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RESEARCH ARTICLE

MicroRNA-mediated Krüppel-like factor 4 upregulation induces alternatively activated macrophage-associated marker and chemokine transcription in 4,4′-methylene diphenyl diisocyanate exposed macrophages

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ABSTRACT

1. Occupational exposure to 4,4′-methylene diphenyl diisocyanate (MDI) is associated with occupational asthma (OA) development. Alveolar macrophage-induced recruitment of immune cells to the lung microenvironment plays an important role during asthma pathogenesis. Previous studies identified that MDI/MDI-glutathione (GSH)-exposure downregulates endogenous *hsa-miR-206-3p/hsa-miR-381-3p*. Our prior report shows that alternatively activated (M2) macrophage-associated markers/chemokines are induced by MDI/MDI-GSH-mediated Krüppel-Like Factor 4 (KLF4) upregulation in macrophages and stimulates immune cell chemotaxis. However, the underlying molecular mechanism(s) by which MDI/MDI-GSH upregulates KLF4 remain unclear.
2. Following MDI-GSH exposure, microRNA(miR)-inhibitors/mimics or plasmid transfection, endogenous *hsa-miR-206-3p/hsa-miR-381-3p,* KLF4, or M2 macrophage-associated markers (*CD206*, *TGM2*), and chemokines (*CCL17, CCL22, CCL24*) were measured by either RT-qPCR, western blot, or luciferase assay.
3. MDI-GSH exposure downregulates *hsa-miR-206-3p/hsa-miR-381-3p* by 1.46- to 9.75-fold whereas upregulates *KLF4* by 1.68- to 1.99-fold, respectively. *In silico* analysis predicts binding between *hsa-miR-206-3p/hsa-miR-381-3p* and *KLF4.* Gain- and loss-of-function, luciferase reporter assays and RNA-induced silencing complex-immunoprecipitation (RISC-IP) studies confirm the posttranscriptional regulatory roles of *hsa-miR-206-3p/hsa-miR-381-3p* and *KLF4* in macrophages. Furthermore, *hsa-miR-206-3p/hsa-miR-381-3p* regulate the expression of M2 macrophage-associated markers and chemokines *via* KLF4.
4. In conclusion, *hsa-miR-206-3p/hsa-miR-381-3p* play a major role in regulation of MDI/MDI-GSH-induced M2 macrophage-associated markers and chemokines by targeting the *KLF4* transcript, and KLF4-mediated regulation in macrophages.

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Introduction

Diisocyanates (dNCOs) are highly reactive chemicals used for polyurethane production in many applications (Allport et al. 2003). 4,4′-methylene diphenyl diisocyanate (MDI) is the world's most produced and utilised dNCO with evolving industrial applications, and also in consumer products (e.g. spray-on truck bed liners, spray foam insulation, car instrument panels, self-expanding memory foam mattresses, three-dimensional printing, etc.) (USEPA 2011; Jones et al. 2017). As such, both global production and the market for MDI are predicted to continue to grow (Statista 2022). Workplace exposure to MDI can lead to development of occupational asthma (OA) and even death in exposed workers (Bernstein et al. 1993; NIOSH 1994a, 1994b; Redlich and Karol 2002; Lofgren et al. 2003; NIOSH 2004; Jan et al. 2008; Engfeldt et al. 2013; Wisnewski et al. 2022); however, the detailed molecular mechanism(s) that participated in the pathogenesis of MDI-OA remain unclear.

Recruitment of immune cells such as eosinophils and T-cells into the lung microenvironment following irritation or allergen exposure in the airways, as well as the interactions between infiltrated immune cells and the airway cells in the lung microenvironment play critical roles during asthma pathogenesis (Barnes 2008; Holgate 2008; Boonpiyathad et al. 2019). Alveolar macrophages have been implicated in the development of asthma (Fricker and Gibson 2017), and alternatively activated (M2) macrophage populations have been found to be elevated in the airways of asthmatic patients (Girodet et al. 2016). By using an MDI-OA animal model, Wisnewski and colleagues showed that many immune cell types, including macrophages, T-cells, and eosinophils can be recruited into the lung microenvironment with induced alternatively activated (M2) macrophage-associated gene signatures after MDI/MDI-GSH exposure (Wisnewski et al. 2015; 2020). Our previous report showed that *in vivo* MDI exposure and *in vitro* MDI-glutathione (GSH) conjugate exposure upregulates some M2 macrophage-associated markers as well as

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chemokine expression through induction of M2 macrophage-associated transcriptional factor, Krüppel-Like Factor 4 (KLF4) (Lin et al. 2023). KLF4 belongs to the zinc-finger transcription factor family and is expressed in a wide variety of mammalian tissues, where it contributes to regulation of proliferation, differentiation, development, tissue homeostasis and apoptosis (Ghaleb and Yang 2017). In macrophages, KLF4 regulates macrophage differentiation and M2 macrophage polarisation (Park et al. 2016); however, the detailed molecular mechanism(s) by which MDI/MDI-GSH exposure induces KLF4 is currently unclear.

MicroRNAs (miRs) are single-stranded, noncoding RNA molecules ranging from 19 to 24 nucleotides in length with the capacity to inhibit protein translation and promote mRNA degradation by imperfectly binding to their target RNA transcripts. Through posttranscriptional regulation of their target genes, miRs are involved in regulating various cellular processes such as the cell cycle, proliferation, apoptosis, and differentiation in many different cell types (Bartel 2018). Additionally, endogenous miR expression profile changes have been implicated in the pathogenesis of several lung diseases including asthma (Sittka and Schmeck 2013; Weidner et al. 2020). Other reports have determined that *KLF4* can be post-transcriptionally regulated by miRs in both normal and disease conditions (Ghaleb and Yang 2017; Li et al. 2023). Furthermore, KLF4 has been previously shown to be regulated by *hsa-miR-206-3p* in many different cell types (Parasramka et al. 2012; Ren et al. 2014; Wang et al. 2021). In asthmatic children, there is a negative correlation between KLF4 and *hsa-miR-206-3p* levels in peripheral blood mononuclear cells (PBMCs) (Qinglei et al. 2015). The murine *Klf4* transcript is *in silico* predicted to be co-targeted by murine miRs *mmu-miR-206-3p* and *mmu-miR-381-3p* (Lin et al. 2019). Our previous reports determined that exposure to MDI-GSH conjugate downregulates endogenous levels of *hsa-miR-206-3p* and *hsa-miR-381-3p* in macrophages (Lin et al. 2020, 2021). Therefore, we hypothesise that MDI-mediated downregulation of *hsa-miR-206-3p* and/or *hsa-miR-381-3p* may participate in the regulation of KLF4 expression following MDI exposure.

