

Materials and Methods

Chemicals and Reagents

Butyric acid, high-performance liquid chromatography (HPLC) grade acetone, 3 Å molecular sieves (4-8 mesh), phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO), 98% 4,4'-methylene diphenyl diisocyanate (MDI), phorbol 12-myristate 13-acetate (PMA), ionomycin salt and reduced-glutathione (GSH) were acquired from MilliporeSigma (St. Louis, MO). Tacrolimus (FK506) was purchased from Selleckchem (Houston, TX). Recombinant human (rh) proteins including interleukin (IL)-4, granulocyte macrophage colony stimulating factor (GM-CSF), and tumor necrosis factor (TNF)- α were purchased from R&D Systems (Minneapolis, MN). Roswell Park Memorial Institute (RPMI)-1640 culture medium and penicillin-streptomycin-glutamine (PSG; 100 \times) were purchased from ThermoFisher 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 sieves for a minimum of 24 h to adsorb water.

Bronchoalveolar lavage cells (BALCs) from MDI aerosol exposed mice

Candidate macrophage-secreted mediator RNA expression studies were performed on the stored RNAs from prior studies, the BALCs RNAs were isolated from the BALB/c mice exposed to MDI aerosol using an in house developed nose-only aerosol inhalation exposure system as described previously. (Hettick et al., 2018; Lin et al., 2019). Animals were exposed to $4580 \pm 1497 \mu\text{g}/\text{m}^3$ MDI aerosol or pure house air control (Ctl) for 1 h followed by bronchoalveolar lavage at 24 h post-exposure.

Cell culture and cell differentiation

THP-1 (ATCC® TIB-202™), HL-60 (ATCC® CCL-240™), 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 $0.5-1 \times 10^6/\text{ml}$ in RPMI-1640 media supplement with 10% FBS, and 1 \times PSG (Complete RPMI media) at 37°C in a humidified atmosphere with 5% CO₂. Enhanced differentiated THP-1 macrophages were prepared using PMA to

induce differentiation as previous described (Lin et al. 2020). All macrophage *in vitro* experiments were performed using enhanced differentiated THP-1 macrophages. Other immune cell types used in chemotaxis experiments were differentiated as follows: For dendritic cell differentiation, a total of 3×10^7 naïve undifferentiated THP-1 cells were harvested by centrifugation at $300\times g$ for 5 minutes, washed twice with PBS and resuspended in 30 ml of serum-free RPMI-1640 culture medium supplemented with 100 ng/ml rhGM-CSF, 10 ng/ml rhTNF- α , and 200 ng/ml ionomycin and seeded into three 10 cm culture dishes for 3 days as described by Berges and colleagues (Berges et al. 2005). For neutrophil differentiation, HL-60 cells (5×10^5 cells/ml) were cultured in complete RPMI-1640 media containing 1.5% DMSO for 7 days as described by Millius and colleagues (Millius and Weiner 2010). For eosinophil differentiation, HL-60_C15 cells (5×10^5 cells/ml) were cultured in complete RPMI-1640 media containing 0.5 mM butyric acid for 7 days as per previous reports (Badewa et al. 2002; Fischkoff 1988; Tiffany et al. 1995).

MDI-GSH conjugation and exposure

MDI-GSH conjugates were prepared as previously described (Lin et al. 2020). Briefly, a 10 mM GSH solution was prepared in sodium phosphate buffer (200 mM; pH= 7.4). 50 μ l of freshly prepared stock solution of 10% MDI (w/v) in dry acetone were added to 25 ml of GSH solution dropwise with stirring, to a final MDI concentration of 800 μ M, after which the tube was subjected to end-over-end mixing for 1 h at 25 °C. Samples were centrifuged at $10,000\times g$ and filtered with a 0.2 μ m syringe filter. MDI-GSH conjugates were prepared immediately prior to use and used to treat 1×10^6 enhanced differentiated THP-1 macrophages in a 6 well plate. After 24 h, the cell culture supernatant (conditioned media) was collected, centrifuged, and stored at -20°C until ready to use. Cells were washed 2 times with warm PBS and cell lysates were prepared by adding 600 μ l of lysis/binding solution from the *mirVana*TM miR Isolation Kit (ThermoFisher Scientific) and stored at -80 °C until ready to use.

