HHS Public Access

Author manuscript

Nanotoxicology. Author manuscript; available in PMC 2023 June 13.

Published in final edited form as:

Nanotoxicology. 2023 April ; 17(3): 249–269. doi:10.1080/17435390.2023.2204161.

Multi-walled carbon nanotubes induce arachidonate 5-lipoxygenase expression and enhance the polarization and function of M1 macrophages *in vitro*

Chol Seung Lim[a], **Brandon Veltri**[b], **Michael Kashon**[c], **Dale W. Porter**[d], **Qiang Ma**[a]

[a]Receptor Biology Laboratory, Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, WV, USA

[b]Department of Microbiology, Immunology, and Cell Biology, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV, USA

[c]Bioanalytics Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, WV, USA

[d]Pathology and Physiology Research Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, WV, USA

Abstract

Fibrogenic carbon nanotubes (CNTs) induce the polarization of M1 and M2 macrophages in mouse lungs. Polarization of the macrophages regulates the production of proinflammatory and pro-resolving lipid mediators (LMs) to mediate acute inflammation and its resolution in a time-dependent manner. Here we examined the molecular mechanism by which multi-walled CNTs (MWCNTs, Mitsui-7) induce M1 polarization *in vitro*. Treatment of murine macrophages (J774A.1) with Mitsui-7 MWCNTs increased the expression of Alox5 mRNA and protein in a concentration- and time-dependent manner. The MWCNTs induced the expression of CD68 and that induction persisted for up to 3 days post-exposure. The expression and activity of inducible nitric oxide synthase, an intracellular marker of M1, were increased by MWCNTs. Consistent with M1 polarization, the MWCNTs induced the production and secretion of proinflammatory cytokines tumor necrosis factor-α and interleukin-1β, and proinflammatory LMs leukotriene B4

CONTACT Qiang Ma, qam1@cdc.gov, Receptor Biology Laboratory, Toxicology and Molecular Biology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, WV, USA.

Author contributions

Q.M., D.W.P., and C.S.L. conceived the study; Q.M., D.W.P., and C.S.L. designed the study; C.S.L. and B.V. performed experiments; C.S.L. and M.K. performed statistical data analyses. C.S.L. provided a draft manuscript. Q.M. finalized the manuscript. All authors contributed to manuscript preparation.

Disclosure statement

No potential conflict of interest was reported by the author(s). The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

Supplemental data for this article can be accessed online at https://doi.org/10.1080/17435390.2023.2204161.

(LTB4) and prostaglandin E2 (PGE2). The cell-free media from MWCNT-polarized macrophages induced the migration of neutrophilic cells (differentiated from HL-60), which was blocked by Acebilustat, a specific leukotriene A4 hydrolase inhibitor, or LY239111, an LTB4 receptor antagonist, but not NS-398, a cyclooxygenase 2 inhibitor, revealing LTB4 as a major mediator of neutrophil chemotaxis from MWCNT-polarized macrophages. Knockdown of Alox5 using specific small hairpin-RNA suppressed MWCNT-induced M1 polarization, LTB4 secretion, and migration of neutrophils. Taken together, these findings demonstrate the polarization of M1 macrophages by Mitsui-7 MWCNTs *in vitro* and that induction of Alox5 is an important mechanism by which the MWCNTs promote proinflammatory responses by boosting M1 polarization and production of proinflammatory LMs.

Keywords

Pulmonary inflammation; MWCNT; macrophage polarization; Alox5; LTB4

Introduction

Mammalian lungs respond to inhaled particles and nanoparticles by eliciting inflammation (Muller et al. 2005; Donaldson et al. 2006; Dong and Ma 2015). Macrophages are major host cells that defend against infection and injury in the immune system. They localize to specific tissues and can adopt distinct phenotypes depending on stimulating factors. In the lung, these cells differentiate into polarized macrophages to mediate pulmonary inflammation and to defend against inhaled pathogens and particulate and chemical insults in an inducer- and context-dependent manner (Pollard 2009; Dong and Ma 2018a; Ma 2020).

Classically activated macrophages, called M1 macrophages, exhibit a T helper (Th) 1-associated phenotype, promoting acute inflammation, extracellular matrix destruction, and cell death. Differentiation of classically activated macrophages requires an initial exposure to interferon-γ (IFN-γ) and subsequent activation with stimuli such as bacterial lipopolysaccharides (LPS). Differentiation of alternatively activated macrophages, called M2 macrophages, is induced by interleukin (IL)-4 and/or IL-13. These macrophages exhibit a Th2-associated phenotype, promoting tissue repair, fibrosis, angiogenesis, and resolution of inflammation (Wynn 2015; Dong and Ma 2018b). In the context of inflammation, M1 and M2 cells often exhibit opposing activities to regulate the dynamic process of inflammation locally and systemically by producing and secreting regulatory molecules. Notably, polarization of macrophages is highly plastic in phenotypes and temporal variations and is inducer- and context-dependent (Hussell and Bell 2014; Murray et al. 2014). Nonetheless, polarization of M1 and M2 macrophages predominates at the initiation of inflammation and the transition from acute neutrophilic to monocyte/macrophage-predominate, fibro-proliferative responses, respectively, in mouse lungs exposed to MWCNTs (Dong and Ma 2018a; Dong and Ma 2018b; Ma 2020). This temporal polarization of M1 and M2 also coincides with the activation and polarization of T helper cell responses (Dong and Ma 2016a). The tight regulation of the initiation, progression, and resolution of inflammation by macrophages and other immune cells ensures timely and sufficient inflammatory

responses for the control of infection and lesions while avoiding the potentially damaging effects of inflammatory mediators on host tissues. Among the many regulating molecules, proinflammatory and pro-resolving lipid mediators (LMs) from polarized macrophages have been recognized as key regulators of inflammation and its resolution in health and disease (Chiang et al. 2012). It has been shown that M1 macrophages produce proinflammatory LMs in the lung and the bronchoalveolar lavage (BAL) fluid to enhance inflammatory responses to nanoparticles, whereas M2 macrophages produce pro-resolving LMs to boost the resolution of lung inflammation induced by nanoparticles (Lim et al. 2020). Dysregulation of LM production and function may contribute to the failure of particle clearance and tissue repair, leading to chronic progression of fibrosis and possibly cancer in lungs exposed to inflammatory and fibrogenic particles and nanoparticles (Ma 2020).

Arachidonate lipoxygenases (ALOXs) play a central role in the production of proinflammatory and pro-resolving LMs. Arachidonate 5-lipoxygenase (ALOX5), also known as 5-lipoxygenase or 5-LOX, is a monomeric soluble enzyme that associates with intracellular membranes upon activation and converts ω-3 and ω-6 polyunsaturated fatty acids (PUFAs) to a range of biologically active products (Rouzer, Matsumoto, and Samuelsson 1986; Haeggstrom 2018). In the context of inflammation, ALOX5 appears to play a dual role in promoting both the proinflammatory and the pro-resolving responses. At the early phase of inflammation, ALOX5, along with other factors, catalyzes the conversion of arachidonic acid (AA, a ω-6 PUFA) to leukotrienes (LTs). LTs stimulate the initiation and progression of inflammation and are implicated in inflammatory and allergic responses in asthma, arthritis, and psoriasis (He, Chen, and Cai 2020). Upon initiation of acute inflammation, ALOX5 promotes the production of pro-resolving lipid mediators through concerted actions with other enzymes such as ALOX15. In particular, resolvins of the E series from eicosapentaenoic acids (EPAs, ω-3 PUFAs) and the D series from docosahexaenoic acid (DHAs, ω-3 PUFAs) are produced to promote the active resolution of acute inflammation under various physiological and disease conditions (Chiang et al. 2012; Serhan 2014). Aberrant functions of ALOX5 have been associated with asthma, atherosclerosis, and several cancers (Radmark et al. 2015). Besides ALOX5, the ALOX5 activating protein (ALOX5AP, 5-lipoxygenase activating protein, FLAP) is required for leukotriene synthesis; ALOX5AP facilitates the translocation of ALOX5 from the cytoplasm to nuclear and endoplasmic membranes and activation of ALOX5 to produce LTs (Dixon et al. 1990; Bell and Harris 1999; Mashima and Okuyama 2015). The ALOX pathways are regulated to control LT-dependent inflammatory events and are differentially activated in polarized macrophages (Haeggstrom 2018; Werz et al. 2018). Polarization of macrophages induces ALOX5 and ALOX5AP in M1 macrophages, resulting in increased biosynthesis of proinflammatory LMs to boost acute inflammation and defence against pathogens, whereas polarization of M2 induces ALOX5 and ALOX15 to stimulate the resolution of acute inflammation during infection of pathogens (Serhan 2014). In mouse lungs exposed to nanoparticles, M1 macrophages exhibited preferential induction of Alox5 and Alox5ap, while M2 macrophages had a high-level expression of Alox5 and Alox15 (Lim et al. 2020). The mechanism by which ALOX5 is regulated and what role it plays in the polarization of macrophages remains to be elucidated.

