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The ETS domain-containing hematopoietic transcription factor PU.1 mediates the induction of arachidonate 5-lipoxygenase by multi-walled carbon nanotubes in macrophages in vitro

Chol Seung Lim[1] · Ja Kook Gu[2] · Qiang Ma[1]

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Abstract

Exposure to fibrogenic multi-walled carbon nanotubes (MWCNTs) induces the production of proinflammatory lipid mediators (LMs) in myeloid cells to instigate inflammation. The molecular underpinnings of LM production in nanotoxicity remain unclear. Here we report that PU.1, an ETS domain-containing master regulator of hematopoiesis, critically regulates the induction of arachidonate 5-lypoxygenase (Alox5) and the production of LMs. MWCNTs (Mitsui-7) at 2.5 or 10 μg/mL induced the expression of Alox5 in murine and human macrophages at both mRNA and protein levels, accompanied by marked elevation of chemotactic LM leukotriene B4 (LTB4). Induction is comparable to those by potent M1 inducers. Carbon black, an amorphous carbon material control, did not increase *Alox5* expression or LTB4 production at equivalent doses. MWCNTs induced the expression of a heterologous luciferase reporter under the control of the murine *Alox5* promoter. Deletional analysis of the 2 kb promoter uncovered multiple inhibitory and activating activities. The proximal 250 bp region had the largest activation that was further increased by MWCNTs. The *Alox5* promoter contains four PU box-like enhancers. PU.1 bond to each of the enhancers constitutively, which was further increased by MWCNTs. Knockdown of PU.1 using specific small hairpin-RNA blocked the basal and induced expression of *Alox5* and the production of LTB4 as well as prostaglandin E2. The results demonstrate a critical role of PU.1 in mediating MWCNTs-induced expression of *Alox5* and production of proinflammatory LMs, revealing a molecular framework where the hematopoietic transcription factor PU.1 is activated to orchestrate multiple proinflammatory responses to sterile particulates.

Keywords MWCNT · PU.1 · Alox5 · Inflammation · Macrophage

Introduction

Exposure to respirable particles and nanoparticles instigates inflammation in the lung contributing to disease progression (Donaldson et al. 2006; Dong and Ma 2015, 2019). Inflammation is actively regulated by macrophages through potent mediators such as proinflammatory and pro-resolving lipid mediators (LMs) (Chiang and Serhan 2020; Lim et al. 2020). In the lung, these cells differentiate into phenotypically polarized cells to mediate pulmonary inflammation and defend against inhaled particulates in an inducer- and context-dependent manner, implicating a complex interplay among myeloid differentiation, regulation of gene expression, and metabolism of LMs in the inflammatory response to microbes and sterile particles (Ma 2020; Wynn et al. 2013).

Macrophages polarize into M1 macrophages (classically activated macrophages) or M2 macrophages (alternatively activated macrophages) (Locati et al. 2013; Murray et al. 2014). M1 and M2 cells exhibit opposing activities during inflammation to regulate dynamic inflammatory processes locally and systemically (Murray and Wynn 2011). M1 macrophages promote acute inflammation, extracellular matrix destruction, and cell death, whereas M2 macrophages foster

✉ Qiang Ma
qam1@cdc.gov

[1] 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 26505, USA

[2] Bioanalytics Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention, Morgantown, WV 26505, USA

tissue repair, fibrosis, angiogenesis, and resolution of inflammation. In mouse lungs exposed to fibrogenic multi-walled CNTs (MWCNTs), polarization of M1 and M2 macrophages predominates at the initiation of inflammation and the transition from acute neutrophilic to monocyte/macrophage-predominate responses, respectively (Dong and Ma 2018a, b). This tight temporal and spatial regulation of the initiation, progression, and resolution of inflammation by macrophages and other immune cells ensures timely and sufficient inflammatory responses to control infection and, at the same time, evades excessive inflammatory damage to host tissue (Dong and Ma 2016; Ma 2020). Proinflammatory and pro-resolving lipid mediators (LMs) secreted from polarized macrophages are key regulators of inflammation and resolution of inflammation in health and disease (Serhan and Savill 2005). We have previously found that M1 macrophages produce proinflammatory LMs, such as leukotriene B4 (LTB4) and prostaglandin E2 (PGE2), in the bronchoalveolar lavage (BAL) fluid and lung tissue to boost the inflammatory response to MWCNTs, whereas M2 macrophages produce specialized pro-resolving LMs (SPMs), such as resolvin D1 (RvD1) and lipoxin, to promote the resolution of lung inflammation induced by MWCNTs (Lim et al. 2020).

Production of proinflammatory LMs and SPMs requires arachidonate lipoxygenases (ALOXs), such as arachidonate 5-, 12-, or 15-lipoxygenase (ALOX5, 12, or 15, respectively), to convert polyunsaturated fatty acids (PUFAs) to a range of bioactive lipids (Dyall et al. 2022). Among them, ALOX5 plays a pivotal role in the regulation of proinflammatory and pro-resolving responses by catalyzing the production of specific LMs (Haeggstrom 2018; Lim et al. 2023). At the early phase of inflammation, ALOX5 catalyzes the conversion of arachidonic acid (AA) to leukotrienes (LTs). LTs stimulate the initiation and progression of inflammation and are implicated in pathologic conditions, such as rheumatoid arthritis and bronchial asthma. LTB4 is a potent LT involved in the initiation of inflammation, regulation of immune response, hematopoiesis, and M1 macrophage facilitation. LTB4 is secreted from M1 macrophages to promote neutrophilic inflammation in response to MWCNTs (Lim et al. 2023).

The ALOX pathways are differentially activated in polarized macrophages to control LM-dependent inflammatory events (Haeggstrom 2018; Serhan 2014). M1 polarization induces ALOX5 and the ALOX5 activating protein (ALOX5AP, 5-lipoxygenase activating protein, FLAP), to increase the biosynthesis of proinflammatory LMs to boost acute inflammation against pathogens. M2 polarization induces ALOX5 and ALOX15 to produce SPMs to promote the resolution of inflammation during infection (Chiang et al. 2012; Werz et al. 2018). In mouse lungs exposed to MWCNTs, M1 macrophages exhibited preferential induction of Alox5 and Alox5ap, while M2 macrophages had high levels of expression of Alox5 and Alox15 (Lim et al. 2020). Furthermore, the knockdown of *Alox5* suppressed M1 polarization and chemotactic migration of neutrophils in addition to blockage of the production and secretion of LTB4 (Lim et al. 2023). The molecular mechanism and pathways mediating Alox5 induction and its function in macrophage and neutrophil activation induced by nanoparticles remain unknown.

In this study, we investigated the induction of *Alox5* gene expression by Mitsui-7 MWCNTs and identified PU.1, an ETS (erythroblast transformation-specific) domain-containing master regulator of hematopoiesis, as a critical transcription factor mediating *Alox5* induction and LM production in macrophages. Specifically, we found that MWCNTS and M1 inducers, but not carbon black, induced *Alox5* expression and LTB4 production from murine and human macrophages. Induction occurred with heterologous reporters under the control of the *Alox5* promoter. Induction involved binding of the hematopoietic transcription factor Pu.1 to four PU-box enhancers found in the *Alox5* promoter. Knockdown of PU.1 using specific small hairpin-RNA blocked the basal and induced expression of *Alox5* and production of LTB4 as well as PGE2. The study demonstrates a critical role of PU.1 in mediating MWCNTs-induced expression of *Alox5* and production of proinflammatory LMs revealing a molecular mechanism by which the hematopoietic master regulator PU.1 is activated to orchestrate multiple proinflammatory responses to sterile nano stimuli in macrophages.

