**MATERIALS AND METHODS**

**Chemicals and Reagents**

4,4’-methylene diphenyl diisocyanate (MDI, 98%), high-performance liquid chromatography (HPLC) grade acetone, 3Å molecular sieve (4-8 mesh), tris buffered saline (TBS), Tween-20, dimethyl sulfoxide (DMSO), phorbol 12-myristate 13-acetate (PMA), butyric acid, bovine serum albumin (BSA) and reduced-glutathione (GSH) were acquired from MilliporeSigma (St. Louis, MO). Phosphate buffered saline (PBS), Roswell Park Memorial Institute (RPMI)-1640 culture medium, and Penicillin-Streptomycin-Glutamine (PSG; 100×) were acquired from Thermo Fisher Scientific (Waltham, MA). Hyclone™ fetal bovine serum (FBS) was obtained from Cytiva Life Sciences (Marlborough, MA). Dry acetone was prepared by incubating 10 ml HPLC grade acetone on 3 Å molecular sieve for a minimum of 24 hours to adsorb water.

**Bronchoalveolar lavage cells (BALCs) from MDI exposed mice**

Candidate gene expression studies for M2 macrophage-associated markers, chemokines, and transcription factors that mediate M2 polarization were performed on stored murine BALCs obtained from prior study (Lin et al. 2019). Briefly, mice were dosed on the dorsal surface of each ear with 25 ml of 1% MDI/acetone (w/v) or acetone control on days 1, 2, 3, 14, 15 and 16. On day 21, the animals were nose-only exposed to 4580 ± 1497 µg/m3 MDI aerosol or house air control for 1h using the in-house constructed nose-only inhalation exposure system (NOIES) followed by bronchoalveolar lavage at 24 h post-exposure. Bronchoalveolar lavage fluid (BALF) was collected via 3× 1ml ice-cold PBS lavages after the exposed lungs were first perfused with 10-ml ice cold PBS. Bronchoalveolar lavage cells (BALCs) from the BALF were collected by centrifugation at 300 × g for 10 min at 4°C and stored in a −80°C freezer until total RNA isolation. Animal experiments were performed in the AAALAC, International-accredited National Institute for Occupational Safety and Health animal facility in accordance with an institutionally approved animal care and use protocol.

**Cell culture and differentiation**

THP-1 (ATCC® TIB-202™), Clone 15 HL-60 (HL-60\_C15; ATCC® CRL-1964™), and Jurkat Clone E6-1 (Jurkat\_E6-1; ATCC® TIB-152™) cells were obtained from American Type Culture Collection (ATCC; Manassas, VA) and maintained at 0.5-1 × 106/ml in RPMI-1640 media supplement with 10% FBS, and 1× PSG (Complete RPMI media) at 37°C in a humidified atmosphere with 5% CO2as previous described (Lin et al. 2021). THP-1 cells (2×106 cells) were differentiated into macrophages using 10 ng/ml PMA in 10-cm culture dishes for 72 h. Differentiation was further enhanced by removal of PMA-containing media, washing twice with PBS and incubation of the cells in fresh complete media for another 72h. Reducing PMA differentiation concentration to 10 ng/ml has been shown to enhance responsiveness to polarizing stimuli (Maeß et al. 2014; Baxter et al. 2020). All *in vitro* cell experiments described in this study used enhanced-differentiated THP-1 macrophages. For eosinophil differentiation used in chemotaxis experiments, HL-60\_C15 cells (5 × 105 cells/ml) were cultured in complete RPMI-1640 media containing 0.5 mM butyric acid for 7 days as per previous reports (Fischkoff 1988; Tiffany et al. 1995; Badewa et al. 2002).

**MDI-GSH conjugation**

MDI-GSH conjugates were prepared as previously described (Lin et al. 2020). Briefly, 10 mM GSH solution was prepared in 200 mM sodium phosphate buffer (pH= 7.4). 50 μl of freshly prepared stock solutions of 10% MDI (w/v) in dry acetone were added to 25 ml of GSH solution dropwise with stirring, to achieve an approximate MDI concentration of 800 μM. The reaction was incubated at 25 °C with end-over-end mixing for 1 h, followed by centrifugation at 10,000 × g and filtered with 0.2 μm syringe filter. Reaction products containing MDI-GSH conjugates were immediately added into enhanced differentiated THP-1 macrophages at the indicated concentrations.

**KLF4 overexpression and knockdown**

Expression plasmids pCMV6-Entry-KLF4 (Origene ID: RC206691) and pCMV6-Entry (ID: PS100001) were purchased from Origene (Rockville, MD). For KLF4 overexpression associated RNA expression studies, 1×106 enhanced-differentiated THP-1 macrophages were reverse transfected with 2.5 μg of either pCMV6-Entry-KLF4 expression plasmid or pCMV6-Entry empty vector using Mirus *Trans*IT®-2020 transfection reagent (Mirus Corporation; Madison, WI, USA) in a 6-well plate for 48 h. After 48 h, total RNA was isolated using *mirVana™* miR Isolation Kit (Thermo Fisher Scientific) according to manufacturer’s instructions for RT-qPCR expression analyses. For KLF4 overexpression associated protein analysis, 5 ×106 enhanced-differentiated THP-1 macrophages were reverse transfected with 10 μg of either pCMV6-Entry-KLF4 expression plasmid or pCMV6-Entry empty vector using Mirus *TransI*T®-2020 transfection reagent in 10-cm dish for 48 h. After 48h, cell lysates were prepared for western blotting.