Carbon nanotubes (CNTs) are nanomaterials made of single-atom graphene sheets that roll up and are welded into seamless tubes (Kumar et al. 2017). CNTs have unique physicochemical characteristics that are targets of great interest for industrial and commercial applications (De Volder et al. 2013). On the other hand, some properties like the nano-scaled size, fiber-like shape, large surface area, low solubility, and high bio-persistence may impose health risks to exposed workers and consumers, as these properties are characteristically associated with the fibrogenic and tumorigenic effects of inhaled particles and fibers, exemplified by silica and asbestos (Castranova and Vallyathan 2000; Bonner 2010; Donaldson et al. 2010; Wang et al. 2011; Dong and Ma 2016b, Dong and Ma 2019b). Animal studies revealed that pulmonary exposure to certain CNTs indeed caused fibrosis and malignancy in the lungs and the pleural cavity (Porter et al. 2010, Dong et al. 2015, Hindman and Ma 2019). How CNT-induced pulmonary inflammation is resolved has not been well understood. Whether the resolution or failed resolution of inflammation upon continued exposure to inflammatory and fibrogenic CNTs contributes to the pathogenesis of chronic disease phenotypes have not been well investigated.

Filamentous multi-walled carbon nanotubes (MWCNTs) can cause acute inflammation and chronic pulmonary lesions, including granulomatous inflammation, interstitial fibrosis, lung cancer and mesothelioma (Castranova and Vallyathan 2000; Bonner 2010; Dong and Ma, 2016b). Inflammation is a common early response to exposure to MWCNTs in the lung that, if left unresolved, would evolve into chronic fibrosis and possibly cancer (Tang et al. 2018; Dong and Ma 2019b). The molecular and cellular events leading to the inflammatory response induced by MWCNTs have not been well elucidated. In particular, the signaling pathways that mediate these early events remain largely unknown. We have previously shown that pulmonary exposure to inflammatory and fibrogenic MWCNTs (Mitsui-7) elicits acute inflammation accompanied by elevated levels of proinflammatory cytokines and LMs (Porter et al. 2010; Dong and Ma 2017; Lim et al. 2020). MWCNTs further increased M1- or M2-specific functions in polarized macrophages in a time-dependent manner. MWCNTs have not been shown to induce a full-scale polarization of macrophages to M1 or M2 phenotypes *in vitro* with two-dimensional cultures of macrophages, though mouse bone marrow-derived macrophages grown as a three-dimensional epithelioid cell aggregates have been shown to polarize into M1 and M2 phenotypes upon prolonged stimulation with carbon nanotubes and asbestos fibers (Sanchez et al. 2011).

In the present study, we investigated whether MWCNTs (Mitsui-7) induce the polarization of macrophages to M1 cells in a two-dimensional culture of macrophages and how MWCNT-polarized macrophages elicit proinflammatory responses *in vitro*. Specifically, we examined the mechanisms by which the production and functions of cytokines and LMs are regulated, with a focus on the induction of Alox5, which catalyzes the synthesis of proinflammatory LMs and promotes macrophage-dependent neutrophilic inflammation.

Materials and methods

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 (Porter et al. 2010; Lim et al. 2020). 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 μm (95% confidence interval of 4.08–4.88 μm) 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 μg/ml) using the Pierce LAL chromogenic endotoxin quantification kit (Thermo Scientific, Pittsburgh, PA).

Carbon black (CB) are amorphous, carbonaceous particulates and therefore are 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 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 (He et al. 2011; Hindman and Ma 2018). Stock solutions were further diluted with the culture media and sonicated immediately before use.

As a 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 the 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 were 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 (7.90×10^9/ml) and by 1.5% at 3 day (7.87×10^9), compared to preparations not incubated with the medium (7.99×10^9). The particle number of carbon black also showed a slight decrease upon incubation in the culture medium, showing 1.02×10^{10}/ml at 1 day and 1.01×10^{10} at 3 days, compared to unincubated (1.15×10^{10}).

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 (Lim et al. 2020). Polarized J774A.1 cells respond to MWCNTs with increased M1 or M2 responses similar to polarized human peripheral blood monocyte-derived macrophages in response to bacterial pathogens (Werz et al. 2018; Lim et al. 2020).

The cells were grown in DMEM with 10% FBS. For differential polarization of M1 and M2, cells at a density of 5×10^5 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 the indicated time (typically three days). Characterization of M1 and M2 phenotypes of J774A.1 cell by the classical inducers have been reported previously (Lim et al. 2020). 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 untreated. All cells were treated as duplicates and all treatment experiments were conducted three times. 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 centrifuged at 4 °C to remove particles, cells, and debris. The cell-free medium was used for the 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 which 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 μg/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 (Millius and Weiner, 2010). 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 2.5×10^6 cells/ml in serum-free media was used for cell migration assay.

Quantitative real-time PCR

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.

Immunoblotting

J774A.1 cells were treated as indicated and were lysed in a lysis buffer (10 mM Tris, pH 7.4, 1% SDS) with 1× 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 nonspecific 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, horse-radish 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 onto 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.

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

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.

Enzyme-linked immunosorbent assay

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.

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 the 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).

Alox5 gene silencing

After one day of 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 a control. After a media change with complete growth media, cells were incubated for an additional day, and then a portion of the cells was 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.

Statistical analysis

Dependent measures were analyzed using mixed-model one-way analyses of variance (ANOVA), with each analysis incorporating the 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 * $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; $^{\#}$, $p < 0.01$. All analyses were carried out using JMP version 16 software (SAS Institute, Cary, NC, USA).

Results

MWCNTs induced Alox5 expression in macrophages

To study M1 polarization and induction of M1-dependent proinflammatory responses by MWCNTs *in vitro*, we first examined the induction of Alox5, a key enzyme in the biosynthesis of LMs critical for the initiation and progression of inflammation. Figure 1 describes the characterization of the induction of Alox5. Untreated J774A.1 cells were used to establish a baseline for induction. Cells treated with the vehicle for suspension of particles (control media) served as a negative control. Treatment with a prototypical M1 inducer, i.e. IFN-γ at 20 ng/ml plus LPS at 100 ng/ml, was used as a positive control for induction. CB is a carbon-based particulate. In contrast to the MWCNTs that have a fiber-like shape with high rigidity, CB does not have a distinctive morphology. CB has been shown previously to be of low toxicity and incapable of inducing acute inflammation at a concentration at which the MWCNTs induced significant acute inflammation in mouse lungs (Dong and Ma 2016a). Therefore, CB was used as an amorphous particulate control for the induction of Alox5. Induction was examined at 1 day (Figure 1(A)) and 3 days (Figure 1(B)) post-exposure.

The basal level *Alox5* mRNA was low in J774A.1 cells cultured for 1 day or 3 days as determined by RT-qPCR (Figure 1(A and B), untreated). Cells treated with the vehicle (control media) had *Alox5* mRNA levels at nearly the same level as those of untreated at 1 day and 3 days post-exposure. As expected, the M1 inducer (IFN-γ + LPS) increased the *Alox5* mRNA level by 4.2-fold at 1 day, and by 5.5-fold at 3 days, post-exposure, compared to untreated control. Treatment of J774A.1 macrophages with MWCNTs at a concentration of 2.5 μg/ml increased the expression of *Alox5* mRNA by 1.9-fold at 1 day. At 10 μg/ml, MWCNTs increased the *Alox5* mRNA expression by 3.4-fold. At 3 days post-exposure, treatment with MWCNTs increased the expression of *Alox5* mRNA by 2.7-fold at a concentration of 2.5 μg/ml and by 4.9-fold at 10 μg/ml of MWCNTs. On the other hand, treatment with CB at concentrations of 2.5, 10, and 30 μg/ml did not induce *Alox5* mRNA significantly at either 1 day or 3 days post-exposure, compared to untreated control.

Expression of the Alox5 protein was analyzed by immunoblotting (Figure 1(C and E)) with quantification (Figure 1(D and F)). The Alox5 protein was detected at a low level in untreated cells cultured for 1 or 3 days. The M1 inducer increased the Alox5 protein level by 288.4% at 1 day and by 335.0% at 3 days post-exposure. Treatment with MWCNTs induced the Alox5 protein expression significantly, increasing the Alox5 protein level by 171.2% at a concentration of 2.5 μg/ml and by 211.5% at 10 μg/ml MWCNTs at 1 day, respectively. At 3 days post-exposure, MWCNTs increased the Alox5 protein level by 203.8% at 2.5 μg/ml and by 270.2% at 10 μg/ml, respectively. Similar to *Alox5* mRNA expression, treatment with CB at all 3 concentrations examined did not increase the Alox5 protein level at either 1 day or 3 days post-exposure, compared to untreated control. Taken together, these results reveal that treatment of J774A.1 macrophages with MWCNTs induced the M1-type expression of Alox5 at both the mRNA and protein levels in macrophages, whereas CB did not induce Alox5 expression to a significant level at concentrations as high as 3 times of the high concentration of MWCNTs (30 versus 10 μg/ml). These findings indicate that

the MWCNTs, but not CB, induces Alox5 expression in J774A.1 cells *in vitro*. This result is consistent with a previous report that the MWCNTs, but not the CB, induces acute inflammation in mouse lungs at the dose of 40 μg/mouse (Dong and Ma 2016a).

