**4,4’-Methylene Diphenyl Diisocyanate Exposure Induces Expression of Alternatively Activated Macrophage-Associated Markers and Chemokines Partially Through Krüppel-Like Factor 4 Mediated Signaling in Macrophages**

**Dataset Number RD-1077-2023-0**

**Introduction**

Exposure to 4,4’-methylene diphenyl diisocyanate (MDI), the most widely used monomeric diisocyanate, in the occupational setting may lead to the development of occupational asthma (OA). Currently, the underlying molecular mechanism(s) by which MDI induces OA remain an active area of research. Alveolar macrophage dysfunctions play important roles in asthma pathogenesis. Upon exposure to outside stimuli, macrophages are polarized into either classically activated (M1) or alternatively activated (M2) phenotypes. Macrophage polarization is associated with asthma development where M2 macrophage populations are usually pronounced in lungs of asthmatic patients. Recent *in vivo* studies demonstrated that M2 macrophage associated markers and chemokines were induced by MDI exposure, indicating that MDI may induce M2 macrophage polarization; however, the underling molecular mechanism(s) by which MDI causes induction of M2 associated markers and chemokines is unclear. M2 macrophage polarization can be regulated by activation of several M2 associated transcription factors; however, whether MDI exposure regulates these transcription factors is unknown. We hypothesize that MDI exposure induces M2 macrophage associate markers and chemokines through upregulation of M2 macrophage-associated transcription factors in macrophages. The first aim of this study is to verify whether MDI-exposure can induce M2 macrophage-associated markers and chemokine expression that were observed in previous studies and to identify candidate M2 macrophage-associated transcription factors in response to MDI exposure that may account for the in M2 macrophage polarization. After identification of the candidate transcription factor(s) that can be regulated by MDI-exposure, we investigate the roles of the candidate transcription factor in regulation of these candidate M2 macrophage associate markers and chemokines in relation to the exposure to MDI.

**Methods Collection**

1. Bronchoalveolar lavage cells (BALCs) RNA from mice with combined dermal/respiratory MDI exposure
   * Total RNA was isolated from stored BALCs from previous studies using animals’ dermal exposure to MDI or vehicle followed by MDI-aerosol or house air inhaled mice.
   * MDI-dermal exposed BALB/c mice were exposed to either 4580 ± 1497 µg/m3 MDI aerosol or pure house air (control; Ctl) for 1 h followed by bronchoalveolar lavage at 24 h post-exposure.
2. Cell culture and cell differentiation
   * THP-1, Jurkat T-cells clone E6-1, and Clone 15 HL-60 cells were obtained from ATCC.
   * Enhanced differentiated THP-1 macrophages were prepared using media containing 10 ng/ml phorbol 12-myristate 13-acetate (PMA) to induce differentiation for 3 days and then enhanced by refeeding fresh media after removing PMA containing media for additional 3 days.
   * Differentiated eosinophils were prepared by culturing Clone 15 HL-60 cells in RPMI-1640 media containing 0.5mM butyric acid for 7 days.
3. MDI-GSH conjugation
   * 10 mM GSH solution was prepared in 200 mM sodium phosphate buffer (pH= 7.4).
   * MDI-GSH conjugation was prepared by adding MDI/acetone directly into GSH solution. End-to-end mixing for 1 h at 25 °C.
4. KLF4 overexpression and knockdown
   * Plasmid DNAs were transfected into THP-1 macrophages using Mirus *Trans*IT®-2020 transfection reagent according to manufacturer’s instructions.
   * KLF4 siRNAs and nontargeting siRNA control were transfected into THP-1 macrophages using Lipofectamine RNAiMAX transfection reagent according to manufacturer’s instructions.
5. M2 macrophage-associated transcription factors, markers, and chemokines expression (transcripts and proteins)
   * Total RNA was isolated using *mirVana*™ miR isolation kit according to manufacturer’s instructions.
   * TaqMan gene expression assays for *CD206, TGM2, CCL17, CCL22* and *CCL24* were obtained from ThermoFisher Scientific.
   * Real-time PCR assays were performed on Applied Biosystems 7500 RT-PCR System.
   * Endogenous M2 macrophage markers CD206 and TGM2 proteins were determined by western blot.
   * Secreted M2 macrophage-associated chemokines CCL17, CCL22, and CCL24 proteins in cell-free conditioned media were determined by ELISA. The human CCL22, and CCL24 ELISA kits were obtained from ThermoFisher Scientific. Human CCL17 ELISA kit was obtained from R&D systems. ELISA were performed according to manufacturer’s instructions.
6. Chemotaxis assays and quantification of migrated cells
   * 3 mm pore size Transwell™ assay insert was used for chemotaxis/cell migration assays.
   * Either Jurkat T-cells or differentiated Clone 15 HL-60 cells were added into Transwell™ assay inserts and allowed those cells responded to conditioned media obtained from THP-1 macrophages transfected with KLF4 overexpression plasmid, KLF4 siRNAs or else KLF4 siRNA transfected THP-1 macrophages exposed to MDI-GSH conjugates.
   * Migrated immune cells were quantified by using CyQUANT® GR proliferation assay according to manufacturer’s instructions.

**Citations –** Publications based on the dataset

Lin, CC, Law, BF, and Hettick, JM (2023). 4,4’-Methylene Diphenyl Diisocyanate Exposure Induces Expression of Alternative Activated Macrophage-Associated Markers and Chemokines Partially Through Krüppel-Like Factor 4 Mediated Signaling in Macrophages. *To be submitted to Xenobiotica. Currently in internal review and clearance.*

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