MATERIALS AND METHODS

Chemicals and Reagents

High-performance liquid chromatography (HPLC) grade acetone, 3Å molecular sieve (4-8 mesh), phosphate buffered saline (PBS), Tris Buffered Saline (TBS), Tween-20, Dimethyl sulfoxide (DMSO), 4,4'-methylene diphenyl diisocyanate (MDI, 98% purity), phorbol 12-myristate 13-acetate (PMA) and reduced-glutathione (GSH) were acquired from MilliporeSigma (St. Louis, MO). Roswell Park Memorial Institute (RPMI)-1640 culture medium, radioimmunoprecipitation assay (RIPA) buffer, Penicillin-Streptomycin-Glutamine (PSG; 100×), and Fetal Bovine Serum (FBS) were purchased from ThermoFisher Scientific (Waltham, MA). Kenpaullone was purchased from Santa Cruz Biotechnology (Dallas, TX). Dry acetone was prepared by incubating 10 ml HPLC grade acetone on 3 Å molecular sieve for a minimum of 24 h to adsorb water.

Cell culture and differentiation

THP-1 (ATCC® TIB-202™) was obtained from American Type Culture Collection (ATCC; Manassas, VA) and maintained at 0.5-1 × 10^6/ml in RPMI-1640 media supplemented with 10% FBS, and 1 × PSG (Complete RPMI media) at 37°C in a humidified atmosphere with 5% CO_2 as previously described (Lin et al. 2023). THP-1 cells (2 × 10^6 cells) were differentiated into macrophages by addition of 10 ng/mL PMA in 10 cm culture dishes for 72 h. Differentiation was further enhanced by removal of the PMA-containing media, washing twice with PBS and then incubating the cells in fresh complete media for another 72h. PMA differentiation at 10 ng/mL to THP-1 monocytes 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.

MDI-GSH conjugation reactions

MDI-GSH conjugates were prepared as previously reported (Lin et al. 2020, 2023). 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 MDI concentration of approximately 800 μM. Upon addition of MDI to GSH solution, reactions were incubated at 25 °C with end-over-end mixing for 1 hr. The reaction mixture was then centrifuged at 10,000 × g and filtered with a 0.2 μm syringe filter. Reaction products containing MDI-GSH conjugate were immediately added into either differentiated THP-1 macrophages, or THP-1 macrophages with kenpaullone treatment at 10 μM of MDI-GSH.

Plasmid construction

pMIR-REPORT firefly luciferase vector was obtained from ThermoFisher Scientific (Waltham, MA). pRL-TK Renilla luciferase reporter was obtained from Promega (Madison, WI). Expression plasmids pCMV6-Entry-KLF4 (Origene ID: RC206691) and pCMV6-Entry (ID: PS100001) were obtained from Origene (Rockville, MD). To construct a wildtype (WT) *KLF4*-3'UTR luciferase translational reporter, a 0.9-kb cDNA fragment representing the 3′UTR of human *KLF4* (NM_004235.6) was generated by PCR using a *MluI* restriction site containing forward primer (ccgacgcgtATCCCAGACAGTGGATATGACCCA) and a *PmeI* restriction site containing reverse primer (ccgtttaaacTTCAGATAAAATATTATAGGTTTA) on THP-1 cell cDNA. The PCR-amplified *KLF4*-3′UTR cDNA fragment was treated with *MluI* and *PmeI*. This fragment was inserted into pMIR-REPORT vector that was prepared by sequential enzyme treatments with *MluI, PmeI*, and calf intestinal alkaline phosphatase (CIP).

Transient transfection and translational reporter assays

For KLF4 overexpression, 1×10^6 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 *TransIT*®-2020 transfection reagent in a 6-well plate for 48 h. After 48 h, total RNA was

isolated using *mirVana*™ miR Isolation Kit (ThermoFisher Scientific) according to manufacturer's instruction for RT-qPCR expression analyses. For miR functional analyses, the following *mirVana*™ miRNA inhibitors (MH) and, miR-mimics (MC) were obtained from ThermoFisher Scientific and diluted to 20 μM in nuclease-free water: *hsa-miR-206-3p* (MH10409, MC10409), *hsa-miR-381-3p* (MH10242, MC10242) MH-negative control #1 (4464076), and MC-negative control #1 (4464058). Cells were subjected to reverse transfection and 24 h later, forward transfection was performed as previously described (Lin et al. 2011). Twenty-four hours after the start of the forward transfection, cell extracts were prepared for RT-qPCR expression analyses. Translational luciferase reporter assays were performed following just one transfection, at 24 h after the start of the reverse transfection. miR-inhibitors or -mimics were co-transfected with *KLF4*-3'UTR luciferase translational reporter plasmid, including the pRL-TK control, and Dual-Luciferase Reporter Assays (Promega) were performed as previously described (Lin et al. 2011).