MWCNTs induced M1 polarization in vitro

MWCNTs have been shown to induce the polarization of M1 and M2 macrophages in mouse lungs in a time-dependent fashion (Dong and Ma 2018a). However, studies on the induction of M1 and/or M2 polarization *in vitro* by nanoparticles have yielded mixed and sometimes controversial results (Sanchez et al. 2011; Meng et al. 2015). Therefore, we investigated whether the MWCNTs used in the current study would induce M1 macrophage polarization in commonly used, 2-dimensional cultures in relation to the induction of Alox5. Polarization of M1 phenotypes was analyzed for the expression of CD68, a cell surface marker, and Nos2, an intracellular and functional marker of M1 macrophages (Figure 2). Polarization was examined first using immunoblotting and quantification of the surface marker CD68 protein (Figure 2(A and B)). While untreated cells showed a low but detectable level of CD68, treatment with MWCNTs at a concentration of 2.5 μg/ml increased the expression of CD68 by 150.5% at 1 day and 198.7% at 3 days post-exposure, respectively, compared to untreated control. At 10 μg/ml, MWCNTs increased the CD68 protein level by 172.8% at 1 day and 194.0% at 3 days post-exposure. The M1 inducer (IFN-γ + LPS) increased the CD68 protein level by 201.7% at 1 day and 262.0% at 3 days post-exposure, respectively, as a positive control for induction of M1 polarization (Figure 2(B)).

M1 macrophages exhibit elevated expression of Alox5ap which is important for the Alox5-dependent biosynthesis of LMs. Therefore, we examined the induction of Alox5ap as an M1 response after MWCNT exposure. Treatment with MWCNTs at a concentration of 2.5 μg/ml slightly increased the expression of Alox5ap protein at 1 day but increased the level by 217.1% at 3 days post-exposure. At 10 μg/ml, MWCNTs increased the Alox5ap protein level by 174.5% at 1 day and 251.3% at 3 days post-exposure. As a positive control, the M1 inducer increased the Alox5ap protein level by 190.5% at 1 day and 275.5% at 3 days post-exposure, respectively (Figure 2(A and C)). Therefore, Alox5ap was evidently induced in M1 macrophages.

Induction of the intracellular M1 marker Nos2 after MWCNT treatment was examined using a Nos2 expression detection assay. Treatment of J774A.1 macrophage with MWCNTs at a concentration of 2.5 μg/ml increased the expression of Nos2 protein level by 2.8-fold at 1 day and 6.1-fold at 3 days post-exposure, compared to untreated or control media-treated samples. At 10 μg/ml, MWCNTs increased the Nos2 protein expression by 3.3-fold at 1 day and 8.4-fold at 3 days post-exposure. The M1 inducer increased the Nos2 protein level by 4.4-fold at 1 day and 9.9-fold at 3 days post-exposure, compared to untreated control, respectively (Figure 2(D)). To further corroborate the induction of Nos2 with macrophage polarization, a known M2 inducer was tested. IL-4 at 20 ng/ml did not induce Nos2 expression, supporting the notion that Nos2 is specifically induced in M1 macrophages.

We then examined the temporal biosynthesis of proinflammatory cytokines and LMs in macrophages and how MWCNTs affect this process. Macrophages were treated with

MWCNTs (2.5 or 10 μg/ml), M1 inducers, or M2 inducers for one or three days, respectively. The cell-free culture media were assayed for TNF-α and IL-1β by ELISA. TNF-α was induced throughout the polarization phase, with a steady increase from M1, but not M2, macrophages. MWCNTs at a concentration of 2.5 μg/mL elevated the TNF-α production to 57.23 pg/ml at day 1 (a 2.4-fold increase over untreated control) and to 97.04 pg/ml on day 3 (4.1-fold). At 10 μg/mL, MWCNTs increased the TNF-α production to 100.65 pg/ml on day 1 (4.2-fold) and by 157.48 pg/ml on day 3 (6.6-fold) (Figure 2(E)). A similar pattern was observed for IL-1β production by MWCNT or M1 inducer. MWCNTs at a concentration of 2.5 μg/ml elevated the IL-1β production to 27.23 pg/ml on day 1 (2.1-fold) and to 43.71 pg/ml on day 3 (3.4-fold). At 10 μg/ml, MWCNTs increased the IL-1β production to 43.99 pg/ml on day 1 (3.4-fold) and to 57.48 pg/ml on day 3 (4.5-fold) (Figure 2(F)). There is no increase in IL-1β production by M2 inducer IL-4. These findings indicate that MWCNTs differentially augment the biosynthesis of proinflammatory cytokines in M1 macrophages, and induction and activation of specific Alox5 pathways are likely to play a role in MWCNT-induced M1 polarization.

MWCNTs enhanced M1-mediated neutrophilic inflammatory response in vitro

Next, we measured the proinflammatory LMs, LTB4 and PGE2, which are expected to be produced by M1, but not M2, macrophages upon activation by MWCNTs. MWCNTs at 2.5 μg/ml increased the LTB4 production to 12.40 pg/ml at day 1 (a 2.2-fold increase over control) and to 36.87 pg/ml at day 3 (a 6.5-fold increase over untreated control). At 10 μg/ml, MWCNTs increased the LTB4 production to 22.57 pg/ml on day 1 (a 4.0-fold increase) and to 50.19 pg/ml on day 3 (an 8.8-fold increase) (Figure 3(A)). M1 inducers increased LTB4 production to 34.04 pg/ml on day 1 (6.0-fold) and 58.15 pg/ml on day 3 (10.2-fold), whereas no apparent increase in the LTB4 level was observed in the culture media of M2 inducer (IL-4)-treated macrophages (Figure 3(A)).

We examined if MWCNTs increase the production of another proinflammatory LM PGE2 (Figure 3(B)). MWCNTs at 2.5 μg/ml strongly increased the PGE2 production to 43.39 pg/ml on day 1 (a 3.0-fold increase over untreated control) and to 155.13 pg/ml on day 3 (10.7-fold increase). At 10 μg/ml, MWCNTs increased the PGE2 production to 98.31 pg/ml on day 1 (6.8-fold) and to 230.84 pg/ml on day 3 (16.0-fold) (Figure 3(B)). M1 inducers increased PGE2 production to 148.60 pg/ml on day 1 (10.3-fold) and 241.26 pg/ml on day 3 (16.7-fold), whereas no increase in PGE2 production was observed in the culture media of M2 inducer-treated macrophages. Taken together, these findings indicate that MWCNTs differentially augment the biosynthesis of proinflammatory LMs in M1 macrophages, and induction and activation of specific Alox5 pathways likely mediate increased synthesis of some of the LMs from endogenous substrates.

M1 macrophages support the initiation and continued inflammation by recruiting neutrophils through chemotaxis. To examine if MWCNT-induced production of proinflammatory LMs has a functional role in acute and continued proinflammatory responses to MWCNTs, we performed a chemotactic cell migration assay where the cell-free culture media of macrophages exposed to MWCNTs (at 2.5 or 10 μg/ml) or M1 inducer IFN-γ + LPS were used to induce chemotaxis of differentiated HL-60 cells that have neutrophil-like

phenotypes *in vitro* (Figure 3(C)). Cultured media from cells treated with MWCNT for 3 days induced neutrophilic cell migration by 2.2-fold at 2.5 μg/ml and by 3.7-fold at 10 μg/ml compared with untreated and control media-treated samples. As a positive control, the M1 inducer increased neutrophilic cell migration by 6.4-fold compared with control through the culture media at 3 days post-treatment. In a separate experiment, we tested whether MWCNTs have a direct chemotactic effect on neutrophil migration (Figure 3(D)). Normal cell culture media was mixed with the control media, MWCNTs (2.5 or 10 μg/ml), or 10% FBS (as positive control) and used for the cell migration assay. The result showed that there was no observable, direct effect of MWCNTs on neutrophil migration in the absence of macrophages (Figure 3(D)). Therefore, MWCNTs induced the production and secretion of the proinflammatory LMs from activated M1 macrophages, which correlated with the induced neutrophilic cell chemotaxis by M1 macrophages *in vitro*.

