

Supplemental Material

Multi-Walled Carbon Nanotubes Induce Arachidonate 5-Lipoxygenase Expression and Enhance the Polarization and Function of M1 Macrophages *in vitro*

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Running title: Induction of Alox5 by MWCNTs

Supplementary materials and methods

Characterization of nanoparticles

MWCNTs and carbon black were imaged using transmission electron microscopy (TEM). Stock samples were prepared as described under Materials and Methods and were further diluted in double distilled H₂O to 1:300 for most of the samples. One drop of the diluted samples was placed onto a formvar-coated 200 mesh copper grid and allowed to dry overnight. The samples were imaged in a JEOL 1400 TEM (Tokyo, Japan) at 80 kV. 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 carbon black diameter, two perpendicular measurements were collected on each particle.

For analysis of zeta potentials, the Malvern spectrometer Zetasizer Nano Z (Malvern Panalytical Inc., Westborough, MA, USA) was used following standard procedures. For measurement of particle stability, MWCNTs and carbon black were mixed with DMEM+10% FBS at 10 µg/ml. After incubation at 37°C for 1 day or 3 days, total number of particles were counted using NanoSight NS300 (Malvern Panalytical Inc) according to vendor's instructions.

Cell toxicity assay

Cells were cultured in 48- or 96-well plates at a density of ~3000–5000 cells/well in a complete growth medium for 24 h. The medium was replaced with fresh culture medium (~100–200 µl/well) containing 3.0% FBS and MWCNTs or carbon black. Concentrations of 1.0, 2.5, 10, 25, or 50 µg/ml were selected to perform the study. After 1 day or 3 day-incubation, the culture was washed with 1X PBS and cell viability was assessed using the Cell Counting Kit-8 (Dojindo, Rockville, MD). Briefly, ~10–20 µl of the CCK-8 solution was added to wells. The plates were incubated at 37°C, 5% CO₂ for 4 hours. The culture medium was collected and examined in a microplate reader at a wavelength of 450 nm. The difference in optical density (OD) relative to untreated control was taken as a measure of cell viability. The percentage of viable cells was calculated by comparing the OD at 450 nm for MWCNT or carbon black-treated and untreated control samples.

Immunoblotting

For inhibition assays, NS-398, a specific inhibitor of cyclooxygenase 2, i.e., Cox-2, prostaglandin-endoperoxide synthase, PTGS, at 2 or 10 µM; Acebilustat, an inhibitor of leukotriene A₄ hydrolase or LTA₄H, at 1 or 5 µM; or dimethyl sulfoxide or DMSO as a vehicle was used to treat cells for 6 hours prior to MWCNTs or IFN-γ+LPS exposure. NS-398 and Acebilustat were purchased from Cayman Chemical, Ann Arbor, MI, USA. DMSO was from Sigma. After treatment with MWCNTs or IFN-γ+LPS, cells 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 determined using a Bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Lysate proteins (10-20 μ g each sample) were resolved on 8 or 10% 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. Primary antibodies used were 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 secondary antibody, horseradish peroxidase-conjugated goat anti-mouse (1:5,000, Jackson ImmunoResearch laboratories, 115-035-146) 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). Scanned images were used to quantify band intensities using the ImageJ software (NIH, 1.52e) and each band was normalized to β -actin.

Statistical analysis

Dependent measures were analyzed using mixed-model one-way analyses of variance (ANOVA), with each analysis incorporating experiment as a random factor. For each experiment, the 2 replicate samples were averaged resulting in a single value for each treatment combination for each of the 3 independent experiments. For some variables, data were log transformed to reduce heterogeneous variance and meet the assumptions of the analysis. Post-hoc comparisons were carried out using Fishers LSD test. All differences were considered significant at *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; or #, $p < 0.01$. All analyses were carried out using JMP version 16 software (SAS Institute, Cary, NC, USA).

Supplemental Figure Legends

Figure S1. Morphology of MWCNTs and carbon black. Representative TEM micrographs of MWCNTs at low (a) or high (b) magnification were shown to illustrate the distinctive fiber-like shape and multi-walled structure of MWCNTs. Representative TEM micrographs of carbon black at low (c) or high (d) magnification were shown to show their distinctive spherical-like morphology.

Figure S2. Cell viability of J774A.1 exposed to MWCNTs or CB. Viabilities of untreated cells, cells treated with MWCNTs (A) or CB (B) at 1.0, 2.5, 10, 25, 50 $\mu\text{g/ml}$ for 1 day or 3 days were determined. Data are expressed as the percentage of untreated cells at each day. Mean \pm S.E.M. of three independent experiments; *, $p < 0.05$; **, $p < 0.01$; ns, not significant; compared to untreated samples of day 1 and day 3, respectively.

