis, MO, USA) at 20 ng/ml plus LPS (Sigma Aldrich) at 100 ng/ml for 1 day or 3 days. M2 polarization was induced by incubation of the cells with IL-4 (Sigma Aldrich) at 20 ng/ml for indicated time (typically three days). Characterization of M1 and M2 phenotypes of J774A.1 cells by the classical inducers has been reported previously. MWCNTs at 2.5 or 10 µg/ml or carbon black at 2.5, 10, or 30 µg/ml were tested for the indicated time. Control media (DMEM plus 1% FBS) were prepared and used to establish a negative control response and to validate that there was no effect on the cells from the dispersion medium. Some cells were kept as untreated. All cells were treated as duplicates and all treatment experiments were conducted 3 time. After treatment with a single dose of MWCNTs, the cells were incubated for 1 day or 3 days in the same media till harvest for analysis. The cell culture medium was collected and were centrifuged at 4^{o}C to remove particles, cells, and debris. The cell-free medium was used for detection of cytokines and LMs by ELISA and the chemotaxis assay. Potential cytotoxicity of MWCNTs and CB on cultured cells was assessed using the Cell Counting Kit-8 that uses highly water-soluble tetrazolium salt, WST-8 for improved sensitivity and efficiency (Dojindo, Rockville, MD). Under the experimental condition, the MWCNTs and CB did not show significant cell toxicity within their concentration range (≤10 mg/mL) and time frame (≤3 days) used (Supplemental Figure S2).

HL-60 (CCL-240, ATCC) is a neutrophil-like cell line and is used to examine directed cell migration owing to its robust response to chemotactic signals. The cells were grown in the RPMI 1640 medium with 10% FBS at 37 °C and 5% CO_2. To differentiate HL-60 cells into neutrophil-like cells, the cells at a density of 2 to 3 × 10^5 cells/mL were incubated with 2 µM all-trans retinoic acid (ATRA, Sigma Aldrich) for 3 days. A cell suspension containing 2.5 x 10^6 cells/ml in serum free media was used for cell migration assay.

1.3. Quantitative real-time PCR (RT-qPCR)

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germantown, MD, USA) per the manufacturer's protocol. For reverse transcription, 1 µg of total RNA was reversely transcribed using a high-capacity RNA-to-cDNA kit (Thermo Fisher Scientific) at 37°C for 1 hour. Real-time qPCR was performed for 35 cycles with SYBR Green 1 PCR Master Mix on a 7500 real-time PCR machine (Thermo Fisher Scientific) using specific primers against mouse Alox5 (Qiagen, PPM28755C) or β-actin (Qiagen, PPM02945B). Reactions were run in triplicate for each sample and a dissociation curve was generated. Threshold cycles (Ct) for Alox5 or Alox5ap amplification were normalized to the housekeeping gene β-actin (ΔCt) and every experimental sample was referred to its control (ΔΔCt). Relative expression change values were calculated as $2^{-\Delta\Delta Ct}$ and expressed as fold changes in comparison with untreated control.

1.4. Immunoblotting

J774A.1 cells were treated as indicated and were lysed in a lysis buffer (10 mM Tris, pH 7.4, 1% SDS) with 1x proteinase inhibitor cocktail (Thermo Fisher Scientific) at the end of the experiment. Cell lysates were collected and sonicated for 10 seconds. The supernatant was collected, and the protein concentration was determined using a Bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Lysate proteins (10-20 µg each sample) were resolved on 8, 10, or 12% SDS-

PAGE gel and transferred onto a 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. Primary antibodies used were rabbit anti-Alox5 (1:1,000, Abcam, ab169755, Waltham, MA, USA), rabbit anti-Alox5ap (1:500, Abcam, ab85227), mouse anti-CD68 (1:200, Novus Biologicals, NB100-683, Centennials, CO, USA), rabbit anti-cyclooxygenase (COX-2, 1:1,500, Abcam, ab179800), rabbit anti-leukotriene A4 hydrolase (LTA4H, 1:2,000, Abcam, ab133512), or mouse anti-β-actin (1:4,000, Sigma Aldrich, A5441) antibodies. After incubation with a second antibody, horseradish peroxidase-conjugated goat anti-mouse (1:5,000, Jackson ImmunoResearch laboratories, 115-035-146, West Grove, PA) or goat anti-rabbit IgG (1:5,000, Jackson ImmunoResearch laboratories, 111-035-144), immunoreactive bands were visualized with Enhanced chemiluminescence substrates (Thermo Fisher Scientific). Band signals were captured on to X-ray film by exposure for 30 seconds and the film was developed using a film processor (Konica Minolta, Wayne, NJ, USA). Scanned images were used to quantify band intensities using the ImageJ software (NIH) and each band was normalized to β-actin.