Inhibition of LTB4 biosynthesis suppressed MWCNT-induced M1 polarization and function

The arachidonic acid pathways play important roles in the development of many inflammatory disease conditions. AA is metabolized by cyclooxygenases (COXs), such as COX-2, or lipoxygenases (LOXs), such as ALOX5, to a variety of bioactive mediators including prostaglandins and LTs (Haeggstrom 2018; Rouzer, Matsumoto, and Samuelsson 1986) (Figure 4(A)). Relevant to this study, COX-2 is believed to be responsible for the induced production of PGE2, whereas ALOX5, together with LTA4H, converts AA to LTB4, in acute inflammation. We investigated if LTB4 or PGE2 produced in macrophages treated with MWCNTs functions as a potent chemoattractant for the observed neutrophilic cell migration. We first modulated the biosynthesis of LTB4 and PGE2 by using specific inhibitors of pathways of AA metabolism (Figure 4(A)). Cells were treated with NS-398, a specific inhibitor of COX-2, or Acebilustat, a specific inhibitor of LTA4H prior to exposure to MWCNTs or IFN-γ + LPS, followed by quantification of LTB4 and PGE2. Pretreatment with Acebilustat at 1 μM reduced MWCNT- or IFN-γ + LPS-induced production of LTB4 by 34.4% and 50.2%, respectively. At 5 μM, Acebilustat reduced MWCNT- or IFN-γ + LPS-induced production of LTB4 by 97.1% or 95.7%, respectively. Pretreatment with NS-398 at 2 or 10 μM did not alter MWCNT- or IFN-γ + LPS-induced production of LTB4 significantly (Figure 4(B)). Pretreatment with NS-398 at 2 μM reduced MWCNT- or IFN-γ + LPS-induced production of PGE2 by 40.6% or 56.0%, respectively. At 10 μM, NS-398 reduced MWCNT- or IFN-γ + LPS-induced production of PGE2 by 98.4% or 99.2%, respectively. Pretreatment with Acebilustat at 1 or 5 μM did not alter MWCNT- or IFN-γ + LPS-induced production of PGE2 significantly (Figure 4(C)).

We then examined if the induced production of LTB4 or PGE2 is involved in the chemotactic activity of macrophages induced by MWCNTs using the chemotactic cell migration assay with or without AA metabolism inhibitors before MWCNT treatment (Figure 4(D)). Cultured media from cells pretreated with Acebilustat at 1 μM prior to MWCNT or IFN-γ + LPS exposure reduced neutrophilic cell migration by 50.7% or 58.3%, respectively. At 5 μM, Acebilustat reduced MWCNT or IFN-γ + LPS-induced neutrophilic cell migration by 89.3% or 91.7%, respectively. Cultured media from cells pretreated with NS-398 at 2 μM or 10 μM did not reduce MWCNT- or IFN-γ + LPS-induced neutrophilic cell migration to a significant extent. Inhibition of the LTB4 receptor BLT1 by a specific

inhibitor of the receptor LY293111 would block the chemotactic activity of LTB4 on neutrophils (Jackson et al. 1999). Treatment with LY293111 at 5 nM indeed reduced cell migration activity in the chemotaxis assay by 41.3% with 10 μg/ml MWCNTs, or by 46.0% with M1 inducer, respectively. At 25 nM, LY29311 reduced the cell migration activity by 76.4% with 10 μg/ml MWCNTs, or by 70.9% with M1 inducer, respectively. Therefore, LY293111 significantly reduced MWCNTs- or M1 inducer-induced neutrophilic cell migration *in vitro*, albeit the inhibition was not complete, with substantial residue activities remaining, indicating that additional chemotactic factors from M1 cells may contribute to MWCNT-induced chemotaxis to a certain extent. Together, these results imply that LTB4 is a potent and major mediator of MWCNT-triggered neutrophilic migration from M1 macrophages.

Knockdown of Alox5 inhibited MWCNT-induced M1 polarization

The above observations suggest that Alox5 and its metabolite LTB4 are key regulators in MWCNT-induced M1 polarization. To test this notion, we knocked down the expression of Alox5 in macrophages with specific shRNA against *Alox5* mRNA (Figure 5). Transduction of Alox5-specific shRNA into macrophages reduced the *Alox5* mRNA level by 82.6% as shown by RT-qPCR, compared to untreated cells. As a control, there was no difference in the *Alox5* mRNA level in cells transduced with a control shRNA, compared to untreated cells (data not shown). Immunoblotting revealed that macrophages transduced with Alox5-specific shRNA had 67.9% decrease in the Alox5 protein, compared to untreated cells or cells transduced with control shRNA (Figure 5(A and B)). Macrophages transduced with shRNA were treated with MWCNTs at 10 μg/ml or with M1 inducer for additional three days. Induction of Alox5 protein was apparent in cells transduced with control shRNA, showing a 2.9-fold increase by MWCNT at 10 μg/ml and a 3.6-fold increase by M1 inducer over control, respectively. However, no induction of Alox5 was observed in cells transduced with Alox5 shRNA by either MWCNTs or M1 inducer (Figure 5(A and B)).

We then examined whether Alox5 knockdown affected MWCNT-induced M1 macrophage polarization by examining the expression of M1 marker CD68 and production of LTB4. CD68 expression was dramatically decreased upon Alox5 shRNA transduction into macrophages, with a 55.1% decrease of CD68 protein, compared to untreated cells (Figure 5(A and C)). Treatment with MWCNTs at 10 μg/ml or with M1 inducer for additional 3 days slightly increased the expression of CD68, which was not statistically significant. The effect of Alox5 knockdown on LTB4 levels was examined. Alox5 shRNA effectively reduced LTB4 production by 68.6%, compared to control shRNA-transduced cells. Treatment of Alox5 shRNA transduced cells with MWCNTs at 10 μg/ml or M1 inducer for additional 3 days slightly increased the production of LTB4, which was not statistically significant. In contrast, cells transduced with control shRNA showed a drastic increase of LTB4 production by 8.3-fold upon treatment with MWCNTs at 10 μg/ml or by 10.2-fold with M1 inducer, respectively (Figure 5(C and D)).

To examine the functional impact of Alox5 knockdown, we analyzed the effect of Alox5-specific shRNA on neutrophilic cell migration by using the chemotaxis assay described above (Figure 5(E and F)). Transduction of Alox5 shRNA in macrophages decreased

neutrophilic cell migration by 75.8%, compared to untreated control, whereas no effect on cell migration was observed with control shRNA (Figure 5(E)). Treatment of the cells transduced with Alox5 shRNA with MWCNTs at 10 μg/ml or with M1 inducer for additional 3 days slightly increased neutrophilic cell migration, which was not statistically significant. On the other hand, cells transduced with control shRNA showed a dramatic increase in neutrophilic cell migration by 3.5-fold with MWCNTs at 10 μg/ml or by 6.2-fold with M1 inducer, respectively (Figure 5(F)). These results imply that Alox5 is a key enzyme involved in MWCNT-induced M1 polarization and chemotactic function.

Discussion

In this study, we demonstrate that exposure of macrophages to inflammatory and fibrogenic, fiber-like MWCNTs stimulates M1 polarization and induces the production of proinflammatory LMs, particularly, LTB4, due to the upregulation of Alox5 expression. Further, MWCNTs induced neutrophilic chemotactic migration through M1-produced LTB4, which may underlie the recruitment of neutrophils critical for the initiation and amplification of inflammation *in vivo*. These observations are of importance because of the emerging role of Alox5 expression in MWCNT-induced macrophage polarization and pulmonary inflammation that potentially contribute to the pathogenesis of progressive and lethal outcomes such as organ fibrosis and cancer, in the lung and the pleura observed in humans exposed to inhaled fibrogenic and tumorigenic particles.

Macrophages are among the major host-defending cells that protect against infection and injury in the body. Macrophages adopt distinct phenotypes depending on stimulating factors and can differentiate into at least two types of polarized macrophages that predominate in the response to particles and nanoparticles such as MWCNTs *in vivo*, though polarization of macrophages is noted to exhibit high plasticity and is inducer- and context-dependent (Pollard 2009; Ma 2020). Polarized macrophages differentially regulate pulmonary inflammation (Murray et al. 2014). Polarization of M1 macrophages requires exposure to IFN-γ and subsequent stimuli like LPS. M1 macrophages exhibit a Th1-associated phenotype to promote tissue inflammation and cell death. M2 macrophages are polarized by IL-4 and/or IL-13 and exhibit a Th2-associated phenotype to promote tissue repair, fibrosis, and resolution of inflammation (Wynn 2015). M1 macrophages produce proinflammatory cytokines and LMs in the lung and the BAL fluid to enhance inflammatory responses, whereas M2 macrophages produce anti-inflammatory cytokines, such as IL-10, and pro-resolving LMs, such as lipoxins and resolving Ds and Es, to promote resolution of lung inflammation and tissue repair (Lim et al. 2020; Ma 2020). Dysregulation of LM production and macrophage function contributes to the development of various pathologic inflammatory conditions, including failure of particle clearance and tissue repair, and chronic progression to fibrosis and cancer (Serhan 2014; Ma 2020).

MWCNTs elicited apparent activation and polarization of macrophages in mouse lungs (Dong and Ma 2018a; Lim et al. 2020). Polarization of macrophages by MWCNTs *in vivo* correlated with the induction of Th1- and Th2-associated inflammation (Dong and Ma 2016a; Dong and Ma 2018b). Polarization of macrophages in mouse lungs exposed to MWCNTs is likely to be mediated through specific signalling pathways.