Materials and methods

Particle preparation

The MWCNTs used in this study were obtained from Mitsui & Company (Mitsui-7, XNRI 1, lot # - 0507 2001K28, Tokyo, Japan). Carbon black (CB) was purchased commercially (Printex 90, Degussa Engineered Carbons, L.P., Parsippany, NJ, USA) and was used as a non-fiber, carbon-based particle control for MWCNTs. Some properties of the MWCNTs and CB have been described previously (Lim et al. 2020, 2023; Porter et al. 2010).

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 (Hindman and Ma 2018; Lim et al. 2023). Stock solutions were further diluted with the culture media and sonicated immediately before use. DMEM with 1% FBS was used as a control media. The level of lipopolysaccharides (LPS) in the MWCNTs or CB was determined to be < 0.1 EU/mL (< 0.01 μg/mL) using

the Pierce LAL chromogenic endotoxin quantification kit (Thermo Scientific, Pittsburgh, PA).

Cell culture and treatment

The J774A.1 murine macrophage cell line was purchased from the American Type Culture Collection (TIB-67, ATCC, Manassas, VA, USA). The cells were grown in DMEM with 10% FBS and 1X Antibiotic–Antimycotic (Thermo Fisher Scientific). MWCNTs at 2.5 or 10 µg/mL or CB at 2.5 or 10 µg/mL were used to treat cells followed by continued culture of the cells in an incubator with 5% CO_2 and 37°C till harvest. The DMEM plus 1% FBS media were used to establish a negative control and baseline levels. Some cells were kept as untreated to serve as a cell control in the absence of treatment. Interferon-γ (IFN-γ, Sigma-Aldrich, St. Louis, MO, USA) at 20 ng/mL plus LPS (Sigma-Aldrich) at 100 ng/mL was used as a positive control for induction of *Alox5* mRNA and Alox5 protein. All cells were examined in duplicates and all treatment experiments were conducted 3 times. After treatment with a single dose of MWCNTs or CB, the cells were collected for further analysis.

Isolation of human monocytes and differentiation of monocytes to macrophages

Human whole peripheral blood with EDTA as an anticoagulant was purchased from STEMCELL Technologies (70508, Vancouver, BC, Canada). Human monocytes were isolated from the blood using the EasySep Magnet for column-free immunomagnetic separation and the EasySep direct human monocyte isolation kit according to the manufacturer's protocol (STEMCELL Technologies).

Isolated human monocytes were plated in 6-well tissue culture plates (Fisher Scientific) at 2.5×10^6 cells/well in the ImmunoCult-SF macrophage medium supplemented with M-CSF at 50 ng/mL (both from STEMCELL Technologies). The cells were cultured for 4 days to induce their differentiation into macrophages (human monocyte-derived macrophages or HMDMs). HMDMs were left untreated or were treated with the control medium, MWCNTs at 2.5 or 10 µg/mL, CB at 2.5 or 10 µg/mL, or IFN-γ plus LPS (positive control). After 24 h of incubation, the cells were collected, and total RNA was prepared for quantification of *ALOX5* mRNA. Cell-free supernatants of the culture media were collected and used for quantification of LMs by ELISA.

Quantitative real-time polymerase chain reaction (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 h. Real-time qPCR was performed for 35 cycles with the SYBR Green 1 PCR Master Mix on a 7500 real-time PCR machine (Thermo Fisher Scientific) using specific primers for the mouse *Alox5* (Qiagen, PPM28755C) or *β-actin* (Qiagen, PPM02945B), and human *ALOX5* (Qiagen, PPH02590G-200) or *ACTB* (*β-actin*) (Qiagen, PPH00073G-200). Reactions were run in triplicates for each sample and a dissociation curve was generated. Threshold cycles (Ct) for *Alox5* amplification were normalized to the housekeeping gene *β-actin* (ΔCt). Each experimental sample was referred to its control (ΔΔCt). Relative expression values were calculated as $2^{-\Delta\Delta Ct}$ and expressed as fold change in comparison to untreated control.

Luciferase reporter assay

Luciferase reporter plasmids carrying various lengths of the promoter region of the mouse *Alox5* gene were generated by using PCR and restriction endonuclease digestion as follows. Briefly, the 2-kb promoter sequence upstream of the transcription start site (−2000 bp, 5′-upstream regulatory region or 5′-URR) of mouse *Alox5* was amplified from the C57BL/6 J mouse genomic DNA by using PCR with the forward primer murine Alox5-2000-F and the reverse primer murine Alox5-URR-R (Table 1). The amplified DNA fragment was inserted into the pGL3-Basic vector (Promega, Madison, WI, USA) at the XhoI and HindIII restriction sites to generate the reporter plasmid pGL3-2,000-Luc. Other reporter constructs corresponding to specific deletions in the promoter region (−1500, −1000, −500, and −250) were generated from pGL3-2000-Luc by PCR using corresponding forward primers and Alox5-URR-R as a reverse primer (Table 1). All constructs were confirmed by DNA sequencing analysis. pCMV-sport-β-gal (Thermo Fisher Scientific) was used as a positive control for transfection efficiency in mammalian cells. Cells were co-transfected with 500 ng of

Table 1 Primers for luciferase constructs

Primer name	Primer sequence
mAlox5-2000-F	5′-GAACCTCGAGTAGAGCCATGCAGGCTC TGTG-3′
mAlox5-1500-F	5′-CCTGCTCGAGTACAGGTTCCCCAATAA CAG-3′
mAlox5-1000-F	5′-AGCCCTCGAGAATCAACTGGAGGG CAGGA-3′
mAlox5-500-F	5′-GGGCCTCGAGTTTCTAAGGCTGGTTCC CAG-3′
mAlox5-250-F	5′-TCTTCTCGAGACAGTGAGGGACACTGA AAC-3′
mAlox5-URR-R	5′-GCTCAAGCTTGCGACCGGCCCTGCCTT CATG-3′

a reporter plasmid and 100 ng of the β-gal plasmid using Lipofectamine™ LTX Reagent with PLUS Reagent (Thermo Fisher Scientific) following the manufacturer's instruction. Cells were harvested 24 h after transfection and the luciferase activity was measured by using a GloMax 20/20 luminometer (Promega) following the manufacturer's instruction. Luciferase activities were normalized to the β-gal intensity of the same samples to correct for variations in transfection efficiency among samples.

Chromatin immunoprecipitation assay (ChIP)

ChIP assays were performed using a Magnify chromatin immunoprecipitation kit (Thermo Fisher Scientific, 49–2024) according to the manufacturer's instruction. Cells were treated as described above. Intracellular proteins were crosslinked to DNA using 1% formaldehyde for 10 min at room temperature. Crosslinking was stopped by adding a 10X glycine solution. After two washes with cold 1X PBS, the cells were harvested in the RIPA buffer (150 mM NaCl, 1% IGEPAL CA-630, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris–HCl at pH 8). Nuclei were obtained by centrifugation, followed by treatment with a nucleolytic buffer to release chromosomal DNA. The chromosomal DNA was sonicated using the Covaris S2 ultrasonicator (Woburn, MA, USA) to shear the chromatin to an average size of ~ 200–500 bp. A fraction (10%) of the supernatants was reserved. For immunoprecipitation, the samples were centrifuged at 20,000×*g* at 4 °C for 5 min. One mg of the protein extract underwent pre-clearance with 30 µL of Protein A-Agarose/Salmon Sperm DNA (50% Slurry) for 30 min. After centrifugation to remove the agarose, the supernatant fractions were incubated with five µg of anti-PU.1 rabbit monoclonal antibody (Abcam, ab227835, Waltham, MA, USA) or the same amount of normal rabbit IgG for 4 h at 4°C, followed by an additional incubation with Dynabeads Protein A/G for 1 h at 4°C. The beads were washed with 1X PBS and the immunocomplexes were collected and eluted twice, each with 1% SDS/0.1 M $NaHCO_3$ for 10 min at 65°C. Crosslinks were reversed by adding the reverse crosslinking buffer provided with the kit followed by incubation at 65°C for 4 h. Immunoprecipitated DNAs were purified by treatment with RNase A and proteinase K. Purified DNA was evaluated and analyzed by conventional PCR using the following primer sets specific to the murine *Alox5* gene promoter: mouse *Alox5* promoter P1 (−229/−72; forward primer, 5′-GGCAAGAAGCCATAA AGATGG-3′ and reverse primer, 5′-CATCCTTCTCTC CTGTGGC-3′), P2 (−1024/−863; forward primer, 5′-GAG CAGGGTATTATCGAAGG-3′ and reverse primer, 5′-TGG GCAGCATTAATTTGTCTG-3′), P3 (−1245/−1109; forward primer, 5′-GTAGCAGTCAAAGAGAGGC-3′ and reverse primer, 5′-TCCACAGATCCTTGGCAC-3′)], and P4 (−1788/−1640; forward primer, 5′-TGCGGAATGTCT GACTGTG-3′ and reverse primer, 5′-GGACAAAGCAGA CCTGGC-3′). Amplified DNA fragments were separated in 1.5% agarose gels.