The current report focuses on characterising whether MDI-mediated *hsa-miR-206-3p* and/or *hsa-miR-381-3p* downregulation may participate in the regulation of endogenous KLF4 and KLF4-mediated regulation of downstream M2 macrophage-associated markers and chemokine responses *in vitro* using human THP-1 macrophages. *In vitro* MDI-GSH conjugate exposure exhibits downregulation of endogenous *hsa-miR-206-3p* and *hsa-miR-381-3p* and subsequent upregulation of KLF4 signalling-mediated induction of M2 macrophage-associated markers and chemokines. This report provides a putative miR-regulated mechanism to describe how KLF4 and KLF4-mediated M2 macrophage-associated markers and chemokines are upregulated following MDI/MDI-GSH exposure in macrophages.

Materials and methods

Chemicals and reagents

High-performance liquid chromatography (HPLC) grade acetone, 3 Å 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 Foetal 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 72 h. PMA differentiation at 10 ng/mL to THP-1 monocytes has been shown to enhance responsiveness to polarising 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). Fifty 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 h. 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 analysed as previously described (Lin et al. 2019; 2020). Reactions were normalised 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-206-3p or miR-mimic-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 analysed 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 (https://dianalab.e-ce.uth.gr/html/diana/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).

Statistical analysis

Data were analysed 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 in GraphPad Prism 7.0 (GraphPad Software, La Jolla, CA, USA.). Differences were considered significant when the analysis yielded $p < 0.05$.

Results

Endogenous **hsa-miR-206-3p/hsa-miR-381-3p** *are downregulated and* **KLF4** *is upregulated in MDI-GSH conjugate treated macrophages*

Our previous reports demonstrated that exposure to MDI/MDI-GSH conjugates downregulate endogenous *hsa-miR-206-3p* and *hsa-miR-381-3p* levels (Lin et al. 2020, 2021) and upregulates endogenous *KLF4* levels (Lin et al. 2023) in THP-1 macrophages. To further investigate whether MDI-GSH conjugate exposure simultaneously regulates endogenous KLF4 as well as either *hsa-miR-206-3p* or *hsa-miR-381-3p* expressions, differentiated-enhanced THP-1 macrophages were treated with MDI-GSH conjugates at concentrations of 0, 1, and 10 μM for 24 h. In agreement with our previous observations, the endogenous *hsa-miR-206-3p* was significantly downregulated 1.46-fold to 2.63-fold (Figure 1A) and endogenous *hsa-miR-381-3p* was significantly downregulated 1.47-fold to 9.75-fold (Figure 1B) after MDI-GSH conjugate exposure in THP-1 macrophages. Endogenous *KLF4* mRNA was significantly upregulated 1.68-fold to 1.99-fold (Figure 1C) and the endogenous KLF4 protein was also upregulated after MDI-GSH conjugate exposure in THP-1 macrophages (Figure 1D). These results confirm that exposure to MDI in the form of MDI-GSH conjugates can simultaneously upregulate the endogenous KLF4 and decrease endogenous *hsa-miR-206-3p* and *hsa-miR-381-3p* levels in macrophages.

KLF4-mediated signalling is partially involved in the regulation of MDI-GSH conjugate-mediated induction of M2 macrophage-associated markers and chemokines in differentiated THP-1 macrophages

Previous studies in our laboratory demonstrated that exposure to MDI/MDI-GSH conjugates may induce M2 macrophage-associated markers and chemokines including *Cd206/CD206, Tgm2/TGM2, Ccl17/CCL17, Ccl22/CCL22* and *CCL24* partially through induction of endogenous *Klf4*/KLF4 expression in bronchial alveolar lavage cells (BALCs)/alveolar macrophages (Lin et al. 2023). We utilised specific siRNAs that were designed to target endogenous mature *KLF4* mRNA transcripts within the cytosol to degrade KLF4 transcripts and therefore knockdown KLF4 protein levels in macrophages. The siRNA-mediated knockdown of KLF4 protein revealed that KLF4 plays a role in regulating M2 macrophage-associated markers and chemokines (Lin et al. 2023). One of the major aims for the current study is to investigate whether miRs can target *KLF4* and therefore regulate KLF4 protein expression, however, miRs and siRNAs employ similar RISC-mediated gene inhibition mechanisms to downregulate target gene expression (Lam et al. 2015). To avoid possible competition between *KLF4* siRNAs and *hsa-miR-206-3p/hsa-miR-381-3p* for RISCs in macrophages, we utilised chemical inhibitors of KLF4 to determine whether *KLF4*-mediated transcriptional control after MDI-exposure participates in the regulation of M2 macrophage-associated markers and chemokines including *CD206, TGM2, CCL17, CCL22* and *CCL24*. We treated THP-1 macrophages with kenpaullone, a chemical inhibitor of KLF4 (Lyssiotis et al. 2009; Tien et al. 2015; Montecillo-Aguado et al. 2021), to inhibit the endogenous KLF4 function in macrophages with or without MDI-GSH conjugate exposure (Figure 2). Compared to the vehicle control treated THP-1 macrophages, treatment of 1 μM kenpaullone significantly downregulated endogenous *KLF4, CD206, CCL17* and *CCL22* transcripts by 15.9-, 2.10-, 13.3,- and 2.28-fold (Figure 2A, B, D, and E), respectively, whereas other M2 macrophage-associated markers and chemokines including *TGM2* and *CCL24* transcripts were not significantly changed (Figure 2C, F). In agreement with previous findings, exposure to 10 μM of MDI-GSH conjugate upregulated endogenous *KLF4, CD206, TGM2, CCL17, CCL22,* and *CCL24* transcript expression by 3.06-, 1.57-, 1.91-, 2.87-, 1.78-, and 2.56-fold, respectively (Figure 2A–F). Furthermore, the induction of endogenous *KLF4, CD206, TGM2, CCL17, CCL22,* and *CCL24* transcripts by MDI-GSH conjugate (10 μM) exposure were attenuated by treatment with 1 μM of kenpaullone (Figure 2A–F). These results indicate that MDI-exposure mediated activation of *KLF4* and KLF4-mediated downstream signalling plays a role in regulation of the MDI-mediated induction of M2 macrophage-associated marker and chemokine transcript expression in macrophages.