As an example, MWCNTs activate the nuclear factor-kappa B (NF-κB) pathway to upregulate the production of proinflammatory cytokines and LMs from macrophages and fibroblasts in the acute phase of lung inflammation, whereas activation of the pathway during fibrotic progression is associated with the production of anti-inflammatory and pro-fibrotic mediators, illustrating temporally controlled, dual functions of the NF-κB pathway in pulmonary inflammatory and fibrotic responses to MWCNTs (Dong and Ma 2019a). Polarization of macrophages by MWCNTs *in vitro* has been investigated but with mixed and, in some cases, controversial, results (Meng et al. 2015). To the best of our knowledge, MWCNTs have not been shown to induce a full-scale polarization of macrophages to either an M1 or M2 phenotype *in vitro* in 2-dimensional cultures, though mouse bone marrow-derived macrophages grown as 3-dimensional epithelioid cell aggregates have been shown to polarize into M1 and M2 phenotypes upon prolonged stimulation with carbon nanotubes and asbestos fibers (Sanchez et al. 2011). MWCNTs were found to enhance the function of M1 macrophages polarized by M1 inducers, such as the production of proinflammatory LMs, whereas, in M2 cells polarized by M2 inducers, MWCNTs increased the production of pro-resolving LMs (Lim et al. 2020). In the current study, MWCNTs alone at a high concentration in the absence of a known M1 inducer were used to treat macrophages and M1 polarization was characterized by quantification of cell surface marker CD68 and intracellular metabolic and functional marker Nos2. Cell surface expression of CD68 and intracellular expression of Nos2 are relatively low in unstimulated J774A.1 cells; but there were apparent and significant increases in the expressions of CD68 and intracellular Nos2 following MWCNT treatment, revealing a mature macrophage phenotype with M1 polarization. Full-scale characterization of M1 phenotypes induced by MWCNTs *in vitro*, including various immunologic markers and functions, metabolic signatures, and temporal transformation of M1, is warranted in future studies.

Regulation of ALOX-mediated biosynthesis of LMs during macrophage polarization is well documented. Upon infection, monocytic cells are exposed to endotoxins released from pathogens, which triggers the maturation of monocytes and activates the ALOX5 pathway by increasing the mRNA and protein expression of the gene to result in an enhanced capacity to synthesize biologically active LMs (Poirier et al. 2020). LPS is a component of the prototypical M1 inducer that stimulates M1 macrophage polarization and increases LT biosynthesis in primary human neutrophils and monocytes (Shapouri-Moghaddam et al. 2018). This is well supported by our current study that demonstrates increased Alox5 expression and function in IFN-γ + LPS- or MWCNT-treated J774A.1 cells. This finding also suggests that the J774A.1 macrophage cell line is a useful tool for investigating Alox5 and Alox5ap up-regulation during macrophage polarization, as evidenced by increased biosynthesis of proinflammatory cytokines and LMs in macrophages treated with M1 inducers (IFN-γ + LPS), or pro-resolving cytokines and LMs in macrophages treated with M2 inducers (IL-4). It was noted that COX-2 expression is also induced by LPS in lymphoid cells and that other lipoxygenases like Alox12 or Alox15 may also be induced by LPS to result in a capacity to produce specialized pro-resolving mediators (Nishiokada et al. 2015). Future studies are necessary to determine the full-scale biosynthetic capacity for various LMs, as well as the temporal relationship among different LMs, during the polarization

of macrophage cells, by using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

Polarization of M1 or M2 macrophages alters their ALOX pathways for the biosynthesis of proinflammatory and pro-resolving LMs (Serhan 2014). Pathogenic *E. coli* or *S. aureus* stimulates ALOX5 and COX pathways in M1 macrophages of human origin. They also stimulate M2 macrophages to produce pro-resolving LMs such as resolvins D2 and D5. In this example, M1 and M2 macrophages respond to pathogens differentially and temporally, producing either leukotrienes or resolvins that further distinguish inflammatory or pro-resolving phenotypes (Werz et al. 2018). Polarization of macrophages induced ALOX5AP in the human M1 macrophages resulting in the increased preferential biosynthesis of proinflammatory LMs, whereas induction of ALOX15 by Th2 inducers increased the production of pro-resolving LMs (Abrial et al. 2015). ALOX5 also contributes to the synthesis of pro-resolving LMs. We have previously shown that M1 macrophages exhibited a high-level induction of Alox5ap, whereas M2 macrophages had a preferential increase of Alox15 (Lim et al. 2020). Moreover, MWCNTs at a low concentration significantly induced Alox5 and Alox5ap expression at the mRNA and protein levels in M1 macrophages polarized by M1 inducers, and MWCNTs induced Alox15 in M2 macrophages polarized by IL-4 (Lim et al. 2020). In the current study, we found that MWCNTs alone at a high concentration in the absence of a known M1 inducer significantly induced Alox5 expression at the mRNA and protein levels, accompanied by increased Alox5ap expression at the mRNA and protein levels. Further studies will examine if MWCNTs alone induce Alox5 by increasing the transcription of the *Alox5* gene at its promoter. Such analysis would identify transcription factors that mediate the upregulation of Alox5 expression by MWCNTs. Notably, we found that carbon black at concentrations as high as three times the high concentration of MWCNTs (30 versus 10 μg/ml) did not cause significant induction of Alox5 under the experiment condition. This finding is consistent with previous reports that carbon black particles are of low toxicity and have a significantly lower capacity than MWCNTs to induce inflammation in mouse lungs *in vivo* (Lam et al. 2004; Shvedova et al. 2005; Ma-Hock et al. 2013; Dong and Ma 2016a). Our finding demonstrates that the Mitsui-7 MWCNTs with fiber-like shape and rigidity induce Alox5 and stimulate proinflammatory responses in cultured macrophages. Comparison with CNTs of varying dimensions and rigidity would further delineate the structure-activity relationship of CNTs for induction of Alox5 in future studies. The role of Alox5 in M2 polarization by MWCNTs has not been investigated and awaits the demonstration of M2 polarization by MWCNTs in cultured macrophages in future studies.

Acute inflammation is typically characterized by increased blood flow, capillary expansion, leukocyte infiltration, and enhanced production of chemical mediators (Wasserman 1980; Ramaiah and Jaeschke 2007). LTB4 is a dihydroxy fatty acid derived from the Alox5 pathway of AA metabolism. LTB4 functions as an important proinflammatory mediator of inflammatory responses to pathogens and danger signals. Alox5 catalyzes the formation of LTA4 from AA. LTA4 is unstable and is further metabolized to LTB4 by LTA4H (Murphy and Gijon 2007). A growing body of evidence supports that LTB4 is a potent chemoattractant and cell activator toward leukocytes and monocytes to result in macrophage–dependent neutrophilic inflammation (Czarnetzki 1983; McMillan and Foster

1988; Afonso et al. 2012; Sheppe and Edelmann 2021). LTB4 also plays an important role in chronic inflammatory diseases, such as asthma, arthritis, psoriasis, and atherosclerosis, and in certain types of cancers (McMillan and Foster 1988; He, Chen, and Cai 2020). Treatment with inflammatory and fibrogenic MWCNTs increased LTB4 production in polarized macrophages. Alox5 is the key enzyme involved in this bioconversion of AA to LTB4s by MWCNTs, because genetic knockdown against Alox5 decreased MWCNT-induced LTB4 production as shown in the current study. Interestingly, blocking steps downstream of Alox5, such as the conversion of LTA4 to LTB4 by LTA4H using a specific inhibitor of LTA4H Acebilustat, could almost completely prevent MWCNT-induced LTB4 production and neutrophilic chemoattraction. Although MWCNTs increased the production of PGE2 from M1 macrophages, inhibition of COX-2 that specifically prevents PGE2 production did not block MWCNT-induced neutrophilic chemoattraction. Taken together, these findings revealed that induction and activation of Alox5 and increased LTB4 production are key events for the induction of M1 macrophage polarization and function by MWCNTs. This conclusion does not exclude the possibility that other proinflammatory mediators, including proinflammatory cytokines and PGEs, modulate the polarization of M1 macrophages.

Secreted LTB4 binds to cell surface receptors BLT1 and BLT2, which are G protein-coupled receptors (GPCRs), on polymorphonuclear leukocytes and monocytes. The binding of LTB4 to the GPCRs activates intracellular signalling to enhance leukocyte infiltration and amplify inflammation (He, Chen, and Cai 2020). Pharmacological studies identified anti-leukotriene agents including LT receptor antagonists that are candidate therapeutics to treat lung disease, psoriasis, arthritis, inflammatory bowel disease, asthma, and certain immunological disorders (Kabir and Morshed 2015; Chauhan et al. 2017). The current study clearly showed that MWCNTs increased the production of LTB4 in cell-free culture media from macrophages and enhanced the neutrophilic migration, which was blocked by a specific LTB4 receptor inhibitor LY293111. This result supports the critical role of the LTB4-BLT1 and/or LTB4-BLT2 signalling pathways in MWCNT-stimulated acute and neutrophilic inflammation. Nonetheless, treatment with LY293111 at the concentration that completely inhibited the chemo-attractive activity of LTB4 did not completely block the neutrophilic migration induced by the cell-free media from MWCNT-polarized macrophages in the current study. This result implies that, while LBT4 is a major mediator of neutrophilic chemotaxis, other proinflammatory mediators including cytokines and LMs produced take part in neutrophilic recruitment concomitantly. It is known that polarization of M1 macrophages correlated with the production of type 1 proinflammatory cytokines, such as TNF-α and IL-1β, that are implicated in several fibrotic lung diseases in animal models and humans (Hu et al. 2021; Kishore and Petrek 2021). MWCNTs clearly increased the elevation of TNF-α and IL-1β, which indicates the development of acute type 1 inflammation. Further studies will determine the profiles of various cytokines and LMs produced as well as their functions on MWCNT-mediated activation of Alox pathways, macrophage polarization, and neutrophilic inflammation in mouse lungs.