PU.1 knockdown by short hairpin RNA (shRNA)

After one day of plating, J774A.1 cells were transduced with mouse PU.1-specific 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. Cells transduced with control shRNA with scrambled sequence were included as negative controls. Some cells were left un-transduced to serve as a control. Cells were incubated overnight as recommended by the manufacturer. The media were replaced by a complete growth medium, and the cells were cultured for an additional day. A portion of the cells were lysed for isolation of the total RNA (for RT-qPCR) and protein (for immunoblotting). The remaining cells were treated with MWCNTs or CB and subsequently lysed to obtain RNA or protein as described above. The cell-free culture medium was collected for enzyme-linked immunosorbent assay (ELISA) assays.

Immunoblot analysis

J774A.1 cells were treated as indicated and were lysed in a lysis buffer (10 mM Tris, pH 7.4, 1% SDS) with a 1X proteinase inhibitor cocktail (Thermo Fisher Scientific) at the end of the experiment. Cell lysates were collected and sonicated for 10 s. The supernatant was collected, and the protein concentration was determined using a Bicinchoninic acid protein assay kit (Thermo Fisher Scientific). Lysate proteins of 10–20 µg each were resolved on a 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 h at room temperature to block non-specific binding. Primary antibodies used were rabbit anti-Alox5 (1:1000, Abcam, ab169755), rabbit anti-PU.1 (1:1000, Abcam, ab227835) or mouse anti-β-actin (1:4000, Sigma-Aldrich, A5441) antibodies. After incubation with a second antibody, either horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5000, Jackson ImmunoResearch laboratories, 111-035-144) or goat anti-mouse (1:5000, Jackson ImmunoResearch laboratories, 115-035-146, West Grove, PA, USA), immunoreactive bands were visualized with the enhanced chemiluminescence substrates (Thermo Fisher Scientific). Band signals were captured onto an X-ray film by exposure for 30 s 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 (1.52a, NIH, USA). Each

band was normalized to β-actin and the relative amount of the PU.1 protein was calculated.

Enzyme-linked immunosorbent assay (ELISA)

Proinflammatory LMs like LTB4 and PGE2 were detected in cell-free culture supernatants collected from cells treated with the control medium or MWCNTs after lentiviral particle transduction. Both ELISA kits were from MyBioSource (MBS268428 for LTB4, MBS266212 for PGE2, San Diego, CA, USA) and measurement was performed following the manufacturer's protocol using a microplate reader equipped with GEN5 software (Agilent Technologies, Santa Clare, CA, USA).

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 to result 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 Fisher's least significant difference test. All differences were considered significant at $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; compared to untreated control; $\#p < 0.01$, compared to control-treated group. All analyses were carried out using JMP version 16 software (SAS Institute, Cary, NC, USA).

Results

Induction of Alox5 in murine and human macrophages by MWCNTs

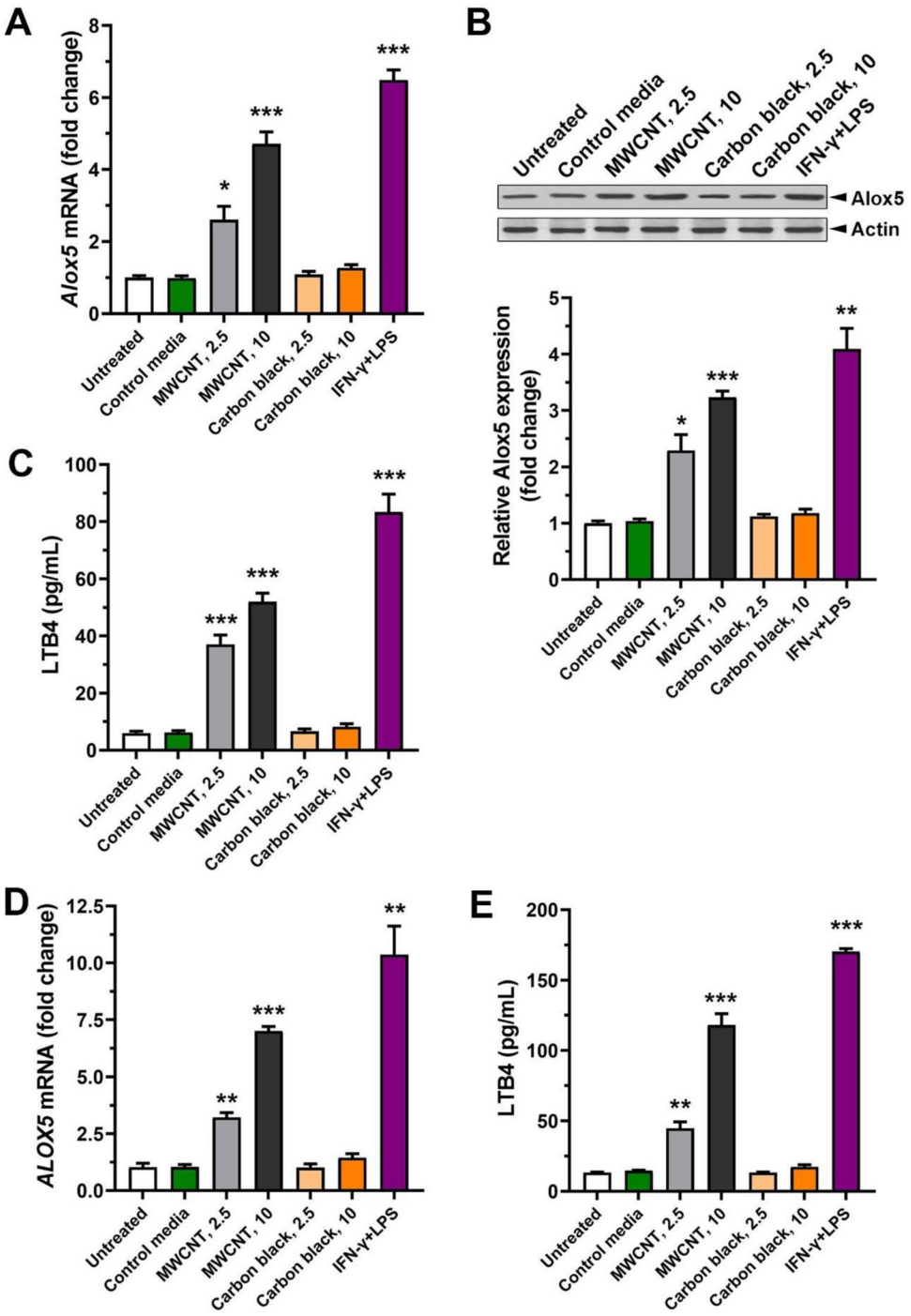
We have previously found that the pulmonary proinflammatory response to nanoparticles by macrophages is dependent on elevated production of proinflammatory lipid mediators in mouse lungs and in vitro, such as the Alox5-dependent production of the leukocyte chemotactic factor LTB4 (Lim et al. 2020, 2023). To elucidate the molecular underpinnings for the regulation of inflammation by MWCNTs, we focused on analyzing the mechanism by which MWCNTs induce *Alox5* mRNA in J774A.1 macrophages.