Inhibition of either **hsa-miR-206-3p** *or* **hsa-miR-381-3p** *upregulates* **KLF4** *and M2 macrophage-associated markers* **CD206, TGM2, CCL17, CCL22** *and* **CCL24** *in macrophages*

We previously reported that KLF4 mediates the induction of M2 macrophage-associated markers CD206 and TGM2 as well as chemokines CCL17, CCL22, and CCL24 after MDI/MDI-GSH exposure in macrophages (Lin et al. 2023) and the induction of KLF4 is associated with downregulation of *hsa-miR-206-3p* and *hsa-miR-381-3p* after MDI-GSH conjugate exposure in macrophages as shown in Figure 1. Furthermore, the murine *Klf4* transcript is *in silico* predicted to be coregulated by murine miRs *mmu-miR-206-3p/mmu-miR-381-3p* (Lin et al. 2019). These observations led us to hypothesise that endogenous *hsa-miR-206-3p* or *hsa-miR-381-3p* may contribute to M2 macrophage-associated marker *CD206, TGM2, CCL17, CCL22,* and *CCL24* regulation in macrophages through

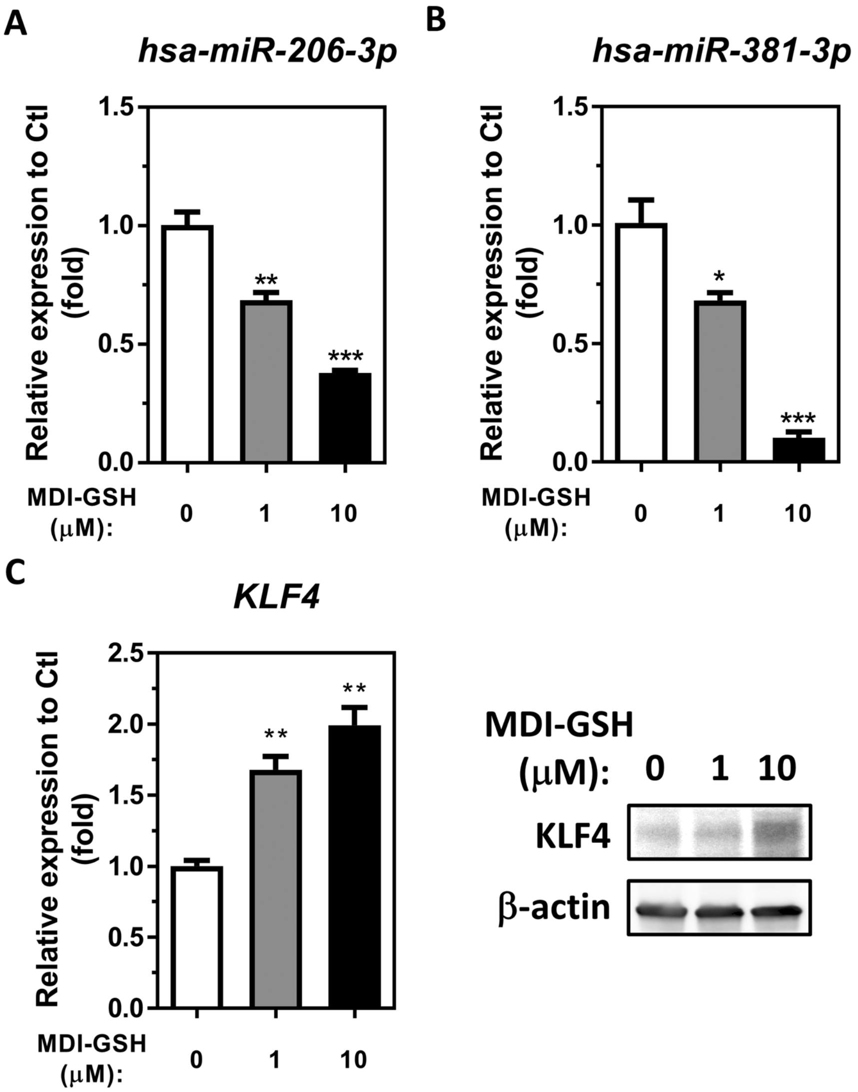

Figure 1. MDI-GSH conjugates downregulate endogenous *hsa-miR-206-3p* and *hsa-miR-381-3p* and upregulate endogenous KLF4 in differentiated THP-1 macrophages. Total RNA was isolated from MDI-GSH treated differentiated THP-1 macrophages at indicated concentration for 24 h by *miRVana*™ miR isolation kit, reverse transcribed, and subjected to TaqMan RT-qPCR assays. Endogenous levels of (A) *hsa-miR-206-3p* and (B) *hsa-miR-381-3p* as well as (C) *KLF4* transcripts were determined at 24 h after MDI-GSH conjugate treatment ($N = 3$; bars, SEM). (D) Endogenous KLF4 protein was analysed by immunoblot. β-actin served as a loading control. MDI: 4,4′-methylene diphenyl diisocyanate. GSH: Glutathione (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

hsa-miR-206-3p/hsa-miR-381-3p-mediated posttranscriptional regulation of *KLF4*. To investigate the role of *hsa-miR-206-3p* and *hsa-miR-381-3p* in regulation of endogenous *KLF4* and M2 macrophage-associated marker transcripts, we utilised loss- or gain-of-function studies by transfecting macrophages with either miR-inhibitors or miR-mimics of *hsa-miR-206-3p* and *hsa-miR-381-3p*. Loss-of-function studies were performed by transfecting miR-inhibitors of either *hsa-miR-206-3p* or *hsa-miR-381-3p* into differentiated THP-1 macrophages to mimic the effects observed as MDI-GSH conjugate treatment downregulates endogenous *hsa-miR-206-3p* and *hsa-miR-381-3p* levels in macrophages (Figure 1A,B). In agreement with the finding that *KLF4* transcripts are upregulated after MDI-GSH conjugate exposure (Figure 1C), transfection of miR-inhibitor-206-3p upregulates endogenous *KLF4* by 3.59-fold and transfection of miR-inhibitor-381-3p upregulates endogenous *KLF4* by 2.16-fold (Figure 3A). Furthermore, transfection of miR-inhibitor-206-3p upregulates endogenous M2 markers *CD206, TGM2, CCL17, CCL22,* and *CCL24* transcripts by 1.56-, 1.42-, 3.22-, 2.58-, and 2.47-fold, respectively (Figure 3B–F). Similarly, transfection of miR-inhibitor-381-3p upregulates endogenous M2 markers *CD206, TGM2, CCL17, CCL22,* and *CCL24* transcripts by 3.48-, 2.02-, 2.48-, 2.56-, and 3.43-fold, respectively (Figure 3B–F). These results indicate that both the endogenous *KLF4* transcript and M2 macrophage-associated marker mRNAs may be regulated by either endogenous *hsa-miR-206-3p* or *hsa-miR-381-3p* in macrophages.

