

## Dataset RD-1016-2020-0

**Title:** Resolution of Pulmonary Inflammation Induced by Carbon Nanotubes and Fullerenes in Mice: Role of Macrophage Polarization

**Dataset Number:** RD-1016-2020-0, <https://www.cdc.gov/niosh/data/datasets/rd-1016-2020-0/>

### Introduction

Exposure to certain engineered nanomaterials (ENMs) causes chronic lesions like lung fibrosis and cancer as a result of unresolved inflammation. Elucidating how ENM-induced inflammation is resolved is necessary for better evaluation of the fibrogenic and tumorigenic potentials of ENMs. This study aimed to identify pro-resolving mechanisms by analyzing the inflammatory and fibrogenic responses to multi-walled carbon nanotubes (MWCNTs, Mitsui-7) and fullerenes (fullerene C60, C60F) in B6C3F1 mice. The findings reveal that MWCNTs at a low dose (40 µg/mouse) and C60F at a high dose (>480 mg/mouse) stimulated acute neutrophilic inflammation, which exhibited rapid initiation and extended resolution. The lesion in MWCNT-exposed mice progressed to fibrotic granulomas by day 28 post-exposure, whereas it remained as alveolar histiocytosis in C60F-exposed mice. The ENMs induced high levels of proinflammatory lipid mediators leukotriene B4 (LTB4) and prostaglandin E2 (PGE2) with peaks at day 1, and high levels of specialized pro-resolving mediators resolvin D1 (RvD1) and E1 (RvE1) with peaks at day 7. Moreover, the ENMs induced time-dependent polarization of M1 macrophages and subsequently of M2 macrophages in the lung, accompanied by elevated levels of type 1 or type 2 cytokines, respectively. The M1 macrophages exhibited preferential induction of arachidonate 5-lipoxygenase activating protein (ALOX5AP), whereas M2 macrophages had a high-level expression of arachidonate 15-lipoxygenase (ALOX15). Polarization of macrophages *in vitro* differentially induced ALOX5AP in M1 macrophages and ALOX15 in M2 macrophages, both of which were further increased by MWCNTs. This study demonstrates that the neutrophilic inflammation induced by ENMs is actively resolved through the differential synthesis of proinflammatory and pro-resolving lipid mediators via distinct ALOX pathways in M1 or M2 macrophages in a time-dependent manner. Incomplete resolution of inflammation contributes to the chronic progression in mouse lungs exposed to persistent and high toxicity ENMs.

### Methods Collection

#### 1. Characterization and preparation of MWCNTs and C60F

- MWCNTs (Mitsui-7) and C60F (Sigma Aldrich) were prepared in a dispersion medium (DM) following a two-step dispersion procedure.
- Transmission electron microscopy (TEM) was used to characterize MWCNTs and C60F.
- Field emission scanning electron microscopy (FESEM) was used to examine morphology of C60F.

#### 2. Animals and treatment

- Six-week old male B6C3F1 mice (Jackson Laboratory), 7 to 10 mice per treatment at each timepoint, were treated with a single dose of 50 µl of DM, MWCNTs (40 µg/mouse), or C60F (640 or 1,280 µg/mouse) in suspensions by pharyngeal aspiration.

- At day 1, 7, or 28 post-exposure, the mice were euthanized for molecular, immunologic, and pathological examinations.
- 3. Macrophage culture, polarization, and treatment**
    - The J774A.1 murine monocyte/macrophage cell line was grown in the Dulbecco's Modified Eagle's Medium with 10% fetal bovine serum.
    - M1 polarization was induced with interferon  $\gamma$  (20 ng/ml) plus lipopolysaccharide (100 ng/ml) and M2 polarization was induced with interleukin 4 (20 ng/ml) for indicated time (typically three days).
    - Polarized M1 or M2-macrophages were treated with DM or MWCNTs (2.5  $\mu\text{g/ml}$ ) for indicated time (i.e., 1, 2, or 3 days post-polarization).
  - 4. Whole lung lavage (WLL) preparation**
    - WLL was performed through the cannula using ice cold  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free phosphate buffered saline, pH 7.4.
    - Total WLL cell counts were obtained using a Coulter Multisizer 3 and cell differentials were determined by LSR II flow cytometry.
  - 5. Enhanced darkfield microscopy**
    - Enhanced darkfield microscope used to visualize MWCNTs and their deposition in lung tissue.
    - Enhanced darkfield images were taken using an Olympus DP-73 digital camera.
  - 6. Histopathology**
    - The lung was removed, fixed with 10 % neutral buffered formalin, and embedded in paraffin.
    - Sections of 5  $\mu\text{m}$  thickness were subjected to hematoxylin and eosin (H&E) staining or Picrosirius red staining.
  - 7. Flow cytometry for immune cell profiling**
    - Cells populations from WLL fluids were gated using LSR II flow cytometer.
    - Data were analyzed using FlowJo software.
  - 8. Cytokine measurement**
    - Measurement of cytokines (IFN- $\gamma$ , IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-4, IL-13, and IL-10) in WLL fluids and lung tissue extract was performed by multiplex immunoassay using ProcartaPlex mouse cytokine/chemokine 36-plex kit on a Luminex 200 instrument system equipped with xPONENT software.
  - 9. Enzyme-linked immunosorbent assay (ELISA)**
    - Production of proinflammatory cytokines (LTB4, PGE2) or SPMs (RvD1, LXA4, RvE1) in WLL fluids were measured using ELISA kits with a microplate reader equipped with SOFTmax PRO 4.0.
  - 10. Immunofluorescent staining and confocal microscopy**
    - To detect cellular expression and localization of F4/80, CD68, CD206, ALOX5AP, or ALOX15, immunofluorescent staining was performed in formalin-fixed, paraffin-embedded lung tissue sections (5  $\mu\text{m}$ ).
    - Images were taken using a Zeiss LSM 780 confocal microscope and captured microscopic images were analyzed using the ImageJ software.
  - 11. Immunoblotting**
    - Detection and quantification of CD68, CD86, CD163, CD206, ALOX5, ALOX15, ALOX5AP, or  $\beta$ -actin.
    - Images were scanned using HP scanjet and were used to quantify each band with ImageJ software.

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