Figure 1 reveals that the basal levels of *Alox5* mRNA and protein were low in macrophages as determined by RT-qPCR and immunoblotting (Fig. 1A and B, untreated). The vehicle control medium did not affect the *Alox5* mRNA and protein levels at 1-day post-exposure. As expected, the M1 inducer (IFN-γ + LPS, positive control) increased the *Alox5* mRNA by 6.5-fold and protein by 4.1-fold at 1-day post-exposure compared to untreated control. Treatment with MWCNTs at a concentration of 2.5 μg/mL increased the expression of *Alox5* mRNA by 2.6-fold and protein by 2.3-fold at 1-day post-exposure. At 10 μg/mL, MWCNTs increased the *Alox5* mRNA expression by 4.7-fold and protein by 3.3-fold. CB is a paracrystalline carbon material that has the hexagonal carbon structure of CNTs but lacks the ordered, three-dimensional structure of CNTs and therefore, was used as an amorphous, carbonaceous material control for MWCNTs. Treatment with CB at a concentration of 2.5 or 10 μg/mL did not induce the *Alox5* mRNA or protein significantly at 1-day post-exposure, compared to untreated control.

Induction and activation of Alox5 stimulate the production of LTB4 to mediate the acute proinflammatory response. We therefore measured the production of LTB4 (Fig. 1C). MWCNTs at 2.5 μg/mL or 10 μg/mL increased the LTB4 production to 36.98 pg/mL (6.2-fold increase over untreated control) and to 51.98 pg/mL (8.8-fold increase), respectively (Fig. 1C). M1 inducers increased LTB4 production to 83.37 pg/mL (14.0-fold increase). No apparent increase in the LTB4 level was observed in the culture media of the control medium- or CB-treated macrophages.

To examine if MWCNTs induce *ALOX5* mRNA in human macrophages, human peripheral blood monocytes were differentiated into adherent macrophages following stimulation with M-CSF for 4 days. Differentiated HMDMs were left untreated or were treated with the control medium, MWCNTs at 2.5 or 10 μg/mL, CB at 2.5 or 10 μg/mL, or IFN-γ plus LPS. After 24 h of incubation, the *ALOX5* mRNA levels were examined by RT-qPCR (Fig. 1D). Like the murine macrophages, untreated human macrophages had low levels of basal expression of *ALOX5* mRNA. The vehicle control medium did not affect the *ALOX5* mRNA expression. Treatment with MWCNTs increased the expression of *ALOX5* mRNA by 3.2-fold at 2.5 μg/mL or 7.0-fold at 10 μg/mL at 1-day post-exposure, respectively. Treatment with CB at concentrations of 2.5 or 10 μg/mL for one day did not induce the *ALOX5* mRNA, compared to untreated control. As expected, the M1 inducer (IFN-γ + LPS) increased the *ALOX5* mRNA by 10.4-fold at 1-day post-exposure, compared to untreated control.

We also measured the production and induction of LTB4 in HMDMs by ELISA assay (Fig. 1E). MWCNTs at 2.5 μg/mL or 10 μg/mL increased the LTB4 production to 44.73 pg/mL (3.4-fold increase over untreated control) or to 118.17 pg/mL (8.9-fold increase), respectively. M1 inducers increased LTB4 production to 170.28 pg/mL (12.9-fold increase), whereas no apparent increase in the LTB4 level was observed in the culture media of the control medium- or CB-treated macrophages (Fig. 1E). These results indicate that the MWCNT-induced Alox5 expressions in murine



macrophages and human macrophages were comparable to each other.

Induced transactivation of the *Alox5* promoter by MWCNT

Induction of *Alox5* mRNA by MWCNTs suggests a mechanism of transcriptional control of *Alox5* mRNA expression by MWCNTs. We therefore examined the transcriptional activation of the *Alox5* gene promoter. The 2 kilobase sequence upstream of the transcription start site of the murine *Alox5* gene was obtained from the genomic DNA of a C57BL/6 J mouse by PCR and was used to construct a luciferase report plasmid (pGL3-2000-Luc) and its 5′-deletion mutants as shown in Fig. 2. The reporter plasmids were transfected to J774A.1 cells together with the pCMV-sport-β-gal plasmid (control for transfection efficiency). The *Alox5* promoter activity was determined using the luciferase

◂**Fig. 1** Induction of Alox5 by MWCNTs. **A** 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 or 10 μg/mL), or M1 inducer (IFN-γ at 20 ng/mL plus LPS at 100 ng/mL) for 1 day. 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 ($n = 3$), $*p < 0.05$, $***p < 0.001$, compared to untreated samples. **B** Cells treated for 1 day as above were lysed and analyzed by immunoblotting against Alox5 or β-actin (loading control). 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 ($n = 3$), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, compared to untreated samples. **C** Culture supernatants collected from the macrophage cells untreated or treated as above for 1 day were used to measure the levels of proinflammatory lipid mediator LTB4 using ELISA. Mean ± SEM ($n = 3$), $***p < 0.001$, compared to untreated samples. **D** HMDMs were untreated or treated with control media (DMEM + 1% FBS), MWCNTs (2.5 or 10 μg/mL), carbon black (2.5 or 10 μg/mL), or M1 inducer (IFN-γ at 20 ng/mL plus LPS at 100 ng/mL) for 1 day. 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 ($n = 3$), $**p < 0.01$, $***p < 0.001$, compared to untreated samples. **E** Culture supernatants collected from HMDMs untreated or treated as above for 1 day were used to measure the levels of proinflammatory lipid mediator LTB4 using ELISA. Mean ± SEM ($n = 3$), $**p < 0.01$, $***p < 0.001$, compared to untreated samples (color figure online)

reporter assay expressed as RLU after normalization with β-gal intensity. The luciferase plasmid without the *Alox5* promoter (pGL3-basic-Luc) showed a detectable, low-level luciferase activity. The pGL3-2000-Luc containing the 2 kb promoter sequence had a promoter activity of larger than 11.2-fold over the background (pGL3-basic-Luc). Deletion of the 500 bp at the 5′-end (pGL3-1500-Luc) reduced the activity of the 2 kb promoter by 34.2%. Further deletions at −1000, −500, and −250 bp steadily increased the activities, with the deletion at −250 bp (i.e., pGL3-250-Luc) producing the highest promoter activity of 15.9-fold over the background, indicating a strong proximal promoter for the basal expression of *Alox5*.

Next, we examined if MWCNTs induce transactivation of the Alox5 promoter. Un-transfected and untreated macrophages were used to provide a baseline activity. Macrophages transfected with the pGL3-250-Luc plus pCMV-sport-β-gal were used to test each treatment. Macrophages transfected with pGL3-basic-Luc plus pCMV-sport-β-gal were used as a control for each treatment. The cells were treated with the control medium, MWCNTs at 10 μg/mL, CB at 10 μg/mL, or the M1 inducer (IFN-γ at 20 ng/mL plus LPS at 100 ng/mL). Luciferase activities were determined at 1-day post-exposure (Fig. 3). Macrophages transfected with pGL3-basic-Luc had low levels of luciferase activities comparable to the baseline activity with or without the treatments. Transfection with the pGL3-250-Luc plasmid produced an activity of 18.5-fold over the pGL3-basic-Luc activity treated with the control medium. MWCNTs at 10 μg/mL further increased the activity to 4.2-fold over the pGL3-250-Luc treated with the control medium, whereas CB at 10 μg/mL had only a marginal increase in luciferase activity over the -pGL3-250-Luc with the control medium. As a positive control, treatment with IFN-γ + LPS produced the highest activity with a more than 5.5-fold increase in activity compared to the control medium.