For KLF4 siRNA knockdown studies, two commercially available *Silencer®* Select siRNAs specific target to the coding region of human KLF4 transcripts (Cat#4392420; Assay ID#s17793, denoted as siKLF4-793 and #s17794, denoted as siKLF4-794) and nontargeting *Silencer®* Select Negative Control #1 siRNA control (Cat#4390843, denoted as siCtl) were acquired from Thermo Fisher Scientific. All siRNAs were diluted to 20 μM in nuclease-free water. To transfect KLF4 siRNAs, differentiated-enhanced THP-1 macrophages were subjected to reverse transfection followed by forward transfection 24h later as previously described (Lin et al. 2020). For studies examining the role of KLF4 in MDI mediated induction of M2 macrophage-associated markers and chemokines, THP-1 macrophages underwent two rounds of siRNA transfection prior to treatment with 10  μM MDI-GSH conjugate or control for 24h, after which cell extracts were prepared for western blotting and/or RT-qPCR expression analyses.

**Expression analyses**

For RT-qPCR assays, total RNA from BALCs or cultured THP-1 macrophages were extracted using *mirVana™* miR Isolation Kit (Thermo Fisher Scientific) according to manufacturer’s instructions. PCR reactions were performed on an ABI 7500 Real-Time PCR System from Thermo Fisher Scientific (Waltham, MA, USA). The mRNA levels were analyzed using the ΔΔCT method as previously described (Lin et al. 2019). Reactions were normalized to either human or mouse beta-2 microglobulin (*B2M/B2m*) for mRNA analysis. Gene expression assays used in this study were acquired from Thermo Fisher Scientific and include: mouse *Chil3* (Cat#4331182; Assay ID: Mm00657889\_mH)*, Chil4* (Mm00840870\_m1)*, Retnlb* (Mm00445845\_m1)*, Clec7a* (Mm01183349\_m1)*, Clec10a* (Mm00546125\_g1)*, Cd163* (Mm00474091\_m1)*, Mrc1/Cd206* (Mm01329359\_m1)*, Pdcd1lg2/Cd273* (Mm00451734\_m1)*, Tgm2* (Mm00436979\_m1), *Klf4* (Mm00516104\_m1), *Pparg* (Mm00440940\_m1), *Stat6* (Mm01160477\_m1), *Irf4* (Mm00516431\_m1), *Spi1* (Mm00488140\_m1), *Cebpb* (Mm07294206\_s1), *Ccl17* (Mm01244826\_g1)*, Ccl22* (Mm00436439\_m1)*, Ccl24* (Mm00444701\_m1), and *B2m* (Mm00437762\_m1);human *CD206* (Hs00267207\_m1), *TGM2* (Hs01096681\_m1), *KLF4* (Hs00358836\_m1), *PPARG* (Hs01115513\_m1), *STAT6* (Hs00180031\_m1), *IRF4* (Hs00180031\_m1), *SPI1* (Hs02786711\_m1), *CEBPB* (Hs00942496\_s1), *CCL17* (Hs00171074\_m1)*, CCL22* (Hs01574247\_m1)*, CCL24* (Hs00171082\_m1), and *B2M* (Hs00187842\_m1).

**Immunoblot and antibodies**

Cell extracts for immunoblot were prepared in RIPA buffer as previously described (Lin et al. 2020). Following electrophoresis, proteins were transferred onto nitrocellulose membranes and probed with diluted antibodies in TBST containing 1% BSA. Specific antibody against human CD206 (Cat#18704-1-AP), and TGM2 (#15100-1-AP) were obtained from Proteintech (Rosemont, IL). Antibody against human KLF4 (Cat#AB4138) was obtained from MilliporeSigma (Burlington, MA). Antibody against b-actin (Cat#sc-47778) was obtained from Sant Cruz Biotechnology (Dallas, TX). Bound antibodies were detected using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific).

**Chemokine ELISA**

Conditioned media was collected 24h after THP-1 macrophages were treated with MDI-GSH conjugates or 24h after THP-1 macrophages were transfected with either KLF4 overexpression plasmid or transfected with KLF4 siRNAs and treated with or without MDI-GSH conjugates as described above. The following enzyme-linked immunosorbent assay (ELISA) kits were obtained from ThermoFisher Scientific: Human CCL22/MDC (Cat#EHADAM11), and human CCL24/Eotaxin-2 (#EHCCL24). Human CCL17/TARC ELISA kit (Cat#DY36405) was obtained from R&D systems (Minneapolis, MN). The assay sensitivity for each chemokine is as follows: CCL17 (7.8 pg/ml), CCL22 (1.5 pg/ml), and CCL24 (2 pg/ml). Human CCL17, CCL22, and CCL24 released into the conditioned media from plasmid or siRNA transfected THP-1 macrophages were measured by ELISA according to manufacturer’s instructions.

**Chemotaxis assays and quantification of migrated cells**

Chemotaxis/migration in response to conditioned media collected from THP-1 macrophages treated with either KLF4 overexpression plasmids or KLF4 knockdown siRNAs with potential M2 macrophage secreted chemokines were performed as previously reported (Lin et al. 2021). The cell chemotaxis/migration assays were performed using a 24-well plate format with Transwell™ inserts containing 3 µm pores sized polycarbonate membrane (Corning™ Transwell™ plates, ThermoFisher Scientific). Chemoattracted/migrated cells were quantified using the fluorescent cell counting dye CyQUANT® GR (ThermoFisher Scientific).

**Statistical analysis**

Data were analyzed using either the unpaired *t*-test (two-tailed) when comparing two groups, or one-way analysis of variance followed by Tukey's multiple comparison *ad hoc* post-test when comparing multiple groups. Statistical analyses were performed using GraphPad Prism 7.0 software (GraphPad Software, La Jolla, CA, USA.). Differences were considered significant when the analysis yielded *P*<0.05.

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