In summary, this study demonstrates that MWCNTs induced the polarization of macrophages to M1 cells under an *in vitro* condition, which mimics certain aspects of the *in vivo* polarization of M1 macrophages in the acute inflammatory response to exposure of MWCNTs in mouse lungs. MWCNTs induced Alox5 expression to increase the synthesis

of proinflammatory LMs and enhance macrophage-activated neutrophilic recruitment. The findings of the present study suggest that induction of Alox5 is a key molecular mechanism to promote acute inflammatory responses to inflammatory and fibrogenic nanoparticles by M1 macrophages, which provides a rational target for pharmacological intervention of M1-mediated neutrophilic inflammation in lungs exposed to inflammatory, fibrogenic, and carcinogenic particles and nanoparticles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank S. Friend for assistance in TEM imaging of MWCNTs and carbon black.

Funding

This work was funded by the NORA Program under Grant 9390BMX (Q.M.) and the NTRC program under Grant 9390HTN (Q.M.), at the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, USA.

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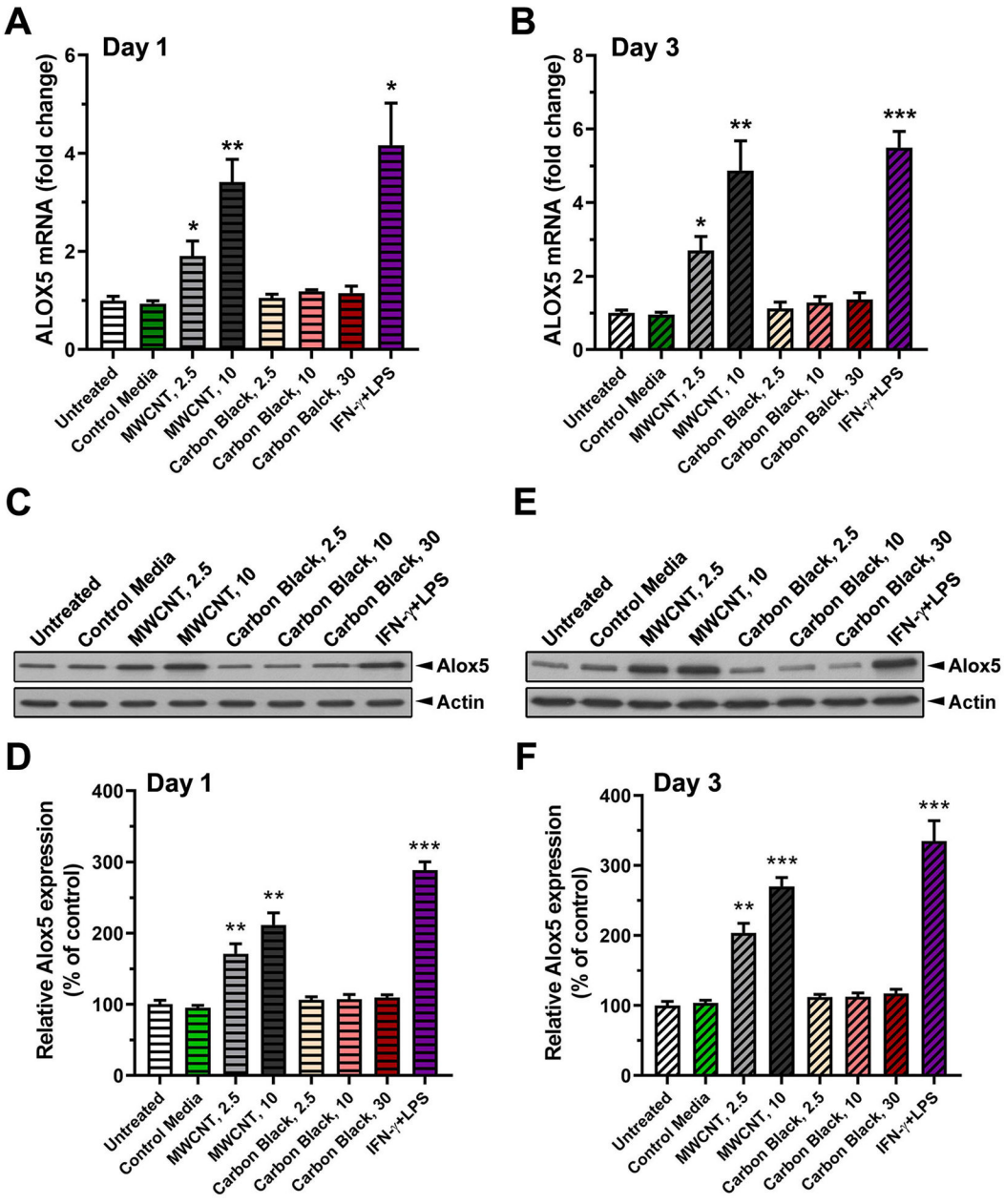

Figure 1.

Induction of Alox5 by MWCNTs. **(A and B)** J774A.1 macrophage cells were untreated or treated with control media (DMEM + 1% FBS), MWCNTs (2.5 or 10 μg/ml), carbon black (2.5, 10, or 30 μg/ml), or M1 inducer (IFN-γ at 20 ng/ml plus LPS at 100 ng/ml) for 1 day (A) or 3 days (B). Total RNA was isolated and mRNA levels of Alox5 were analyzed by RT-qPCR using specific primers and expressed as fold change after normalization with β-actin level for each treatment. Mean ± SEM ($n = 3$), *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, compared to untreated samples of day 1 or day 3, respectively. **(C-F)** Cells treated for 1 day (C and D) or 3 days (E and F) as above were lysed and analyzed by immunoblotting against Alox5 or β-actin (loading control). The representative image was shown from 3 different experiments. The relative amount of Alox5 was normalized to the

amount of β-actin and expressed as % of untreated control at each day and quantification was shown as Mean ± SEM ($n = 3$), **, $p < 0.01$, ***, $p < 0.001$, compared to untreated samples of Day 1 and Day 3, respectively.

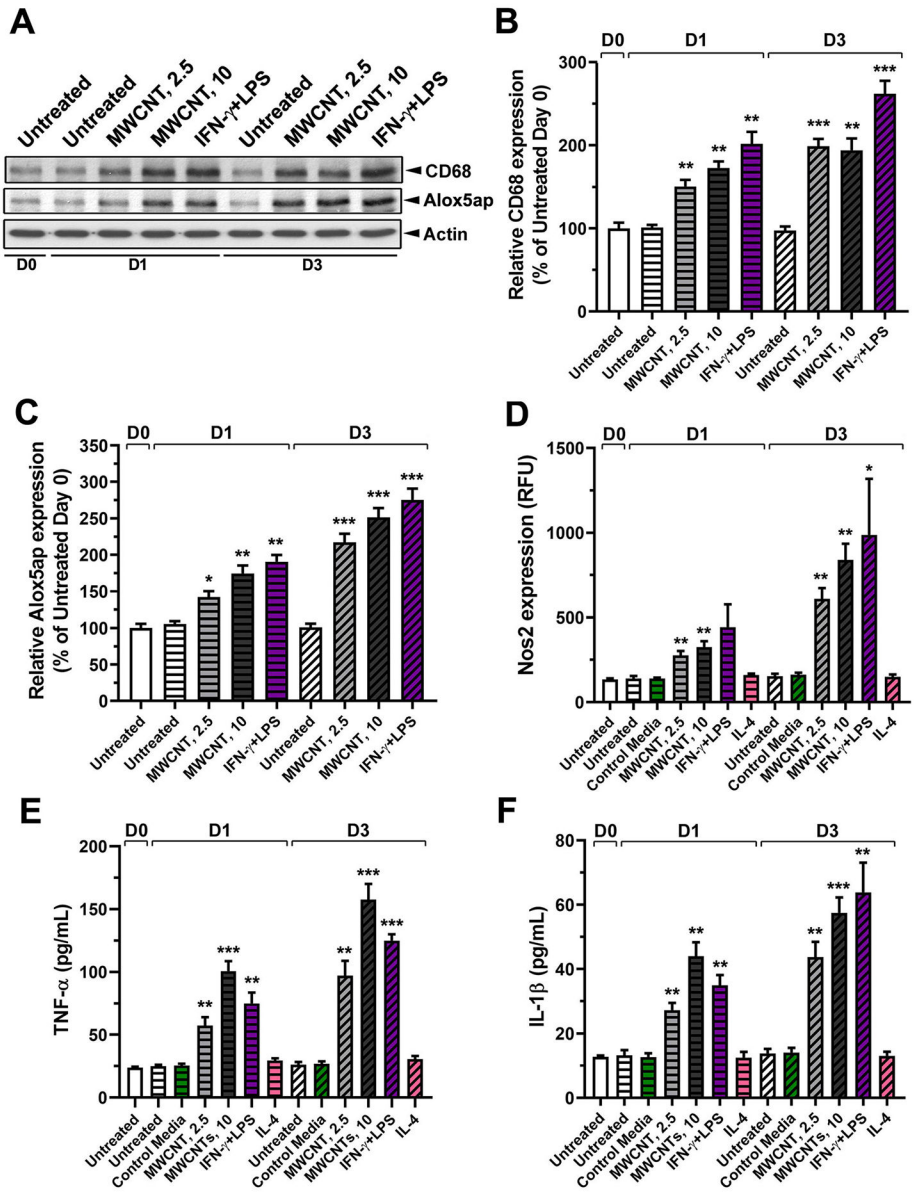

Figure 2.