Induced binding of PU.1 to the *Alox5* promoter at multiple enhancers by MWCNTs

Induced transactivation of the *Alox5* promoter by MWCNTs confirmed the transcriptional activation of *Alox5* expression by the nanotubes. We therefore investigated specific transcription factors that mediate the induction of the gene. Inspection of the 2 kb upstream regulatory region of mouse *Alox5* revealed a TATAA-less promoter with several putative enhancer sequences for specific transcription factors. Among the sequences, four purine-rich (PU) box-like enhancers were identified at positions of −137, −937, −1166, and −1711. The PU box-like enhancer sequence resembles the binding sequence (xxAGGAAxx) for the transcription factor PU.1, an E26 transformation-specific (ETS)-domain-containing transcription factor required for myeloid and B-lymphoid cell development (Klemsz et al. 1990; McKercher et al. 1996; Pham et al. 2013; Scott et al. 1994). Hence, the sequences were named as putative PU.1 binding sites 1, 2, 3, and 4, respectively (Fig. 4). To examine if PU.1 indeed binds to these enhancers in the genomic *Alox5* promoter, we performed a ChIP assay using an anti-mouse PU.1 antibody. The results revealed that PU.1 binds to each of the PU-Box-like enhancers in macrophages constitutively. Moreover, the binding of PU.1 at each enhancer was markedly increased by treatment with MWCNTs at the concentration of 10 μg/mL. The results identified PU.1 as a transcription factor that likely mediates the induced transactivation of the *Alox5* promoter by MWCNTs by binding to the four PU-box-like enhancers within the 2 kb upstream regulatory region of the mouse *Alox5* promoter.

PU.1 as a major mediator of induced transcription of *Alox5* by MWCNTs

To establish the role of PU.1 in the basal and induced expression of *Alox5*, the PU.1 gene was knocked down in macrophages using a PU.1-specific shRNA. Figure 5 showed that the transduction of PU.1-specific shRNA into macrophages reduced the *Alox5* mRNA level by 76.5% compared to cells without the transduction and cells transduced with a control shRNA (Fig. 5A). Immunoblotting revealed that macrophages transduced with PU.1-specific shRNA had a 73.9%

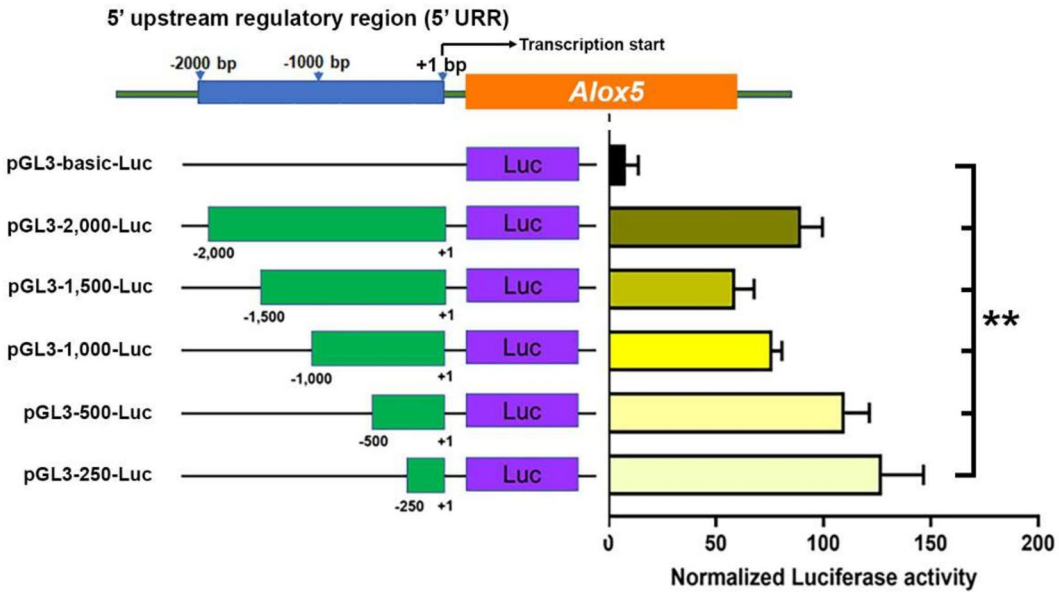


Fig. 2 *Alox5* promoter transactivation in J774A.1 cells. J774A.1 cells were co-transfected with pGL3-basic plasmids or the indicated plasmids with pCMV-sport-β-gal plasmid. After 24 h incubation, the *Alox5* promoter activity was determined by the luciferase reporter gene assay as described in the Methods section. Relative light units (RLU) were determined, normalized with β-gal intensity, and expressed as normalized luciferase activity. Mean ± SEM ($n = 3$), $^{**}p < 0.01$, compared to pGL3-basic-Luc-transfected samples

decrease of the Alox5 protein, compared to cells without the transduction and cells transduced with the control shRNA (Fig. 5B).

We then examined if the PU.1 knockdown affected the MWCNT-induced expression of *Alox5*. Macrophages transduced with PU.1 shRNA were treated with MWCNTs at 10 μg/mL or with the M1 inducer for 1 day. Induction of *Alox5* mRNA was apparent in cells transduced with the control shRNA with a 4.9-fold increase by MWCNT at 10 μg/mL and a 6.3-fold increase by the M1 inducer over untreated control, respectively. However, no induction of *Alox5* mRNA was observed in cells transduced with the PU.1 shRNA by either MWCNTs or the M1 inducer. CB at 10 μg/mL did not induce *Alox5* mRNA in either control shRNA- or PU.1 shRNA-transduced cells (Fig. 5C).

To examine the functional impact of PU.1 knockdown, we analyzed the production of proinflammatory LMs LTB4 and PGE2 (Fig. 6). Transduction of the PU.1 shRNA effectively reduced LTB4 (2.3 pg/mL) production by 63.7% and PGE2 (6.1 pg/mL) production by 58.2%, compared to control shRNA-transduced cells (LTB4, 6.3 pg/mL and PGE2, 14.5 pg/mL, respectively) (Fig. 6A and B). Treatment of PU.1 shRNA-transduced cells with MWCNTs at 10 μg/mL for an additional 1 day slightly increased the production of LTB4 (8.0 pg/mL) and PGE2 (35.6 pg/mL). However, cells transduced with control shRNA showed a drastic increase of LTB4 (52.5 pg/mL) and PGE2 (222.4 pg/mL) production by 15.7-fold and 8.3-fold, respectively, upon treatment with MWCNTs at 10 μg/mL (Fig. 6A and B). These results indicate that PU.1 is a key transcription factor mediating the transactivation of the *Alox5* promoter and induction of *Alox5* mRNA and Alox5 protein by MWCNTs, which account for the increased production of proinflammatory LMs LTB4 and PGE2.

Discussion

In this study, we investigated the induction of Alox5 and the production of proinflammatory LM LTB4 in macrophages by inflammatory and fibrogenic Mitsui-7 MWCNTs. Alox5 and LTB4 are known to have a role in the initiation and progression of acute inflammation and polarization of M1 macrophages (Lim et al. 2023). Here we showed that the hematopoietic master regulator PU.1 critically regulates MWCNT-induced expression of Alox5 leading to increased production of proinflammatory LMs and elevated acute inflammation. Elucidating the mechanism and pathways of Alox5 induction provides new molecular insights into macrophage polarization and lung inflammation that are relevant 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 respirable particles.