1.5. Detection of nitric oxide synthase 2 (Nos2, mouse iNOS) expression

To detect the Nos2 protein in macrophages, an intracellular Nos2 detection assay kit (Abcam) was used following the manufacturer's protocol. Briefly, cells treated as described above were washed and stained with a staining dye mix from the kit. After incubation at 37°C for 1 hour, fluorescence signals were measured using a fluorescence plate reader (Thermo Fisher Scientific) at Ex/Em=485/530 nm, which is proportional to the amount of intracellular Nos2.

1.6. Enzyme-linked immunosorbent assay (ELISA)

Proinflammatory cytokines, i.e., tumor necrosis factor-α (TNF-α) and interleukin-1 β (IL-1β), and LMs, i.e., leukotriene B4 (LTB4) and prostaglandin E2 (PGE2), were detected in cell-free culture supernatants collected from cells treated with control media, MWCNTs, IFN-γ+LPS, or IL-4 by ELISA. All ELISA kits were from MyBioSource (San Diego, CA, USA) and measurement was performed following the manufacturer’s protocol.

1.7. Chemotaxis assay

To determine the effect of MWCNTs on neutrophilic cell migration *in vitro,* a transwell cell migration assay was performed using a cell migration assay kit equipped with a 24-well transwell insert (pore size 3 μm; Cell Biolabs, San Diego, CA). Briefly, after extensive washing, ATRA-differentiated HL-60 (dHL-60) cells were collected and suspended in the RPMI 1640 medium without FBS (2.5×10^5 cell/well/100 μl) and plated on each upper chamber. The lower chambers were filled with cell-free culture supernatants, containing certain amounts of MWCNTs prepared in the basal RPMI 1640 medium containing 1% FBS, or RPMI 1640 medium containing 10% FBS. For the inhibition assay, a specific cyclooxygenase 2 (Cox-2, prostaglandin-endoperoxide synthase, PTGS) inhibitor, NS-398 (at 2 or 10 μM), a leukotriene A4 hydrolase (LTA4H) inhibitor, Acebilustat (at 1 or 5 μM) (both from Cayman Chemical, Ann Arbor, MI, USA), or dimethyl sulfoxide (DMSO, Sigma Aldrich) as a vehicle was treated for 6 hours prior to MWCNTs or IFN-γ+LPS exposure. A specific LTB4 receptor inhibitor LY293111 (5 or 25 nM, Cayman Chemical) or DMSO as a vehicle was added directly into the dHL-60 cell suspension to prevent LTB4-mediated chemotactic effect. Following 6 hours of incubation at 37°C, the cells in the upper chamber of the membrane were removed with a cotton swab, and the cells on the underside were collected and lysed. Each cell lysate was incubated with CyQuant GR dye for 20 min at

room temperature and the fluorescence was detected using a fluorescence plate reader (Thermo Fisher Scientific) at Ex/Em of 480/520 nm and presented as relative fluorescence units (RFU).

1.8. Alox5 gene silencing

After one day plating, J774A.1 cells were transduced with mouse Alox5-specific short hairpin (shRNA) or scrambled control shRNA lentiviral particles (5×10^3 viral particles/µl) (Santa Cruz Biotechnology, Dallas, TX, USA) at a multiplicity of infection (m.o.i.) of 4 to ensure efficient infection. The cells were incubated overnight as recommended by the manufacturer. Untreated cells were included as control. After a media change with complete growth media, cells were incubated for an additional day, and then a portion of the cells were lysed for isolation of total RNA (for RT-qPCR), and protein (for immunoblotting). The remaining cells were treated with MWCNTs and subsequently lysed to obtain RNA or protein as described above. The cell-free culture media was collected for ELISA assays.