Figure S3. Effect of inhibitors on protein expression. (A) Macrophage cells were untreated or pre-treated with DMSO, NS-398 (NS, 10 μM), or Acebilustat (Acb, 5 μM) for 6 hours and then treated with MWCNTs (10 $\mu\text{g/ml}$) or M1 inducer (IFN- γ at 20 ng/ml plus LPS at 100 ng/ml) for 3 days. Cells were lysed and analyzed by immunoblotting against Alox5, COX-2, LTA4H, or β -actin (loading control). Representative image was shown from 3 different experiments. The relative amount of Alox5(B), COX-2 (C), LTA4H (D) was normalized to the amount of β -actin and expressed as % of untreated control at each day and quantification was shown as Mean \pm SEM ($n=3$), *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, compared to untreated control.

Supplemental Figures

Figure S1

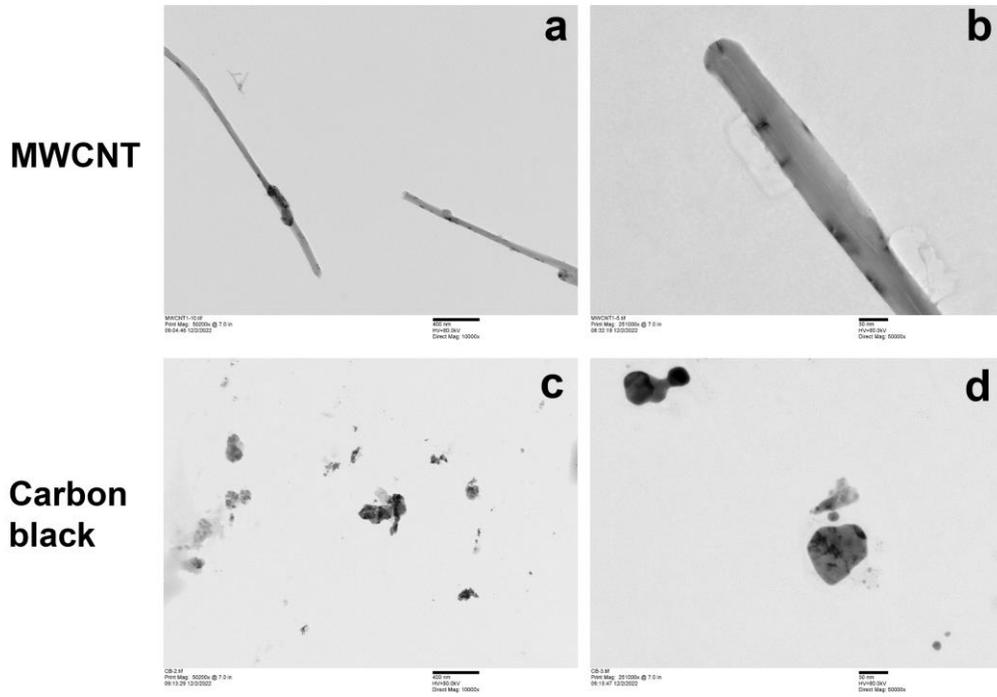


Figure S2

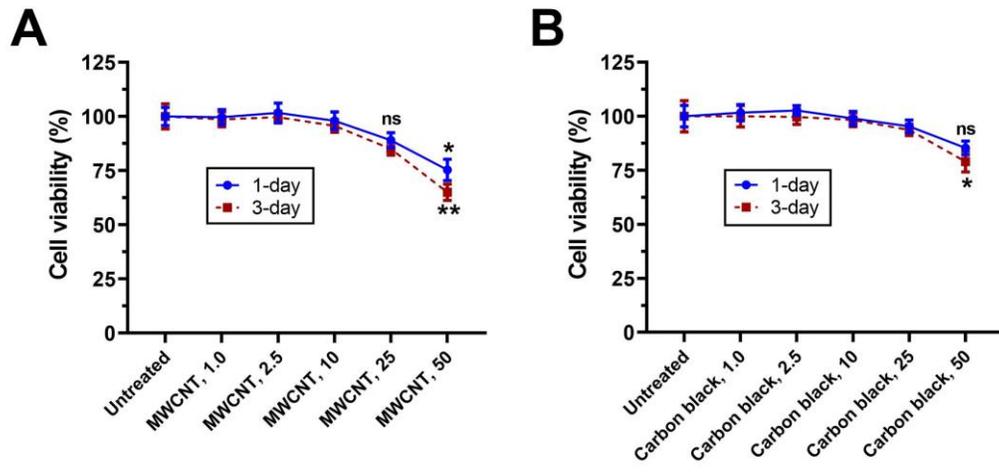
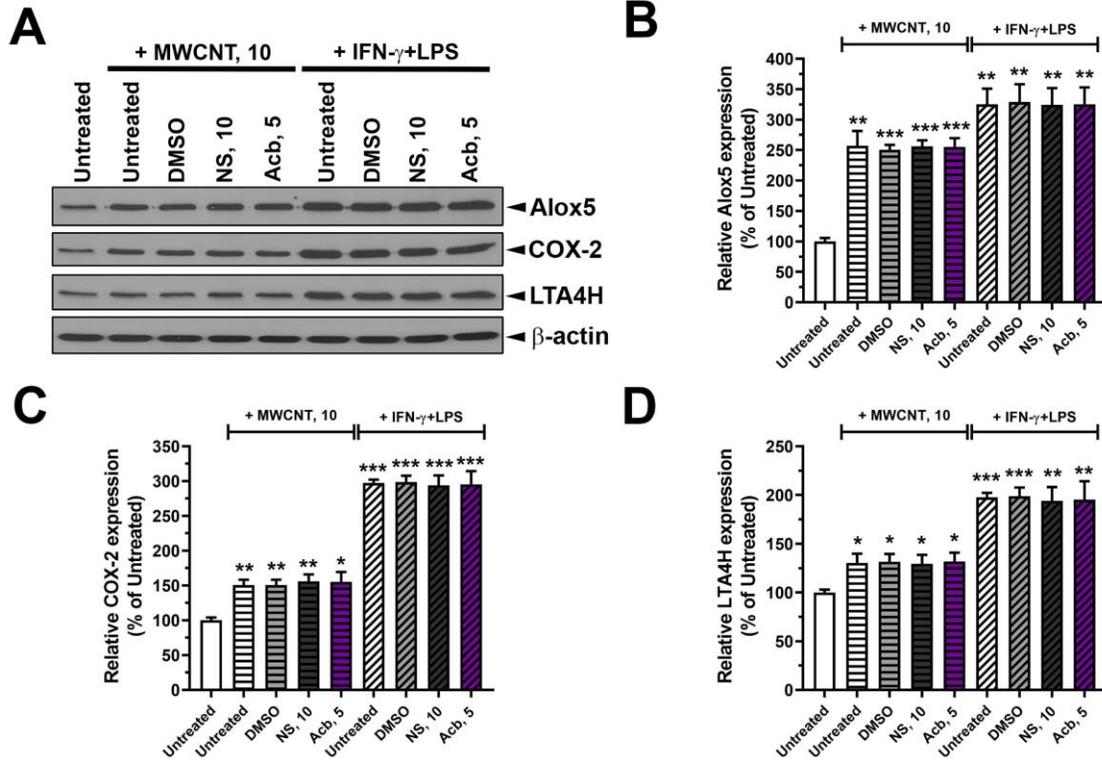


Figure S3



Supplemental Table 1. Zeta potential measurement of MWCNT and carbon black^a