Macrophages are major host-defending cells to protect against inhaled pathogens and sterile particulates in the lung. Upon stimulation by particles, lung macrophages adopt distinct phenotypes or polarization in an inducer- and context-dependent manner (Ma 2020). Polarized macrophages in

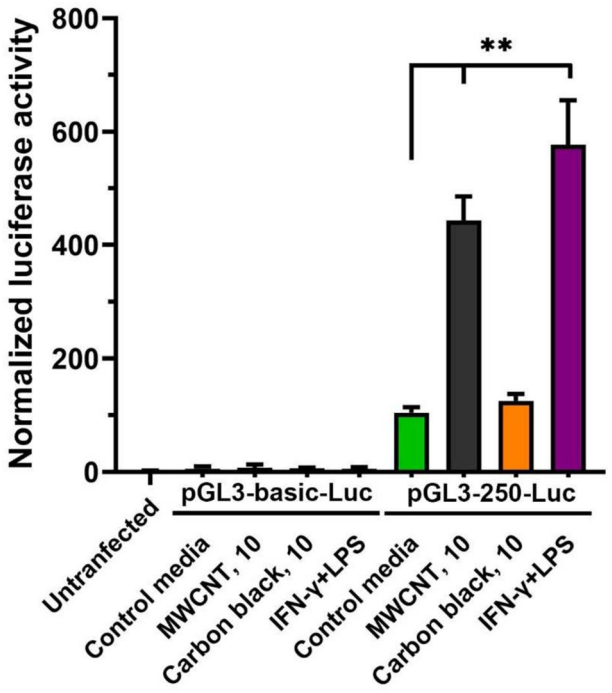


Fig. 3 Transactivation of the *Alox5* promoter with MWCNT exposure. J774A.1 cells were untreated or co-transfected with pGL3-basic-Luc or pGL3-250P-Luc with pCMV-sport-β-gal plasmid. After 24 h incubation, cells were further treated with control media, MWCNT (10 μg/mL), CB (10 μg/mL), or M1 inducer (IFN-γ at 20 ng/mL plus LPS at 100 ng/mL). After 1 day of incubation, luciferase reporter gene assays as described in the Methods section. Relative light units (RLU) were determined, normalized with β-gal intensity, and expressed as normalized luciferase activity. Mean ± SEM ($n = 3$), $^{**}p < 0.01$, compared to control media-treated samples within the pGL3-250-Luc plasmids-transfected group

turn differentially regulate lung inflammation induced by particles (Dong and Ma 2018a; Lim et al. 2020). M1 macrophages are polarized by M1 inducers, such as IFN-γ plus LPS, and exhibit a T helper 1 (Th1)-associated phenotype to promote inflammation, particle clearance, and cell death, whereas M2 macrophages are polarized by M2 inducers, such as IL-4 and IL-13, and exhibit a Th2-associated phenotype to stimulate tissue repair, fibrosis, and resolution of inflammation (Dong and Ma 2016). M1 macrophages produce proinflammatory cytokines and LMs, such as LTB4 and PGE2, that boost acute inflammation, while M2 macrophages produce anti-inflammatory cytokines, such as IL-10 and IL-13, and pro-resolving LMs, such as lipoxins and RvD1, that promote resolution of inflammation and tissue repair (Lim et al. 2020; Werz et al. 2018). Dysregulation of cytokine and LM production contributes to the development of a range of pathologic inflammatory conditions and their progression to fibrosis and cancer (Wynn and Vannella 2016).

The ALOX-mediated biosynthesis of LMs during macrophage polarization has been well characterized (Haeggstrom 2018; Lim et al. 2020; Werz et al. 2018). Polarization of M1 macrophages induces ALOX5 and ALOX5AP in human and mouse M1 macrophages, resulting in increased biosynthesis of proinflammatory LMs. Polarization of M2 macrophages induces ALOX15 along with ALOX5 to increase the production of pro-resolving LMs. Aberrant functions of ALOX5 have been associated with asthma, atherosclerosis, and several cancers (He et al. 2020; Radmark et al. 2015). LTB4 derives from the Alox5 pathway and functions as an important proinflammatory mediator serving as a potent chemoattractant to neutrophils and inducing the adhesion and activation of leukocyte on the endothelium (Afonso et al. 2012; McMillan and Foster 1988; Sheppe and Edelmann 2021). LTB4 also plays a role in the development

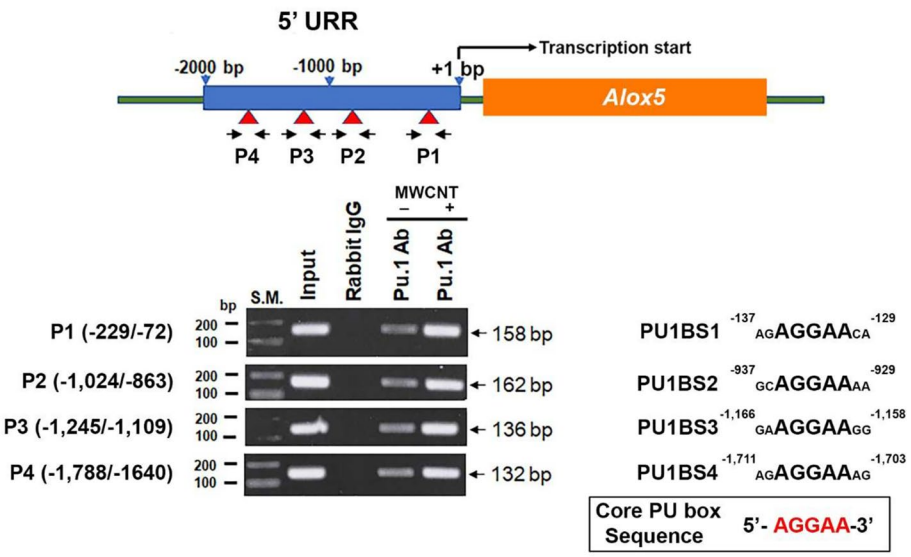


Fig. 4 The transcription factor PU.1 binding to *Alox5* promoter. ChIP assays with J774A.1 cells were performed using anti-PU.1 antibody or rabbit IgG. Co-immunoprecipitated DNA was quantified by PCR with specific primer sets (Table 2) for the indicated region of the mouse *Alox5* gene. Within the indicated promoter region, the putative PU.1 binding sequence (xxAGGAAxx) was identified in bold, and each binding site was presented as a PU.1 binding site 1 (PU1BS1), PU1BS2, PU1BS3, or PU1BS4, respectively. Core PU box sequence was highlighted

Table 2 Primers for ChIP assay

PU.1 binding	Primer name	Primer sequence
Promoter 1 (P1)	mAlox5-chip-F1	5′-GGC AAG AAG CCA TAA AGA TGG-3′
	mAlox5-chip-R1	5′-CAT CCT TCT CTC CTG TGG C-3′
Promoter 2 (P2)	mAlox5-chip-F2	5′-GAG CAG GGT ATT ATC GAA GG-3′
	mAlox5-chip-R2	5′-TGG GCA GCA TTA ATT TGT CTG-3′
Promoter 3 (P3)	mAlox5-chip-F3	5′-GTA GCA GTC AAA GAG AGG C-3′
	mAlox5-chip-R3	5′-TCC ACA GAT CCT TGG CAC-3′
Promoter 4 (P4)	mAlox5-chip-F4	5′-TGC GGA ATG TCT GAC TGT G-3′
	mAlox5-chip-R4	5′-GGA CAA AGC AGA CCT GGC-3′

of chronic inflammatory disease, such as asthma, arthritis, and atherosclerosis, and certain cancers (He et al. 2020; McMillan and Foster 1988).