Mimics of either hsa-miR-206-3p *or* hsa-miR-381-3p *downregulate KLF4 and M2 macrophage-associated markers* CD206, TGM2, CCL17, CCL22 *and* CCL24 *in macrophages*

Using a gain-of-function approach by transfection of miR-mimics, we further explored the regulatory function of

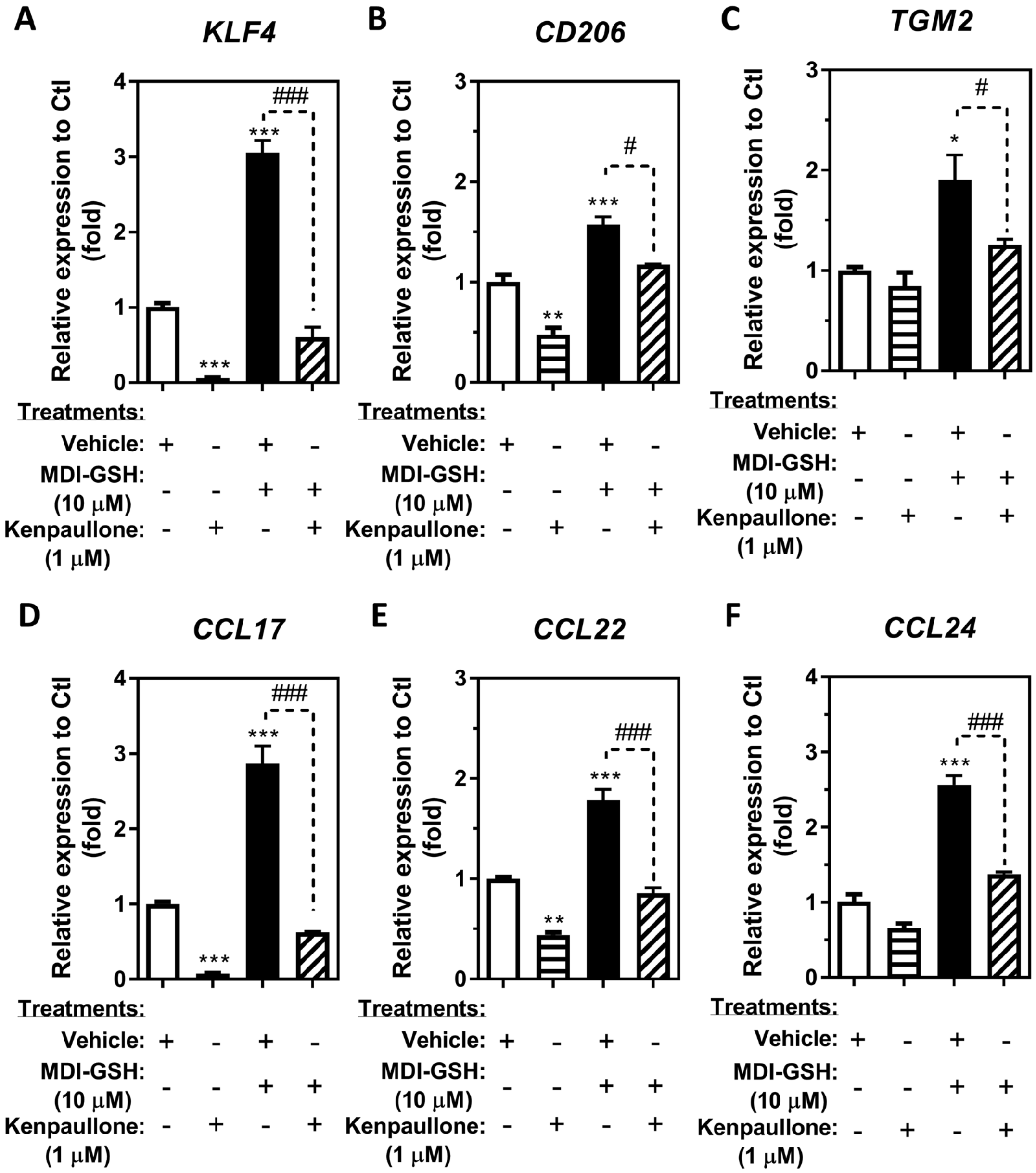

Figure 2. KLF4 inhibition attenuate MDI-GSH mediated induction of M2 macrophage-associated marker and chemokine expression in differentiated THP-1 macrophages. The differentiated THP-1 macrophages were treated either with 1 μM of Kenpaullone or vehicle for 24 h followed by treatment with or without 10 μM MDI-GSH conjugates for 24 h. Total RNA were isolated from THP-1 macrophages with indicated treatment by *miRVana*™ miR isolation kit, reverse transcribed, and subjected to TaqMan stem-loop RT-qPCR. The endogenous M2 macrophage-associated transcription factor (A) *KLF4*, markers (B) *CD206*, (C), *TGM2*, (D) *CCL17*, (E) *CCL22*, and (F) *CCL24* mRNA levels were determined in total RNA isolated from THP-1 macrophages ($N = 3$; bars, SEM). Vehicle (Ctl): RPMI-1640 media containing 10% FBS and 0.01% DMSO. MDI: 4,4′-methylene diphenyl diisocyanate. GSH: Glutathione. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared to vehicle treated macrophages; #$p < 0.05$, ###$p < 0.001$, when compared to macrophages treated with vehicle followed by 10 μM MDI-GSH conjugate exposure.).

hsa-miR-206-3p and *hsa-miR-381-3p* to inhibit the endogenous levels of *KLF4* and M2 macrophage-associated marker transcripts. Transfection of miR-mimic-206-3p downregulated endogenous *KLF4* by 3.09-fold and transfection of miR-mimic-381-3p downregulated endogenous *KLF4* by 6.45-fold (Figure 4A). In addition, transfection of miR-mimic-206-3p downregulated endogenous M2 macrophage-associated markers *CD206, TGM2, CCL17, CCL22,* and *CCL24* transcripts by 8.07-, 2.24-, 1.67-, 2.54-, and 2.95-fold, respectively, whereas transfection of miR-mimic-381-3p downregulated endogenous *CD206, TGM2, CCL17, CCL22,* and *CCL24* transcripts by 4.30-, 2.69-, 3.14-, 2.48-, and 2.22-fold, respectively (Figure 4B-F). Given that miR-inhibitors of both *hsa-miR-206-3p* and *hsa-miR-381-3p* upregulate *KLF4* expression (Figure 3A), and the miR-mimics of both *hsa-miR-206-3p* and *hsa-miR-381-3p* downregulate endogenous *KLF4* (Figure 4A), these results suggest that the *KLF4* transcript may be post-transcriptionally regulated by *hsa-miR-206-3p*/*hsa-miR-381-3p* in macrophages. Furthermore, the endogenous M2 macrophage-associated markers *CD206, TGM2, CCL17, CCL22,* and *CCL24* transcripts are regulated by *hsa-miR-206-3p*/*hsa-miR-381-3p* in macrophages (Figure 3B–F; Figure 4B–F). Together with the observation that *hsa-miR-206-3p*/*hsa-miR-381-3p* downregulate *KLF4,* these data indicate that the elevation of M2 macrophage-associated markers *CD206, TGM2, CCL17, CCL22,* and *CCL24* transcripts in response to MDI-GSH