Transient transfection and PPP3CA overexpression

To investigate chemokine expression in macrophages overexpressing PPP3CA, 1×10^6 enhanced differentiated THP-1 macrophages were transfected with 2.5 μ g of either pcDNA3.1⁺/c-(k)dyk-PPP3CA

expression plasmid (GenScript, Piscataway, NJ) or pcDNA3.1⁺ empty vector (ThermoFisher Scientific) using Mirus *TransIT*-2020 transfection reagent (Mirus Corporation; Madison, WI) in a 6-well plate for 24 h. After 24 h, total RNA was extracted and prepared for RT-qPCR expression analysis to determine expression of candidate chemokines. For miR functional analyses, the following *mirVana*[®] miRNA inhibitors (MH) were obtained from ThermoFisher Scientific (Waltham, MA) and diluted to 20 μ M in nuclease-free water: hsa-miR-206-3p (MH10409), hsa-miR-381-3p (MH10242), and MH-negative control #1 (4464076). A total of 2×10^5 enhanced differentiated THP-1 macrophages were subjected to two rounds of transfection in 6-well plates: an initial reverse transfection followed 24 h later by forward transfection as previously described (Lin et al. 2011). At 24 h after the start of the forward transfection, the cell culture media were collected, centrifuged, and stored at -20°C until ready to use. Cells were washed twice with PBS and cell extracts were prepared for RNA isolation and RT-qPCR expression analysis.

Expression analysis

Gene expression was determined by RT-qPCR analysis. Total RNA from BALCs or cultured THP-1 macrophages was extracted using *mirVana*[™] miR Isolation Kit (ThermoFisher Scientific) as per manufacturer's instructions. The mRNA and miR levels were determined as previously described (Lin et al. 2020; Lin et al. 2019; Lin et al. 2011; Lin et al. 2015; Sharma et al. 2014). Candidate gene/miR expression was normalized to either human or mouse beta-2 microglobulin (*B2M/B2m*) for mRNA analysis, or to *U6* snRNA for miR analysis. Gene expression assays and miR-specific assays used in this study were obtained from ThermoFisher Scientific (Waltham, MA) and include: human *IL1B* (Cat#:4331182/Assay ID:Hs01555410_m1), *TNF* (Hs00174128_m1), *IL6* (Hs00174131_m1), *CCL2/MCP-1* (Hs00234140_m1), *CCL3/MIP-1 α* (Hs00234142_m1), *CCL5/RANTES* (Hs00982282_m1), *CXCL8/IL-8* (Hs00174103_m1), *TGFB1* (Hs00998133_m1), *B2M* (Hs00187842_m1), mouse *Il1b* (Mm00434228_m1), *Tnf* (Mm00443258_m1), *Il6* (Mm00446190_m1), *Ccl2/Mcp-1* (Mm00441242_m1), *Ccl3/Mip-1 α* (Mm00441259_g1), *Ccl5/Rantes* (Mm01302427_m1), *Ccl11* (Mm00441238_m1), *Ccl17/Tarc* (Mm01244826_g1), *Ccl22/Mdc* (Mm00436439_m1), *Tgfb1* (Mm01178820_m1), *Gmcsf/Csf2*

(Mm01290062_m1), *Ptgs2/Cox2* (Mm03294838_g1), *Alox5/5-Lox* (Mm01182747_m1), *Alox5ap/Flap* (Mm00802100_m1) and *B2m* (Mm00437762_m1), hsa-miR-206-3p (Cat# 4427975; Assay ID#000510), hsa-miR-381-3p (#000571), and *U6* snRNA (#001973). PCR reactions were performed on an ABI 7500 Real-Time PCR System from ThermoFisher Scientific (Waltham, MA). Expression of mRNAs and miRs were determined by using the $\Delta\Delta$ CT method as previous described (Lin et al. 2020; Lin et al. 2019; Lin et al. 2011; Lin et al. 2015; Sharma et al. 2014).

Chemokine enzyme-linked immunosorbent assay

Conditioned media was collected from either MDI-GSH conjugate-treated THP-1 macrophages or miR-inhibitor transfected THP-1 macrophages as described above. The following enzyme-linked immunosorbent assay (ELISA) kits were obtained from ThermoFisher Scientific: Human CCL2/MCP-1 (Cat# 88739922), human CCL3/MIP-1 α (#88703522), and human IL8/CXCL8 (#88808622). Human CCL5/RANTES ELISA kit (Cat# DY27805) was obtained from R&D systems (Minneapolis, MN). The assay sensitivity for each chemokine is as follows: CCL2 (7 pg/ml), CCL3 (16 pg/ml), CCL5 (15.6 pg/ml), and IL8/CXCL-8 (2 pg/ml). Human CCL2, CCL3, CCL5 and IL8 released into the conditioned media from THP-1 macrophages were measured by ELISA according to manufacturer's instructions.