Particle	Dilution	ZP (mv)	Mob ($\mu\text{m}^*\text{cm}/\text{Vs}$)	Cond (ms/cm)	Particle	Dilution	ZP (mv)	Mob ($\mu\text{m}^*\text{cm}/\text{Vs}$)	Cond (ms/cm)
MWCNT	Water	-15.34 \pm 0.29	-1.26 \pm 0.07	1.99 \pm 0.17	MWCNT	DMEM	-12.03 \pm 1.24	-0.94 \pm 0.10	16.43 \pm 0.76
Carbon black	Water	-10.58 \pm 0.59	-0.83 \pm 0.13	1.57 \pm 0.06	Carbon black	DMEM	-9.88 \pm 1.62	-0.78 \pm 0.13	17.17 \pm 1.74

^aMWCNTs or Carbon black was prepared in water or DMEM at 10 $\mu\text{g}/\text{ml}$. Zeta potential (ZP, mv), mobility (Mob, $\mu\text{m}^*\text{cm}/\text{Vs}$), and conductivity (Con, ms/cm) were measured using Zetasizer Nano Z following vendor's instruction. Data represent mean \pm STD from three measurements.

Supplemental Table 2. Particle stability (particle #/mL)^a

Treatment	0 day	1 day	3 days
MWCNTs	7.99 x 10 ⁹	7.90 x 10 ⁹	7.87 x 10 ⁹
Carbon black	1.15 x 10 ¹⁰	1.02 x 10 ¹⁰	1.01 x 10 ¹⁰

^aMWCNTs or carbon black was mixed with DMEM+10% FBS at 10 µg/ml. After incubation at 37°C for 1 day or 3 days, total numbers of particles were counted using NanoSight NS300 following vendor's instruction. Data represent mean ± STD from three measurements.