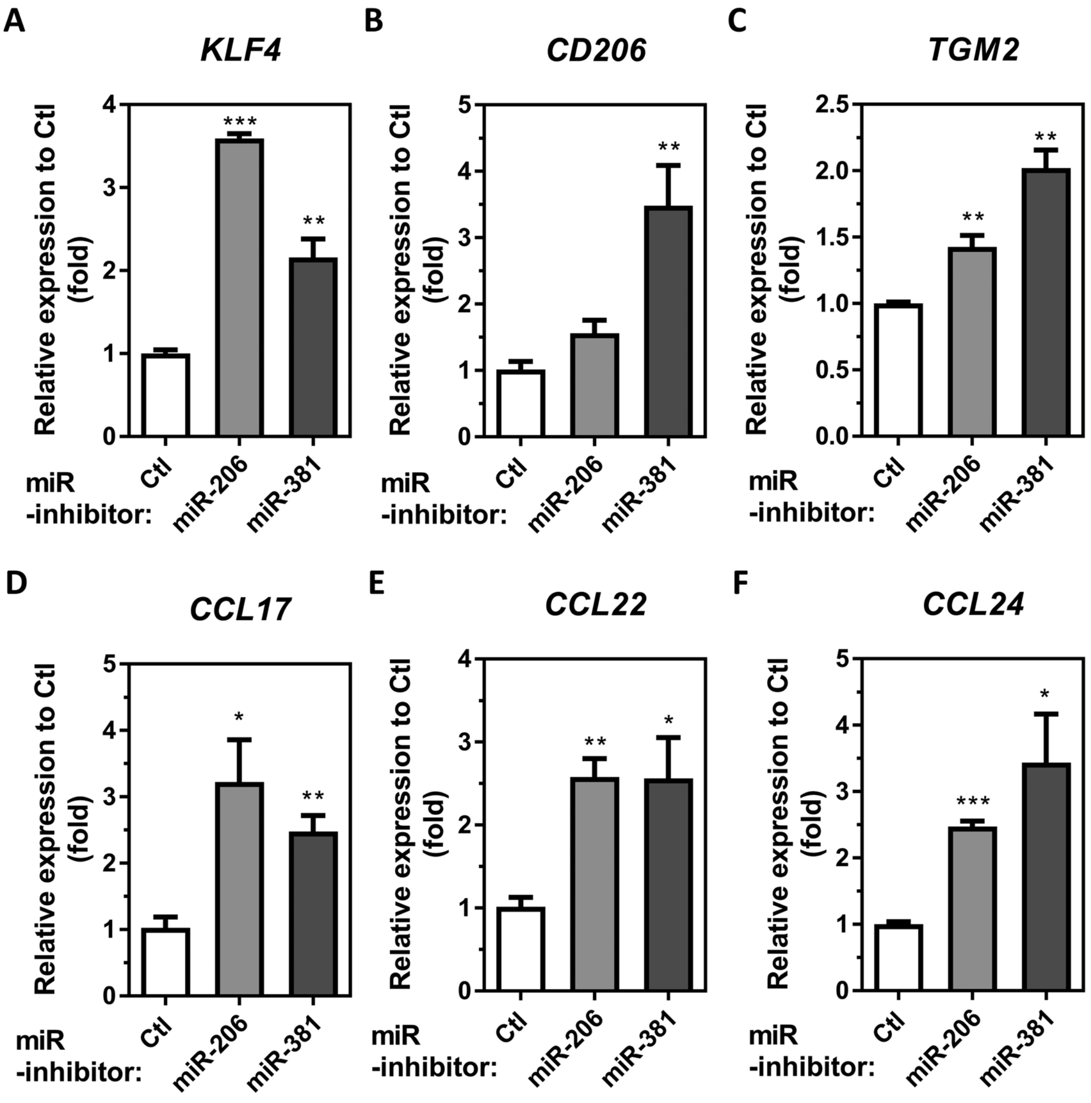

Figure 3. Transfection of miR-inhibitors of *hsa-miR-206-3p* and *hsa-miR-381-3p* induce endogenous M2 macrophage-associated markers and chemokines in differentiated THP-1 macrophages. THP-1 macrophages were transfected with 25 nM of either miR-inhibitor-206-3p, miR-inhibitor-381-3p or nontargeting miR-inhibitor control (miR-inhibitor-Ctl) for 48 h. Total RNA were isolated from the indicated miR-inhibitor transfected THP-1 macrophages by *miRVana*™ miR isolation kit, reverse transcribed, and subjected to TaqMan RT-qPCR. Endogenous mRNA expressions of (A), *KLF4*, (B) *CD206* (C) *TGM2*, (D) *CCL17*, (E) *CCL22*, and (F) *CCL24* were determined by RT-qPCR ($N = 3$; bars, SEM). (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

conjugate exposure is likely to be regulated by *hsa-miR-206-3p*/*hsa-miR-381-3p*-mediated KLF4 transcriptional activation.

Human KLF4 *transcript as a potential target for either* hsa-miR-206-3p *or* hsa-miR-381-3p

Previous study in our laboratory showed that the murine *Klf4* transcript is *in silico* predicted to be regulated by either murine miRs *mmu-miR-206-3p* or *mmu-miR-381-3p* (Lin et al. 2019). Given that murine *Klf4* transcripts are highly conserved in humans and the murine miRs *mmu-miR-206-3p* and *mmu-miR-381-3p* are identical to their human counterparts *hsa-miR-206-3p* and *hsa-miR-381-3p,* we further examined the potential binding of *hsa-miR-206-3p* and *hsa-miR-381-3p* with the human *KLF4* transcript (NM_004235.5) using the *in silico* miR-target prediction tool miRDB (Wong and Wang 2015) and TargetScanHuman 8.0 (Agarwal et al. 2015; McGeary et al. 2019). *In silico* analysis identified one putative *hsa-miR-206-3p* binding site and two *hsa-miR-381-3p* predicted binding sites located in the 3′UTR of the *KLF4* transcript (Figure 5A).

To investigate whether *hsa-miR-206-3p* and/or *hsa-miR-381-3p* can regulate endogenous KLF4 expression in differentiated THP-1 macrophages, we performed gain- and loss-of-function studies by transfecting either miR-mimics or miR-inhibitors of *hsa-miR-206-3p* and *hsa-miR-381-3p* into differentiated/enhanced THP-1 macrophages. Independent transfections of either miR-mimic-206-3p or miR-mimic-381-3p downregulated endogenous KLF4 protein (Figure 5B) and RNA (Figure 5C) compared to the miR-mimic control. Because MDI-GSH conjugate treatment decreased endogenous *hsa-miR-206-3p* and *hsa-miR-381-3p* levels in differentiated THP-1 macrophages (Figure 1A,B), we performed a loss-of-function study by transfecting differentiated THP-1 macrophages with miR-inhibitor-206-3p, miR-inhibitor-381-3p