Chemotaxis assays and quantification of migrated cells

Cell chemotaxis/migration assays were performed using 3 μ m pore Transwell™ inserts in a 24-well plate format (Corning™ Transwell™ plates, ThermoFisher Scientific). Chemoattracted/migrating cells were quantified using the fluorescent cell counting dye CyQUANT® GR (ThermoFisher Scientific). The fluorescent cell counting method used with the Transwell™ assay counts all cells that migrate to the lower chamber -- this method demonstrates a higher sensitivity than the traditional cell counting method using random selected high-power fields to count cells under a microscope (Gildea et al. 2000). Briefly, differentiated immune cells were collected, washed twice with PBS, and resuspended in serum-free RPMI 1640 media. A total of 1×10^6 cells in 100 μ l serum-free RPMI 1640 media were added to each upper chamber and placed on the lower chamber containing chemoattractant (conditioned media). Five hundred

microliters of cell-free conditioned media from either MDI-GSH conjugate treated or miR-inhibitor transfected THP-1 macrophages were placed in the lower chamber as chemoattractant, and the cells allowed to migrate for 6 h at 37°C in a humidified atmosphere with 5% CO₂. After 6 h, the lower chamber media containing migrated cells in suspension were collected in separate tubes and placed on ice. The media from the upper chamber was aspirated and discarded, and the upper chamber transferred to a clean well containing PBS for washing. The upper chamber membrane and lower chamber surface were washed twice with PBS to collect migrated cells that remained attached to surfaces, 500 µl cell detaching media (0.25% Trypsin-EDTA, Cat#25200056, ThermoFisher Scientific) were added back to the lower chambers, and the upper chambers reinstalled. The whole plate was further incubated at 37 °C for 30 min to detach cells. After 30 min, the migrated cells in cell detaching media were combined with conditioned media/migrated cells collected previously, centrifuged at 300 × g for 5 min, washed with PBS twice and stored at -80 °C before quantification using CyQUANT[®] Cell proliferation assay (ThermoFisher Scientific) as per manufacturer's instructions.

References

- Badewa AP, Hudson CE, Heiman AS. 2002. Regulatory effects of eotaxin, eotaxin-2, and eotaxin-3 on eosinophil degranulation and superoxide anion generation. *Exp Biol Med (Maywood)*. 227(8):645-651.
- Berges C, Naujokat C, Tinapp S, Wieczorek H, Hoh A, Sadeghi M, Opelz G, Daniel V. 2005. A cell line model for the differentiation of human dendritic cells. *Biochem Biophys Res Commun*. 333(3):896-907.
- Fischkoff SA. 1988. Graded increase in probability of eosinophilic differentiation of hl-60 promyelocytic leukemia cells induced by culture under alkaline conditions. *Leuk Res*. 12(8):679-686.
- Gildea JJ, Harding MA, Gulding KM, Theodorescu D. 2000. Transmembrane motility assay of transiently transfected cells by fluorescent cell counting and luciferase measurement. *Biotechniques*. 29(1):81-86.
- Lin CC, Law BF, Hettick JM. 2020. Acute 4,4'-methylene diphenyl diisocyanate exposure-mediated downregulation of mir-206-3p and mir-381-3p activates inducible nitric oxide synthase transcription by targeting calcineurin/nfat signaling in macrophages. *Toxicol Sci*. 173(1):100-113.
- Lin CC, Law BF, Siegel PD, Hettick JM. 2019. Circulating mirs-183-5p, -206-3p and -381-3p may serve as novel biomarkers for 4,4'-methylene diphenyl diisocyanate exposure. *Biomarkers*. 24(1):76-90.
- Lin CC, Liu LZ, Addison JB, Wonderlin WF, Ivanov AV, Ruppert JM. 2011. A klf4-mirna-206 autoregulatory feedback loop can promote or inhibit protein translation depending upon cell context. *Mol Cell Biol*. 31(12):2513-2527.
- Lin CC, Sharma SB, Farrugia MK, McLaughlin SL, Ice RJ, Loskutov YV, Pugacheva EN, Brundage KM, Chen D, Ruppert JM. 2015. Kruppel-like factor 4 signals through microrna-206 to promote tumor initiation and cell survival. *Oncogenesis*. 4:e155.

- Millius A, Weiner OD. 2010. Manipulation of neutrophil-like hl-60 cells for the study of directed cell migration. In: Papkovsky DB, editor. Live cell imaging: Methods and protocols. Totowa, NJ: Humana Press. p. 147-158.
- Sharma SB, Lin CC, Farrugia MK, McLaughlin SL, Ellis EJ, Brundage KM, Salkeni MA, Ruppert JM. 2014. Micrnas 206 and 21 cooperate to promote ras-extracellular signal-regulated kinase signaling by suppressing the translation of *rasa1* and *spred1*. *Mol Cell Biol.* 34(22):4143-4164.
- Tiffany HL, Li F, Rosenberg HF. 1995. Hyperglycosylation of eosinophil ribonucleases in a promyelocytic leukemia cell line and in differentiated peripheral blood progenitor cells. *J Leukoc Biol.* 58(1):49-54.