Expression analyses

For RT-qPCR assays, total RNA from THP-1 macrophages was extracted using *mirVana*™ miR Isolation Kit (ThermoFisher Scientific) according to manufacturer's instructions. PCR reactions were performed on an ABI 7500 Real-Time PCR System from ThermoFisher Scientific (Waltham, MA). The mRNA and miR levels were analyzed as previously described (Lin et al. 2019; Lin et al. 2020). Reactions were normalized to human beta-2 microglobulin (*B2M*) for mRNA analysis and *U6* snRNA for miR analysis. Gene/miR expression assays used in this study were acquired from ThermoFisher Scientific and include: human *KLF4* (Cat#4331182; Assay ID: Hs00358836_m1), *TGM2* (Hs01096681_m1), *CD206* (Hs00267207_m1), *CCL17* (Hs00171074_m1), *CCL22* (Hs01574247_m1), *CCL24* (Hs00171082_m1), and *B2M* (Hs00187842_m1), *hsa-miR-206-3p* (Cat# 4427975; Assay ID No. 000510; hsa/Homo sapiens), *hsa-miR-381-3p* (No. 000571), and *U6* snRNA (No. 001973).

Immunoblot and antibodies

Cell extracts for immunoblot were prepared in RIPA buffer as previously described (Lin et al. 2020, 2023). Following electrophoresis, proteins were transferred onto nitrocellulose membranes and probed with diluted antibodies in TBST containing 1% BSA. Specific antibody against human KLF4 (Cat#AB4138) was obtained from MilliporeSigma (Burlington, MA). Antibody against β-actin (Cat#sc-47778) was obtained from Santa Cruz Biotechnology (Dallas, TX). Bound antibodies were detected using Pierce ECL Western Blotting Substrate (ThermoFisher Scientific).

Validation of miR target by Argonaute (Ago) immunoprecipitation

Immunoprecipitation (IP) of the miR-containing RNA inducing silencing complex (miR/RISC) and miR targeting mRNAs was performed using the miRNA target IP kit (Active Motif, Carlsbad, CA) as previous described (Lin et al. 2020). Briefly, enhanced-differentiated THP-1 macrophages were trypsinized and seeded at 8×10^6 cells in 10 cm dishes. The cells were transfected with 25 nM of either miR-mimic-miR-206-3p or miR-mimic-miR-381-3p or miR-mimic negative control #1 for 24 h. Two 10 cm dishes of cells using an equal number of 1.6×10^7 cells were taken for the IP reaction. After cell lysis, the lysates were divided into two equal aliquots. Each lysate aliquot underwent IP by using either a pan-Ago antibody to precipitate the RISC containing Agos/miRs/mRNAs or an isotype IgG antibody control. The precipitated complex was collected, and the RNA was purified from the RISC complex using *mirVana*™ miR Isolation Kit (ThermoFisher Scientific). The RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) and TaqMan qPCR assays of human *KLF4, TGM2* and *CCL22* was used for RT-qPCR. The data was analyzed by comparing the cells transfected with miR-mimics or non-target miR-mimic-control oligonucleotide and the fold enrichment of either *KLF4*, *TGM2* or *CCL22* was calculated from the anti-panAgo and the IgG isotype antibody IP preparations as described by the manufacturer.

In silico* analysis of predicted interactions between *KLF4, CD206, TGM2, CCL17, CCL22, CCL24* transcripts and *hsa-miR-206-3p/hsa-miR-381-3p

Potential interactions between the 3'UTRs of human *KLF4, CD206, TGM2, CCL17, CCL22,* and *CCL24* transcripts and *hsa-miR-206-3p/hsa-miR-381-3p* were first examined using the online *in silico* tool, TargetScanHuman v8.0 (http://www.targetscan.org/vert_80/) (Agarwal et al. 2015; McGeary et al. 2019). Candidate miR-mRNA interactions were further examined with several *in silico* algorithms including miRanda (Enright et al. 2003), PicTar (Krek et al. 2005), PITA (Kertesz et al. 2007), and RNA22 (Miranda et al. 2006) using the web-based tool miRsystem (http://mirsystem.cgm.ntu.edu.tw/) (Lu et al. 2012). Furthermore, two online databases containing the most recent experimentally supported miR-gene interactions were queried to verify candidate miR binding to candidate mRNAs as followed: DIANA-TarBase v.8 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=tarbasev8) (Karagkouni et al. 2018) and miRTarBase (https://mirtarbase.cuhk.edu.cn/~miRTarBase/miRTarBase_2022/php/index.php) (Huang et al. 2020).

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