Induction of M1 macrophage markers with MWCNT treatment. **(A-C)** J774A.1 cells treated as described for Figure 1 were lysed and analyzed by immunoblotting against M1 marker CD68, Alox5ap, or β-actin (loading control). The representative image was shown from 3 separate experiments. The relative amount of CD68 (B) or Alox5ap (C) was normalized with β-actin amount and expressed as % of untreated control at each day and quantification was shown as Mean ± SEM ($n = 3$), *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, compared to untreated samples at Day 1 (D1) and D3, respectively. **(D)** Cells treated as in (A) were used for Nos2 expression detection assay. Relative Nos2 expression was presented as a relative fluorescence unit (RFU). Mean ± SEM ($n = 3$), *, $p < 0.05$, **, $p < 0.01$, compared to untreated at D1 and D3, respectively. **(E and F)** Macrophage cells were untreated or treated with MWCNTs (2.5 or 10 μg/ml), M1 inducer (IFN-γ at 20 ng/ml plus LPS at 100 ng/ml),

or M2 inducer (IL-4 at 20 ng/ml) for 1 or 3 days. Culture supernatants were collected and used to measure the levels of proinflammatory cytokines TNF-α (E) and IL-1β (F) using ELISA. Mean ± SEM ($n = 3$), **, $p < 0.01$, ***, $p < 0.001$, compared to untreated samples at D1 and D3, respectively.

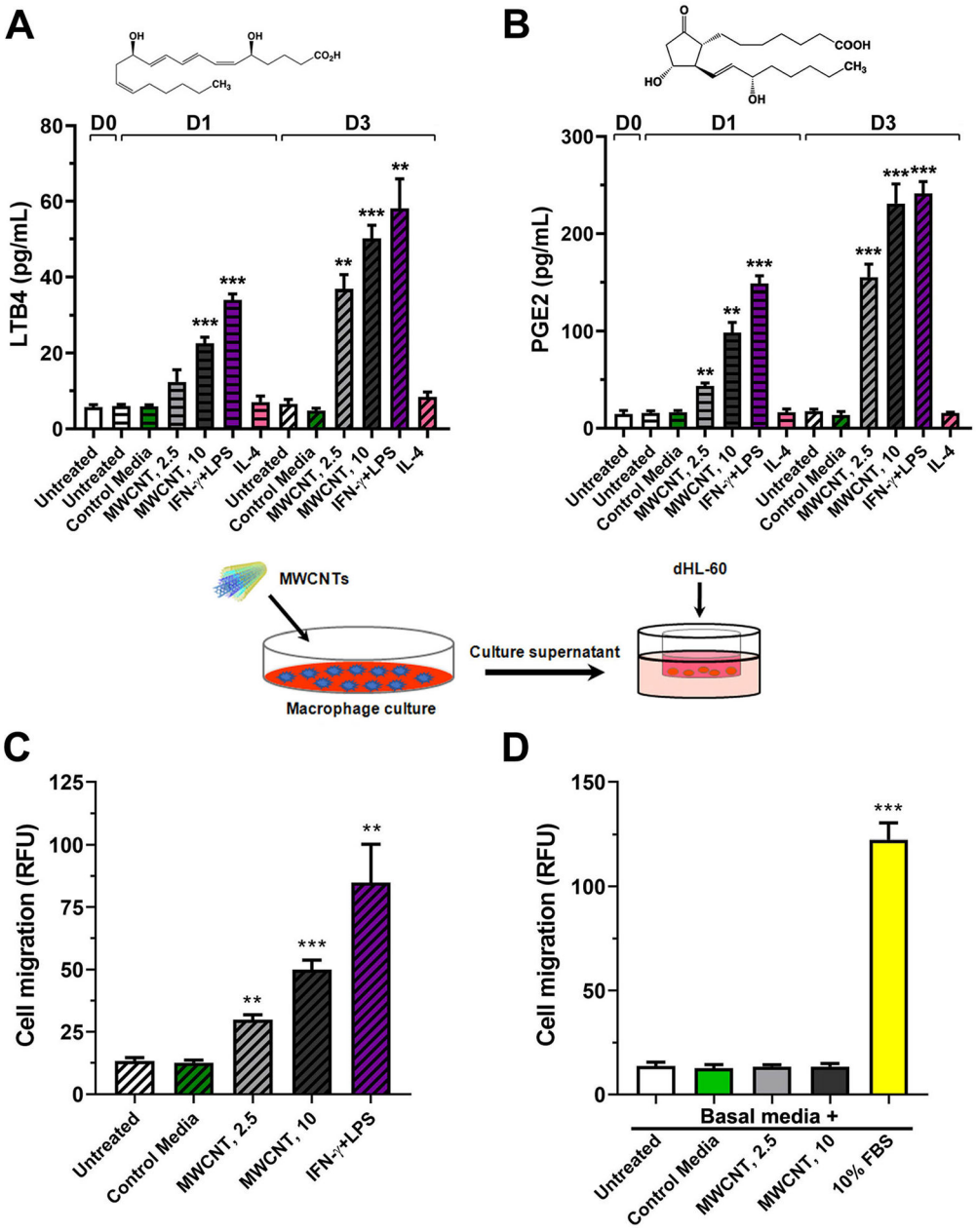

Figure 3.

Increased production of proinflammatory LMs, LTB4 and PGE2, and induction of neutrophil inflammation with MWCNT treatment. **(A and B)** Cells were treated as described in Figure 2 and culture supernatants were collected to measure the levels of proinflammatory LMs LTB4 (A) and PGE2 (B) using ELISA. Mean ± SEM (n = 3). **, $p < 0.01$, ***, $p < 0.001$, compared to untreated samples at D1 and D3, respectively. **(C)** Neutrophilic inflammation was measured using a cell migration activity assy. Culture supernatants from untreated cells or cells treated with control media (DMEM + 1% FBS), MWCNTs (2.5, or 10 μg/ml), or IFN-γ + LPS for 3 days were collected and introduced into lower chamber. ATRA-induced differentiated HL-60 (dHL-60) cell suspension was added into upper insert.

After 6 hours of incubation, migrated cells were collected and lysed. Each cell lysates were incubated with a fluorescence dye and the relative fluorescence unit was measured using a fluorescent plate reader at Ex/Em = 480/520 nm and quantification was shown as RFU. Mean ± SEM ($n = 3$), **, $p < 0.01$, ***, $p < 0.001$, compared to untreated. **(D)** Basal RPMI 1640 media, a mixture of basal media + Control media, basal media + MWCNT (2.5 or 10 μg/ml), or basal media + 10% FBS was used in the lower chamber and cell migration assay was performed as described above. Mean ± SEM ($n = 3$), ***, $p < 0.001$, compared to untreated.