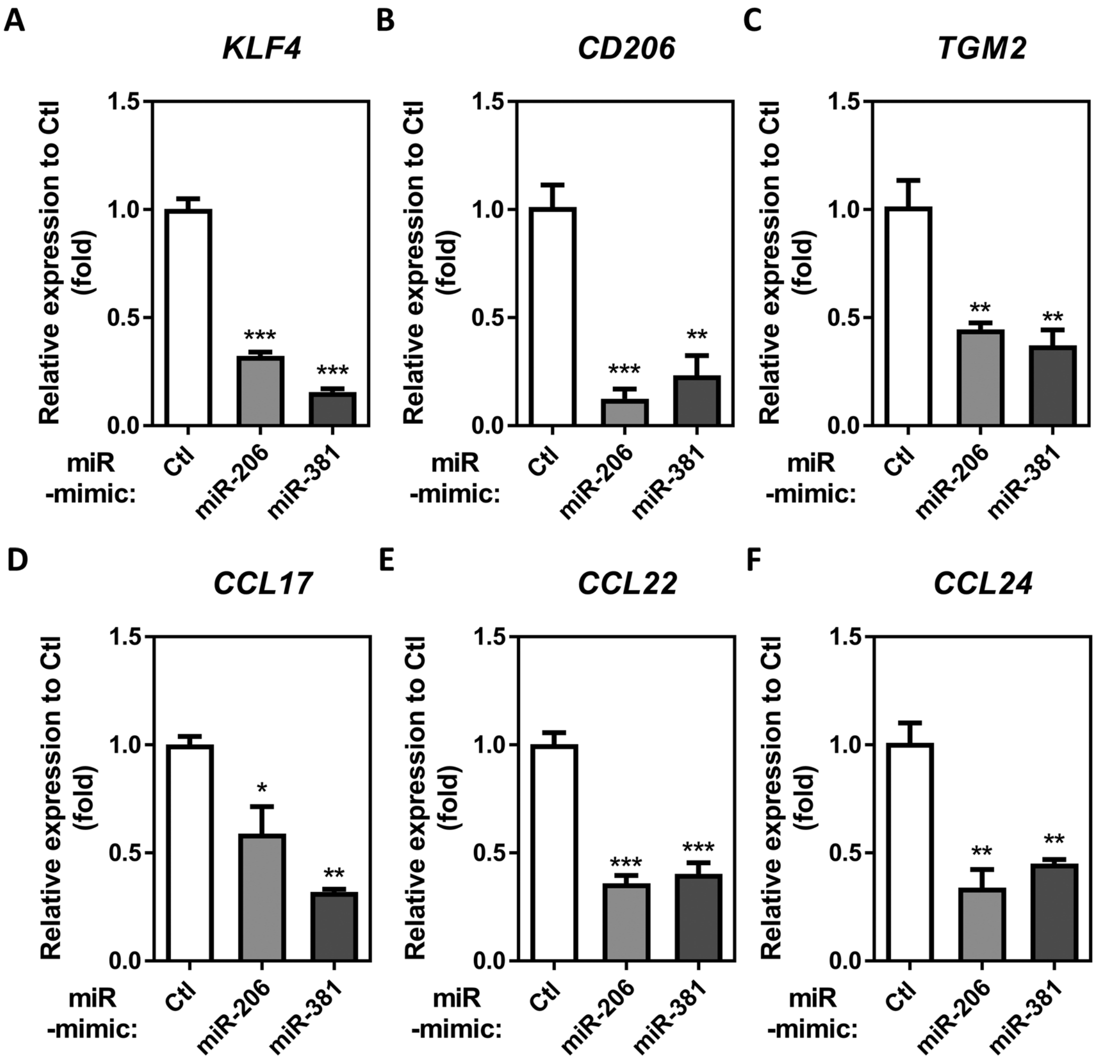

Figure 4. Transfection of miR-mimics of *hsa-miR-206-3p* and *hsa-miR-381-3p* decrease endogenous M2 macrophage-associated markers and chemokines in differentiated THP-1 macrophages. THP-1 macrophages were transfected with 25 nM of either miR-mimic-206-3p, miR-mimic-381-3p or nontargeting miR-mimic control (miR-mimic-Ctl) for 48 h. Total RNA was isolated from the indicated miR-mimics transfected THP-1 macrophages by *miRVana*™ miR isolation kit, reverse transcribed, and subjected to TaqMan RT-qPCR. Endogenous mRNA expressions of (A), *KLF4,* (B) *CD206* (C) *TGM2*, (D) *CCL17*, (E) *CCL22*, and (F) *CCL24* were determined by RT-qPCR ($N=3$; bars, SEM). ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$).

and a non-targeting miR-inhibitor control to investigate whether the endogenous KLF4 level would be affected by either *hsa-miR-206-3p* or *hsa-miR-381-3p*. Consistent with KLF4 as a target of either *hsa-miR-206-3p* or *hsa-miR-381-3p*, independent transfection of either miR-inhibitor-206-3p or miR-inhibitor-381-3p increased endogenous KLF4 protein level (Figure 5D), as well as increased endogenous *KLF4* mRNA level (Figure 5E). These data further suggest that *KLF4* transcript may be a potential direct target of either *hsa-miR-206-3p* or *hsa-miR-381-3p* in differentiated macrophages.

Verification of KLF4 *transcript as a direct target of either* hsa-miR-206-3p *or* hsa-miR-381-3p *in differentiated THP-1 macrophages*

A potential target gene that is regulated by miRs may be either directly regulated by binding of a particular miR or through other miR-regulated pathways (e.g. transcriptional, or posttranscriptional regulation) (Tsang et al. 2007; Inui et al. 2010). The miR-mediated translational repression or mRNA transcript degradation of miR-targeted mRNAs is regulated through the binding of a miR-containing RISC which contains argonaute proteins (AGOs) and other proteins including RNAses or translational repressors to the 3′UTR of the target mRNAs (Gregory et al. 2005). To determine if *KLF4* transcripts are directly regulated by either *hsa-miR-206-3p* or *hsa-miR-381-3p*, we first confirmed the binding of either *hsa-miR-206-3p* or *hsa-miR-381-3p* to *KLF4* transcript by performing a pan-argonaute (AGO) antibody pulldown method to precipitate the RISCs which contain AGOs/miRs/mRNAs (RISC-IP), and followed by confirming the roles of *hsa-miR-206-3p* or *hsa-miR-381-3p* on the translational repression by using a *KLF4*-3′UTR luciferase translational reporter assay. Transfection of miR-mimic-206-3p increased precipitated *KLF4* transcripts by 4.14-fold compared to the non-targeting miR-mimic control, whereas transfection of