ALOX5 is regulated at multiple levels. ALOX5 requires ALOX5AP for activation. ALOX5AP assists ALOX5 to translocate from the cytoplasm to cell membranes where ALOX5 obtains its polyunsaturated fatty acid substrates, such as arachidonic acid, and becomes activated (Bell and Harris 1999; Dixon et al. 1990). We have previously shown that Alox5ap is co-induced with Alox5 by MWCNTs and M1 inducers in murine macrophages, indicating a concerted stimulating effect of MWCNTs on both proteins to activate the Alox5 pathway in M1 macrophages (Lim et al. 2023). In this study, we focused on analyzing the transcriptional regulation of *Alox5* gene expression by MWCNTs. Alox5 has been recognized as a key regulating protein of hematopoietic development. Alox5 is expressed and induced in many cell types developmentally and in adulthood in the developmental state-, tissue-, cell type-, and inducer-dependent manners (Chen et al. 2017; Steinhilber 1994). The human *ALOX5* promoter is characterized as a TATAA-less promoter with several consensus cis-acting elements in the proximal region, including the binding sites for serum protein 1 (Sp1) and early growth response protein 1 (Egr-1) within a GC-rich sequence between 179 and 145 base pairs upstream of the ATG translation codon. This finding suggests that the basal and inducible transcription of the *ALOX5* gene involves an interplay among Sp1, Egr-1, and other transcription factors within the GC-rich promoter region (Hoshiko et al. 1990). Like the human *ALOX5* promoter, the mouse *Alox5* gene promoter lacks a typical TATAA box. The mouse promoter contains several consensus-binding sites for transcription factors, including NF-κB, glucocorticoid receptors, GATA, AP1, and Ets proteins (Silverman et al. 2002). Overall, the functional significance of these factors in pulmonary inflammation remains largely unclear. Particularly, the role of hematopoietic cell-specific transcription factors such as PU.1 in transactivating *Alox5* and other proinflammatory genes has not been well studied.

PU.1, encoded by the *SPI1* gene, is a member of the Ets family of transcription factors (Klemsz et al. 1990). PU.1 contains three functional domains. The ETS domain has a winged-helix-turn-helix structure and is responsible for the binding of PU.1 to DNA sites with a central [(G/A)GAA] sequence within the promoter of target genes (Klemsz et al. 1990). A transactivation domain is required for the M-CSF-dependent proliferation of macrophages (Celada et al. 1996). The PEST domain is rich in proline (P), glutamic acid (E), serine (S) and threonine (T) residues and is involved in controlling protein stability and proteolytic degradation. PU.1 is preferentially expressed in hematopoietic tissues and is essential for the development and maturation of myeloid cells including granulocytes, monocytes, macrophages, and lymphoid cells (Burda et al. 2010; Scott et al. 1994). For macrophages, PU.1 is abundantly expressed in terminally differentiated macrophages and dendritic cells (DC) and is necessary for the differentiation of macrophages (Li et al. 2020). PU.1 regulates the expression levels of genes involved in macrophage differentiation (Dahl and Simon 2003; Li et al. 2020). Cytokine receptors involved in the development of monocytes, including the macrophage colony-stimulating factor receptor (M-CSFR), granulocyte colony-stimulating factor receptor (G-CSFR), granulocyte–macrophage colony-stimulating factor receptor (GM-CSFR), and interleukin-7 receptor (IL-7R), are regulated transcriptionally by PU.1 (Friedman 2002; Turkistany and DeKoter 2011). PU.1 also controls the proliferation of macrophages by upregulating M-CSFR. Recent studies revealed that PU.1 is an essential regulator of pro-fibrotic cells in fibrosis where PU.1 induces the polarization of resting and inflammatory fibroblasts into fibrotic fibroblasts (Wohlfahrt et al. 2019). Expression of PU.1 appears to regulate microglial homeostasis in amyloid-β-induced inflammation in the brain (Cakir et al. 2022).

Given the critical role of PU.1 in myeloid differentiation and function, a role of PU.1 in MWCNT-stimulated macrophage activation is suggested and investigated in this study. Several lines of evidence from this study established PU.1 as a critical transcription factor mediating the basal expression and induction of Alox5 in mouse and human macrophages. It is worth noting that the murine J774A.1 cells used in the study are M1-prone macrophages and exhibit certain features of alveolar macrophages, such as