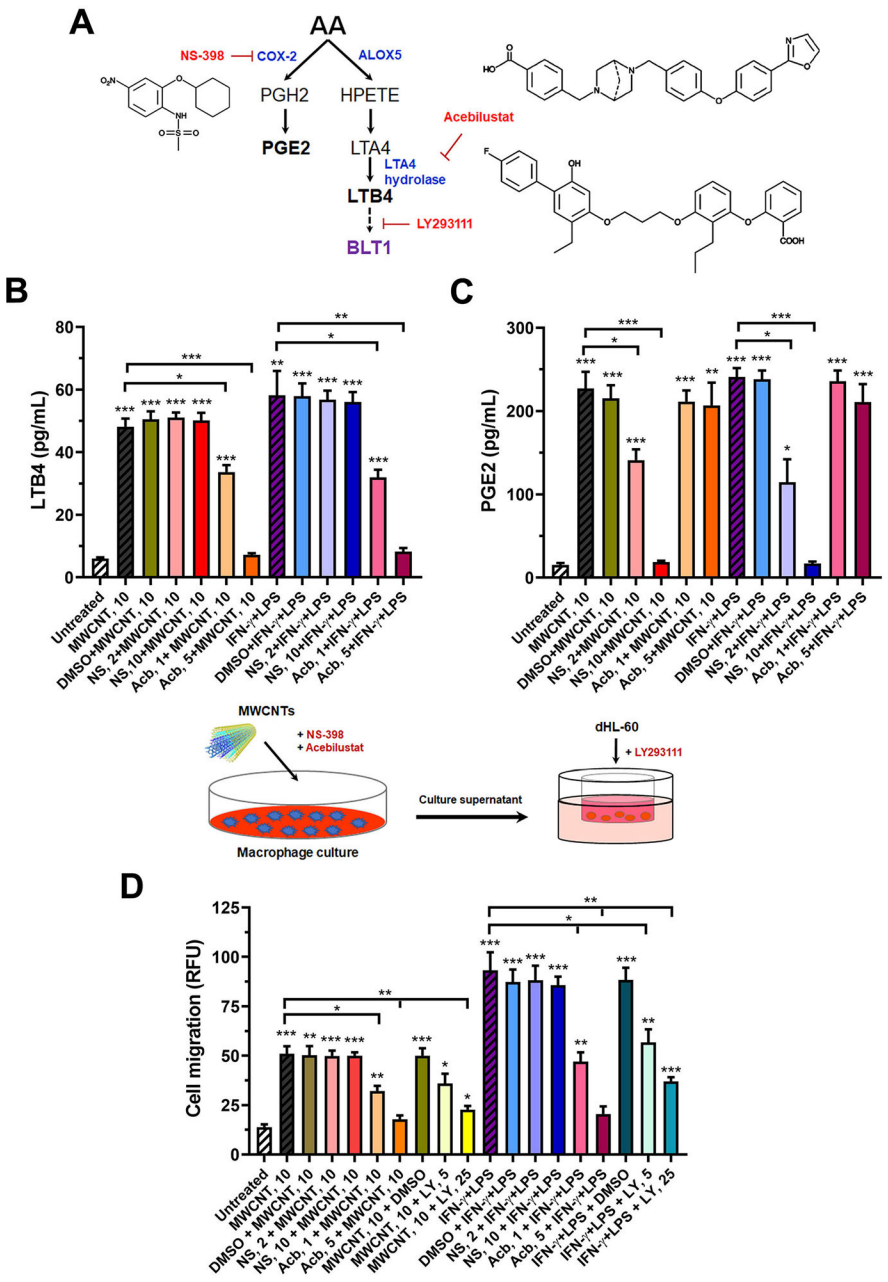

Figure 4.

Specific inhibition of AA metabolic pathways inhibits MWCNT-induced M1 macrophage polarization. **(A)** Schematic representation of the arachidonic acid (AA) metabolism and respective LM biosynthesis along with specific inhibitors. **(B and C)** Macrophage cells were untreated or pretreated with DMSO, NS-398 (NS, 2 or 10 μM), or Acebilustat (Acb, 1 or 5 μM) for 6 hours and then treated with MWCNTs (10 μg/ml) or M1 inducer (IFN-γ at 20 ng/ml plus LPS at 100 ng/ml) for 3 days. Culture supernatants were collected and used to measure the levels of proinflammatory LMs, LTB4 (B) and PGE2 (C) using ELISA. Mean ± SEM ($n = 3$), *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, compared to untreated, MWCNTs, IFN-γ + LPS. **(D)** Culture supernatants from cells treated as described above were used for

cell migration activity assay. A specific LTB4 receptor inhibitor, LY293111 (5 or 25 nM) or DMSO as a vehicle was added directly into the dHL-60 cell suspension. Mean ± SEM (n = 3), *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, compared to untreated.

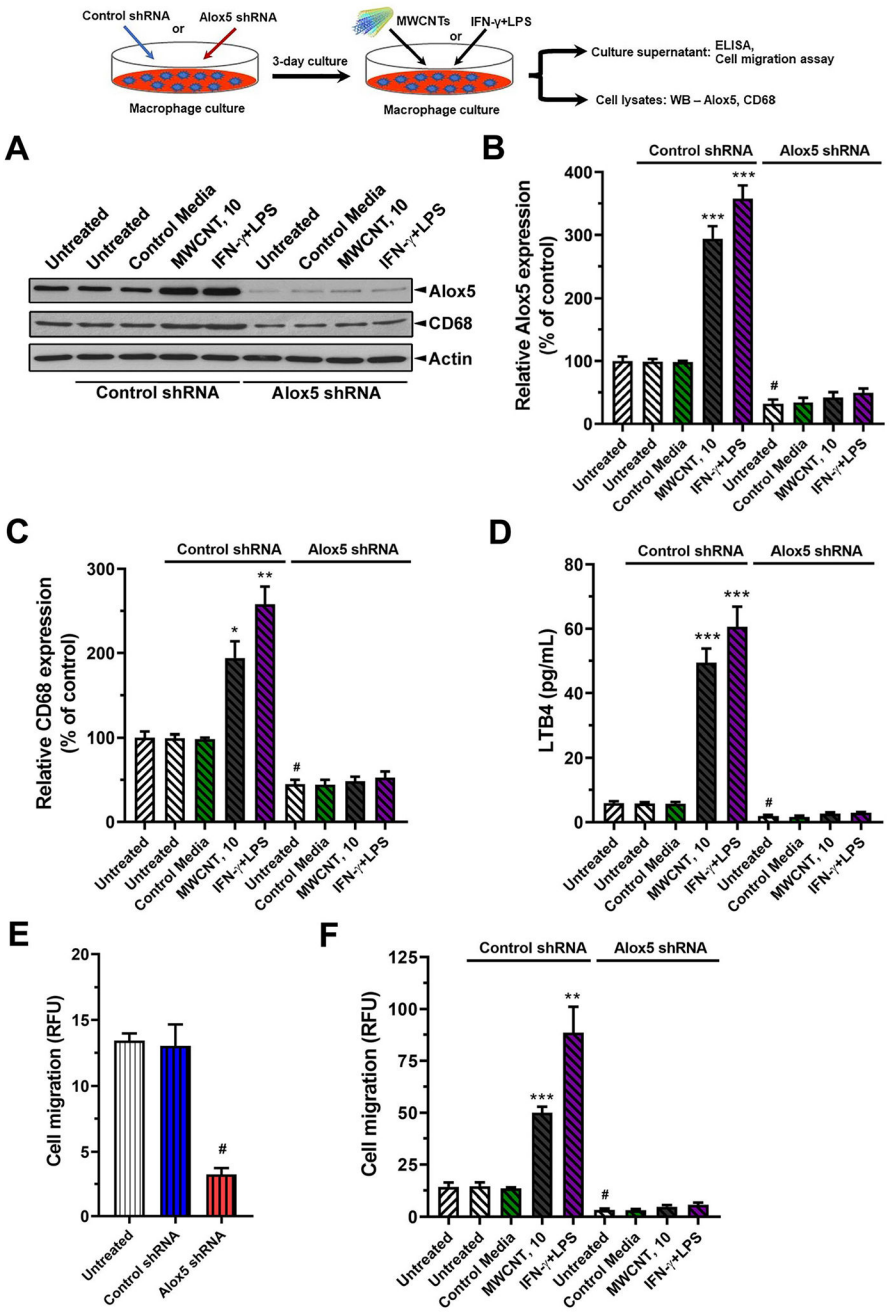

Figure 5.

Knockdown of Alox5 prevents MWCNT-induced M1 macrophage polarization and neutrophilic inflammation. Knockdown of Alox5 and treatment of cells were illustrated in a diagram on top of the figure. **(A-C)** Cells were untreated or transduced with control or Alox5-specific shRNA lentiviral particles for 2 days and then untreated or treated with control media, MWCNT (10 μg/ml), or M1 inducer (IFN-γ at 20 ng/ml plus LPS at 100 ng/ml). After 3 days of incubation, cells were lysed and analyzed by immunoblotting against Alox5, CD68, or β-actin (loading control). The representative image was shown from 3 different experiments. The relative amount of each protein, i.e. Alox5 (B) and CD68 (C),

was normalized to β-actin and expressed as % of untreated control and quantification was shown as Mean ± SEM ($n = 3$), *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, compared to untreated; #, $p < 0.01$, compared to control shRNA-treated cells. **(D)** From cells treated as described above, culture supernatants were collected and used to measure quantitatively the levels of the proinflammatory LM, LTB4, using ELISA. Mean ± SEM ($n = 3$), ***, $p < 0.001$, compared to untreated; #, $p < 0.01$, compared to control shRNA-treated cells. **(E)** Cell migration activity was performed using the culture supernatants from cells untreated or transduced with control or Alox5-specific shRNA particles for 2 days. Mean ± SEM ($n = 3$), #, $p < 0.01$, compared to untreated. **(F)** Culture supernatants from cells as described in (A) were collected and used for cell migration activity assay. Mean ± SEM ($n = 3$), **, $p < 0.01$, ***, $p < 0.001$, compared to untreated; #, $p < 0.01$, compared to control shRNA-treated cells.