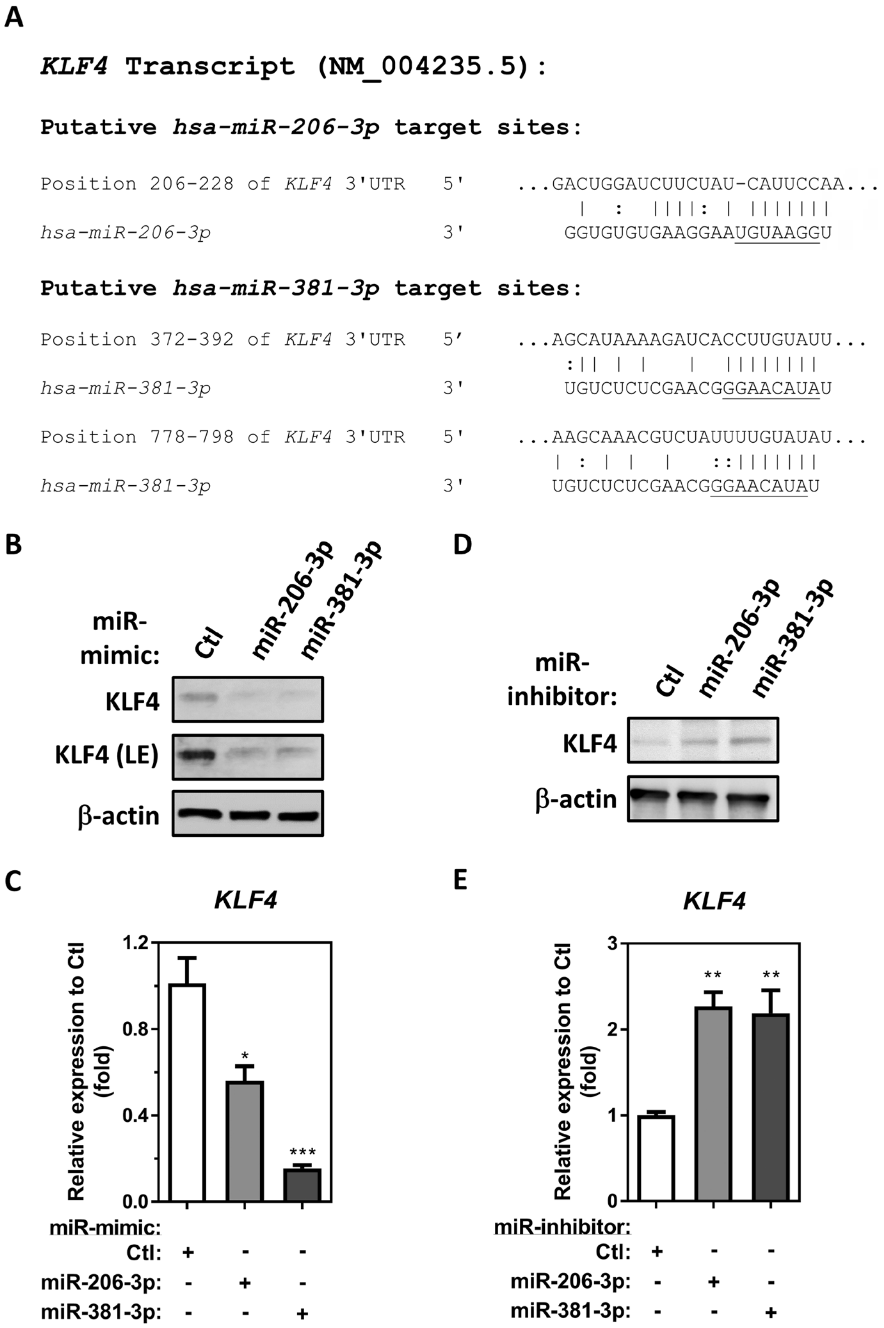

Figure 5. The *KLF4* transcript is a target of either *hsa-miR-206-3p* or *hsa-miR-381-3p*. (A) Alignment of the *KLF4*-3′UTR region indicating putative *hsa-miR-206-3p* and *hsa-miR-381-3p* binding sites. Seed sequences of *hsa-miR-206-3p* and *hsa-miR-381-3p* are underlined. Endogenous KLF4 protein levels in differentiated THP-1 macrophages transfected with 25 nM of miR-mimic (B) or miR-inhibitor (D) were determined by immunoblot. β-Actin served as a loading control. LE: longer exposure. Total RNA isolated from differentiated THP-1 macrophages transfected with 25 nM of miR-mimic (C) or miR-inhibitor (E) determined by *KLF4* TaqMan stem-loop RT-qPCR mRNA assays ($N = 3$; bars, SEM) (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

miR-mimic-381-3p increased precipitated *KLF4* transcripts by 8.65-fold compared to the non-targeting control (Figure 6A). These results indicate that the *KLF4* transcript binds to both *hsa-miR-206-3p* and *hsa-miR-381-3p*-containing RISCs.

Furthermore, we addressed the specificity and translational inhibition ability of either *hsa-miR-206-3p* or *hsa-miR-381-3p* on the *KLF4* mRNA transcript using the *KLF4*-3′UTR luciferase reporter assay. Transfection of miR-mimic-206-3p decreased *KLF4*-3′UTR luciferase reporter activities by 1.60-fold whereas transfection of miR-mimic-381-3p downregulated the KLF4 luciferase reporter activities by 1.67-fold compared to the cells transfected with miR-mimic-Ctl (Figure 6B). In addition, transfection of either miR-inhibitor-206-3p or miR-inhibitor-381-3p increased the KLF4 luciferase reporter activities by 1.90-fold and 1.66-fold, respectively, when compared to transfection of miR-inhibitor-control (Figure 6C). Because both *hsa-miR-206-3p* and *hsa-miR-381-3p* are capable of repressing *KLF4*-3′UTR translational reporter activities, these data indicate that the *KLF4* transcript is, in fact, a direct target of both *hsa-miR-206-3p* and *hsa-miR-381-3p* in differentiated macrophages.