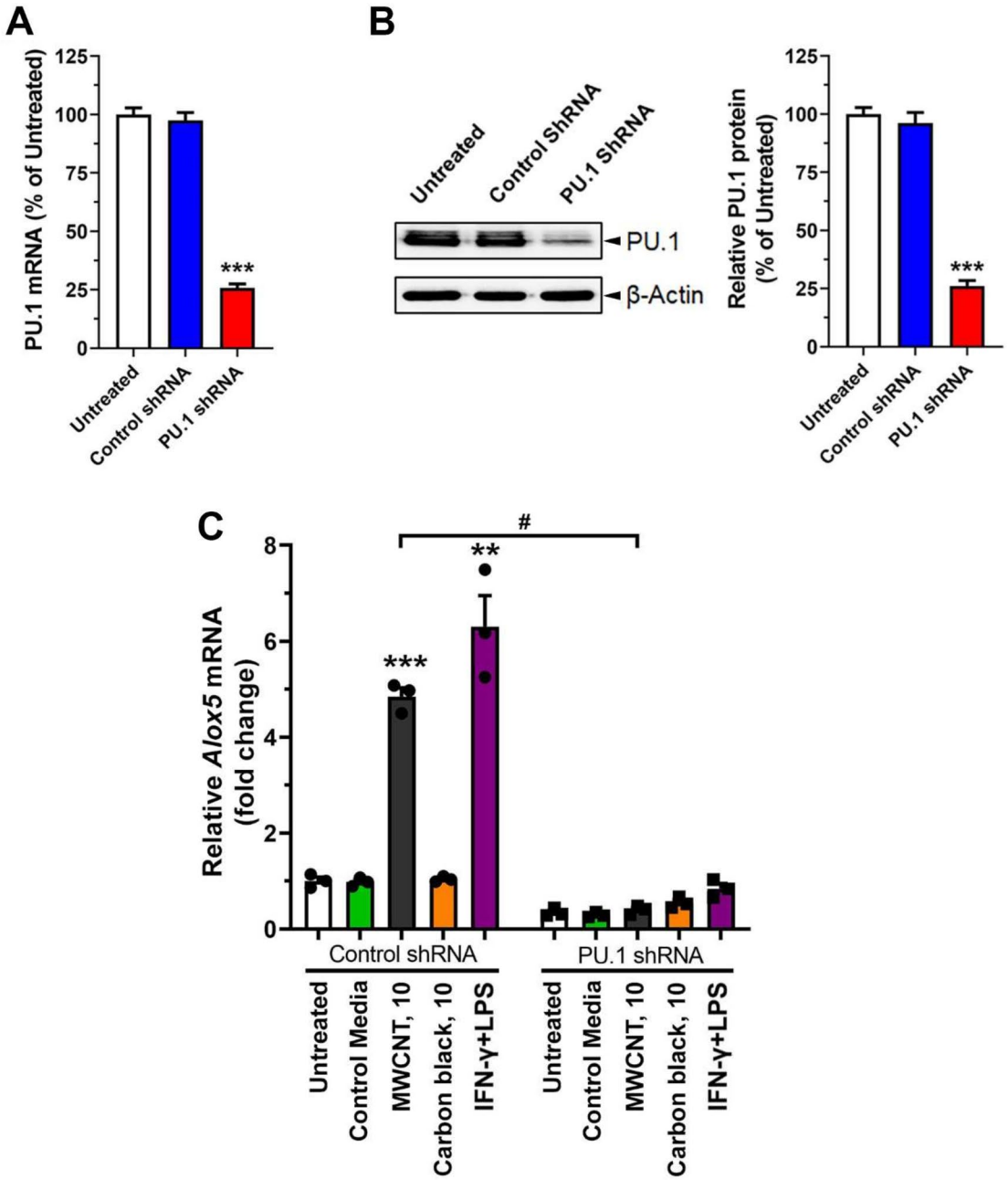


Fig. 5 Effect of PU.1 knock-down on Alox5 expression. **A** Cells were untreated or transduced with control or PU.1-specific shRNA lentiviral particles for 2 days. Total RNA was isolated and mRNA levels of PU.1 were analyzed by RT-qPCR using specific primers and expressed as the percentage of untreated control after normalization with β-actin level for each treatment. Mean ± SEM (n = 3), ***$p < 0.001$, compared to untreated samples. **B** Total proteins were isolated from cells treated like the above used for immunoblotting analysis against mouse PU.1 or β-actin (a loading control). A representative image was shown from 3 different experiments. The relative amount of PU.1 protein was normalized to β-actin and expressed as % of untreated control and quantification was shown as Mean ± SEM ($n = 3$), ***$p < 0.001$, compared to untreated or control shRNA-treated cells. **C** Cells were untreated or transduced with control or mouse PU.1-specific shRNA lentiviral particles for 2 days and then untreated or treated with control media, MWCNT (10 μg/mL), CB (10 μg/mL), or M1 inducer (IFN-γ at 20 ng/mL plus LPS at 100 ng/mL). After 1 day of incubation, total RNA was isolated and mRNA levels of *Alox5* were analyzed by RT-qPCR using specific primers and expressed as fold change of untreated control after normalization with β-actin level for each treatment. Mean ± SEM ($n = 3$), **$p < 0.01$; ***$p < 0.001$, compared to untreated samples; #$p < 0.001$, compared to control shRNA-transduced samples

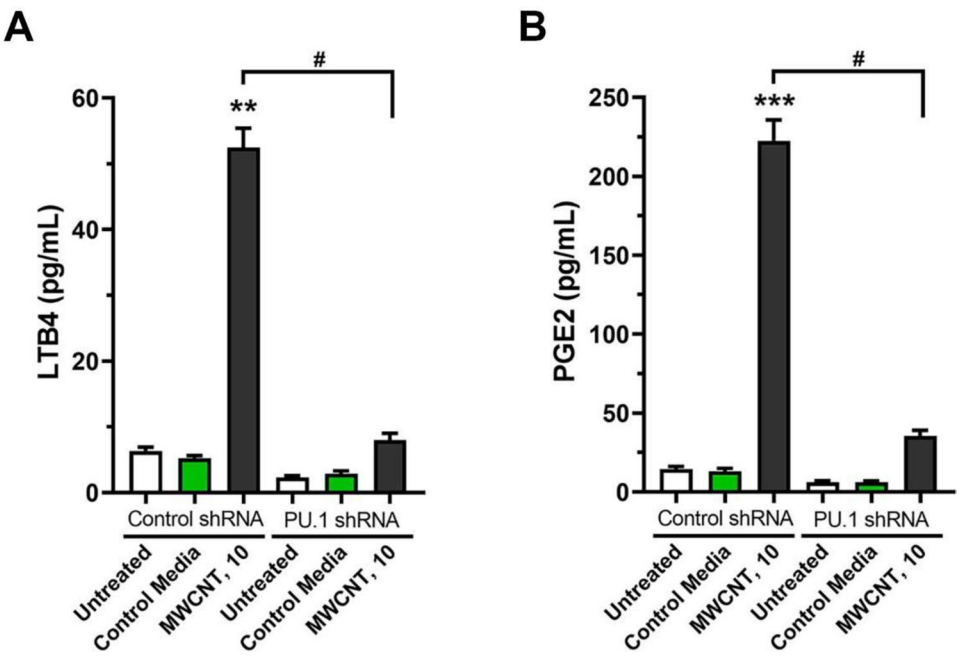


Fig. 6 Effect of PU.1 knockdown on production of proinflammatory lipid mediators. Cells were untreated or transduced with control or mouse PU.1-specific shRNA lentiviral particles for 2 days and then untreated or treated with control media or MWCNT (10 µg/mL). After 1 day of incubation, the culture supernatants were collected and used to measure quantitatively for the levels of the proinflammatory LMs, **A** LTB4 or **B** PGE2, using ELISA. Mean ± SEM ($n = 3$), $^{**}p < 0.01$; $^{***}p < 0.001$, compared to untreated; $\#p < 0.01$, compared to control shRNA-treated cells

surface adhesion, avid phagocytosis of pathogens and particles, and a high capacity to produce and secrete large quantities of proinflammatory cytokines and LMs upon stimulation. The 2 kb promoter of *Alox5* contains four putative PU.1 enhancer sequences in regions that support the basal and MWCNT-induced expression of a heterologous luciferase reporter. ChIP immunoprecipitation confirmed that PU.1 binds to each of the enhancers constitutively and binding is further increased by MWCNTs. Knockdown of PU.1 using specific small hairpin-RNA suppressed the basal and MWCNT-induced expression of *Alox5* and production of LTB4. Knockdown of PU.1 also abolished the induced production of PGE2 in macrophages, indicating a critical role of PU.1 in the synthesis of PGE2 and induction of cyclooxygenase 2 (COX2). Others have also observed induced production of PGE2 and induction of COX2 via Pu.1 (Azim et al. 2007). We will further investigate how PU.1 is activated by MWCNTs.

Mitsui-7 MWCNTs have a rod-like shape with high rigidity. The MWCNTs have been shown to induce Alox5 and stimulate proinflammatory responses in mouse lung and in cultured macrophages (Lim et al. 2020, 2023). In this study, we found that MWCNTs alone at a high concentration significantly induced *Alox5* expression in the absence of a known M1 inducer to result in increased production of LTB4 in mouse and human macrophages. Notably, CB at the same concentrations did not cause significant induction of *ALOX* mRNA in either murine or human macrophages and there was no increase of LTB4 production by CB under the experimental condition. These findings are consistent with the notion that induction is dependent on the rigid, rod-like, three-dimensional structure of the MWCNTs (Lim et al. 2023). The finding suggests a useful, molecular model for investigating the role of structure and physicochemical properties of nanoparticles in nanotoxicity.

In conclusion, we demonstrated that the transcription factor PU.1 plays a critical role in the macrophage-specific expression of Alox5 and its induction by MWCNTs. PU.1 positively regulates *Alox5* transcription by binding to specific PU.1 binding sequences to elevate the promoter activity of the *Alox5* gene promoter. The findings indicate that transcriptional activation of *Alox5* by PU.1 is a key regulation pathway through which MWCNTs increase the synthesis of proinflammatory LMs to promote macrophage-dependent inflammation. The results revealed a complex interplay between PU.1-mediated gene transcription required for myeloid differentiation and nanoparticle-induced polarization of M1 macrophages, induction of Alox5, and production of proinflammatory LMs in the inflammatory response to inhaled sterile particulates in the lung.

Author contributions Q.M. and C.S.L. conceived the study; Q.M., J.K.G., and C.S.L. designed the study; C.S.L. performed the experiments; C.S.L. and J.K.G. performed statistical data analyses. C.S.L. provided a draft manuscript. Q.M. finalized the manuscript. All authors contributed to manuscript preparation.

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Data availability Data supporting our conclusions is included in the main body of the paper.

Declarations

Conflict of interest The manuscript does not contain clinical studies or patient data. The authors declare that they have no conflict of interest. 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.

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