Title

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

Introduction

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) induce M1 polarization *in vitro*, with a focus on the induction of arachidonate 5-lipoxygenase (Alox5), a key enzyme in the biosynthesis of LMs. Treatment of J774A.1 murine macrophages with MWCNTs increased the expression of Alox5 mRNA in a concentration- and time-dependent manner, with the largest induction (5.3-fold over control) occurring at 10 mg/ml and 3 days post-exposure. The Alox5 protein was similarly induced. Alongside Alox5 induction, MWCNTs induced the expression of CD68, a cell surface marker of M1, by approximately 2.0-fold and that induction persisted for up to 3 days post-exposure. Both the expression and activity of inducible nitric oxide synthase, an intracellular marker of M1, were increased by MWCNTs up to 8.4-fold. Consistent with M1 polarization, MWCNTs induced the production and secretion of proinflammatory cytokines tumor necrosis factor-α and interleukin-1β, and proinflammatory LMs leukotriene B4 (LTB4) and prostaglandin E2 (PGE2). Moreover, cell-free media from MWCNT-polarized macrophages induced the migration of neutrophils, which was blocked by Acebilustat, a specific leukotriene A4 hydrolase inhibitor, or LY239111, a LTB4 receptor antagonist, but not the cyclooxygenase 2 inhibitor, NS-398, 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, implicating an important role of Alox5 in MWCNT-induced M1 polarization and function. Taken together, these findings demonstrate the polarization of M1 macrophages by MWCNTs *in vitro* and highlight induction of Alox5 as an important mechanism by which MWCNTs promote proinflammatory responses by boosting M1 polarization and production of pro-inflammatory LMs.

Methods Collection

1. Dispersion of MWCNTs

- MWCNTs (Mitsui-7) were prepared in a control medium (CM) [Dulbecco's modified eagle's medium (DMEM) with 1% fetal bovine serum (FBS)] at a concentration of 2 mg/ml through vortexing and by sonication.
- Stock solutions of MWCNTs were further diluted with the culture media at 2.5 or 10 μg/ml, sonicated immediately before use.

2. Cell culture, polarization, and treatment

- J774A.1 murine monocyte/macrophage cells were grown in DMEM with 10% fetal bovine serum.
- J774A1. Cells (5 x 10^5 cells/ml) were seeded in DMEM with 3% FBS for 1 day and then treated with other reagents for 1day or 3 days.

- M1 polarization was induced with interferon γ (20 ng/ml) plus lipopolysaccharides (100 ng/ml) and M2 polarization was induced with interleukin 4 (20 ng/ml) for indicated time (typically three days).
- Control media (DMEM media with 1% FBS) were prepared and treated to establish a negative control response.
- After treatment with a single dose of MWCNTs (2.5 or 10 μg/ml), the cells were incubated for 1 day or 3 days in the same media until harvested to examine total RNA and protein expression.
- HL-60, a promyelocytic cell line, was grown in the RPMI1640 medium with 10% FBS.
- Differentiated HL-60 cells (dHL-60) were prepared with use of 2 μM all-trans retinoic acid treatment for 3 days.

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

- Detection and quantification of Alox5, Alox5ap, or β-actin at mRNA level.
- Real-time qPCR was performed, and the relative expression change values were calculated as $2^{-\Delta\Delta Ct}$ and expressed as fold change in comparison with untreated control.

4. Immunoblotting

- Detection and quantification of Alox5, CD68, or β-actin at protein level.
- Images were scanned using HP scanjet and were used to quantify each band with ImageJ software.

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

- Detection and quantification of Nos2 expression using an intracellular Nos2 detection assay kit.
- The fluorescence signal was measured using a fluorescence plate reader and the relative fluorescence unit (RFU) was expressed.

6. Enzyme-linked immunosorbent assay (ELISA)

- Production of proinflammatory cytokines (TNF-α, IL-1β) and lipid mediators (LTB4, PGE2) in cell-free culture supernatant were measured using ELISA kits with a microplate reader equipped with SOFTmax PRO 4.0.

7. Chemotaxis assay

- Transwell cell migration assay was performed using a cell migration assay kit to determine the effect of MWCNTs on neutrophilic cell migration *in vitro.*
- ATRA-induced differentiated HL-60 (dHL-60) cells were collected and suspended in 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, indicated amount of MWCNTs in the basal RPMI 1640 medium containing 1% FBS, or RPMI 1640 medium containing 10% FBS.
- For inhibition assay, a cell-free culture media from cells treated with a specific cyclooxygenase 2 inhibitor, NS-398 (at 2 or 10 μM), a leukotriene A4 hydrolase inhibitor, Acebilustat (at 1 or 5 μM), or dimethyl sulfoxide (DMSO) as a vehicle 6 hr prior to MWCNTs or IFN-γ+LPS exposure added at each lower chamber. A specific LTB4 receptor inhibitor LY293111 (5 or 25 nM) or DMSO as a vehicle was added directly into the dHL-60 cell suspension.

- The fluorescence signal was measured using a fluorescence plate reader and the relative fluorescence unit was expressed to show the migrated cells.

8. Alox5 gene silencing

- To knockdown Alox5 gene expression in macrophages, mouse Alox5-specific short hairpin (shRNA) as RNA interference was introduced into macrophages and incubated for 2 days and then cells were used for RT-qPCR and immunoblotting analysis.
- Cells were treated with MWCNTs or M1 inducer and subsequently used for RT-qPCR and immunoblotting analysis, and the cell-free culture media was collected and used for ELISA assay.

Citations: Publications based on the dataset

Lim CS, Veltri B, Kashon M, Ma Q (2022) Multi-Walled Carbon Nanotubes Induce Arachidonate 5-Lipoxygenase Expression and Enhance the Polarization and Function of M1 Macrophages. Nanotoxicology, DOI: 10.1080/17435390.2023.2204161.

Acknowledgements

This work was supported by NIOSH. CAN# 09390BMX.

Authors

Chol Seung Lim[1] (oxr9@cdc.gov)
Brandon Veltri[2] (otu3@cdc.gov)
Michael Kashon[3] (mqk1@cdc.gov)
Qiang Ma[1,4] (qam1@cdc.gov)

[1] Receptor Biology Laboratory, Toxicology and Molecular Biology Branch, HELD, NIOSH, Morgantown, WV 26505, USA
[2] Department of Microbiology, Immunology, and Cell Biology, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV 26505, USA
[3] Bioanalytics Branch, HELD, NIOSH, Morgantown, WV 26505, USA
[4] Corresponding author

Contact

For further information, contact:
TMBB/HELD/NIOSH
304.285.6241