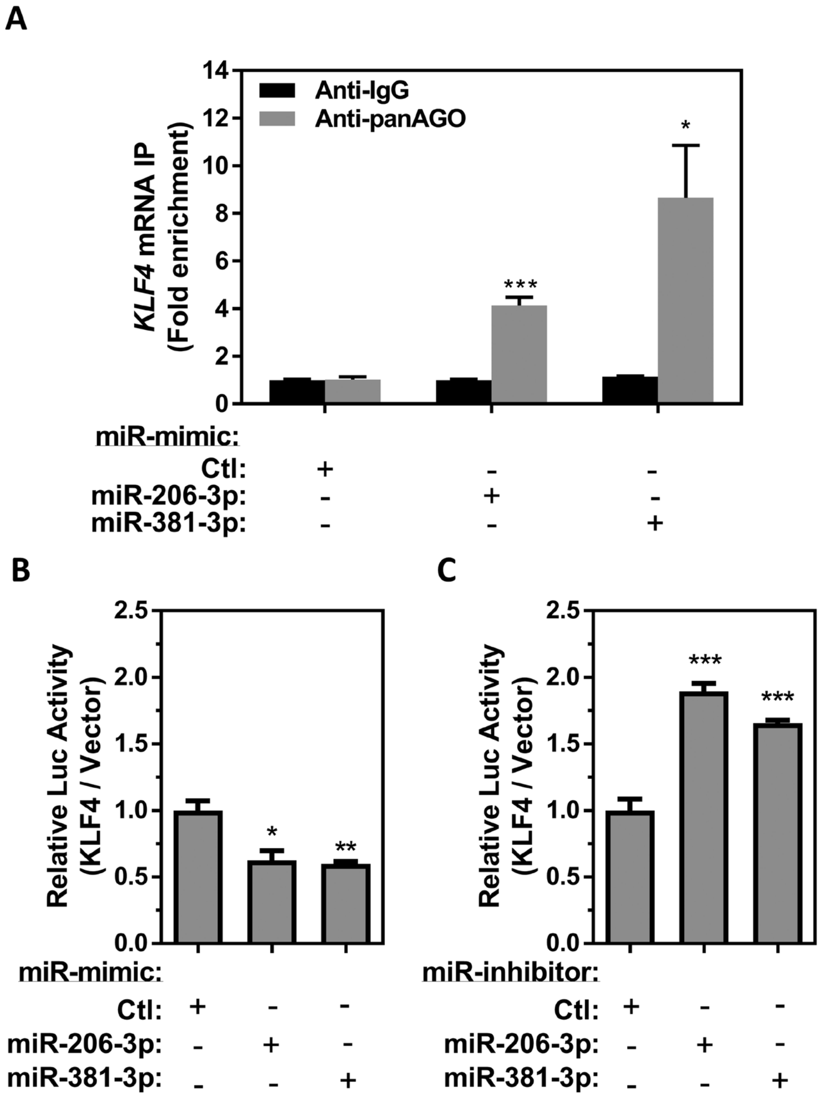

Figure 6. Both *hsa-miR-206-3p* and *hsa-miR-381-3p* bind to endogenous human *KLF4* transcripts in differentiated THP-1 macrophages. (A) Differentiated THP-1 macrophages were transfected with 25 nM of indicated miR-mimics or nontargeting miR-mimic-Ctl for 24 h. The cells were collected and immunoprecipitated using the panAGO or isotype IgG antibody after 24 h transfection. RNA was isolated and the fold enrichment of *KLF4* transcript was measured ($N=3$; bars, SEM). (B) Differentiated THP-1 macrophages were cotransfected with *KLF4*-3′UTR luciferase reporter and 25 nM of indicated miR-mimics or nontargeting miR-mimic-Ctl. After 24 h, the luciferase activities were measured by the Dual-Glo assay kit ($N=3$; bars, SEM). (C) Differentiated THP-1 macrophages were cotransfected with *KLF4*-3′UTR luciferase reporter and 25 nM of indicated miR-inhibitors or nontargeting control. After 24 h, the luciferase activities were measured by the Dual-Glo assay kit ($N=3$; bars, SEM). (*$p<0.05$, **$p<0.01$, ***$p<0.001$); AGO: agornate protein.

Either hsa-miR-206-3p *or* hsa-miR-381-3p *indirectly regulates M2 macrophage-associated markers and chemokines in differentiated THP-1 macrophages*

In the preceding experiments, we demonstrated that both *hsa-miR-206-3p* and *hsa-miR-381-3p* may regulate the expression of M2 macrophage-associated markers (Figures 3 and 4). The ability of *hsa-miR-206-3p* and *hsa-miR-381-3p* to regulate M2 markers *CD206* and *TGM2* as well as *CCL17*, *CCL22* and *CCL24* expression may either be through direct binding to the 3′UTR of these marker and chemokine transcripts or through other indirect regulatory mechanisms. To determine possible direct interactions between *hsa-miR-206-3p/ hsa-miR-381-3p* and the M2 macrophage-associated marker and chemokine transcripts, we first used the *in silico* algorithm TargetScanHuman 8.0 to retrieve candidate miRs that are predicted to target human *CD206, TGM2, CCL17, CCL22,* and *CCL24* transcripts (Supplemental Tables 1–5). TargetScanHuman 8.0 predicted that *hsa-miR-206-3p,* but not *hsa-miR-381-3p,* can target the *TGM2*-3′UTR (Supplemental Table 2) and *CCL22*-3′UTR (Supplemental Table 4), whereas neither *hsa-miR-206-3p* nor *hsa-miR-381-3p* were predicted to bind the 3′UTRs of *CD206, CCL17,* and *CCL24* transcripts (Supplemental Tables 1, 3, and 5). To further support the predicted interaction of the *TGM2*-3′UTR as one of the *hsa-miR-206-3p* targets, we used several *in-silico* algorithms including *DIANA, miRanda, PicTar, PITA*, and *RNA22*. However, the *miRanda, PicTar, PITA*, and *RNA22* algorithms failed to predict any miR-mRNA interaction between the *TGM2*-3′UTR and *hsa-miR-206-3p* (data not shown). Similarly, neither *hsa-miR-206-3p* nor *hsa-miR-381-3p* were predicted to exhibit binding between any 3′UTRs of *CD206, CCL17,* and *CCL24* transcripts when analysed by *DIANA, miRanda, PicTar, PITA*, and *RNA22* (data not shown). Further analysis of the human *TGM2* transcript (NM_004613.4) and *CCL22* transcript (NM_002990.5) using the *in silico* miR-target prediction tools TargetScanHuman 8.0 and miRDB, we have identified one putative *hsa-miR-206-3p* binding site in the 3'UTR of *TGM2* transcript (Figure 7A) and *CCL22* transcript (Figure 7B). To confirm the suggested interaction between *hsa-miR-206-3p* on *TGM2* and *CCL22* transcripts as predicted by TargetScanHuman 8.0 and miRDB in THP-1 macrophages, we performed RISC-IP experiments by using an anti-panAGO antibody. Neither *TGM2* nor *CCL22* mRNA were enriched in RISC-IP of THP-1 macrophages transfected with miR-mimic-206-3p (Figure 7C), suggesting that there is no binding between either *TGM2* or *CCL22* transcripts with *hsa-miR-206-3p* in THP-1 macrophages. Furthermore, we cannot find any experimentally validated record that demonstrates interactions between 3'UTR of either *TGM2* or *CCL22* and *hsa-miR-206-3p* using miR-mRNA interaction databases, DIANA-TarBase v8.0. Based on the combination of *in silico* miR-mRNA interaction analysis and RISC-IP experiments, we conclude that M2 macrophage-associated markers and chemokines *CD206, TGM2, CCL17, CCL22*, and *CCL24* transcripts are not directly targeted by either *hsa-miR-206-3p* or *hsa-miR-381-3p* in THP-1 macrophages. Therefore, the demonstrated ability of *hsa-miR-206-3p/hsa-miR-381-3p* to upregulate M2 macrophage-associated markers and chemokines *CD206, TGM2, CCL17, CCL22*, and *CCL24* transcripts likely utilises indirect regulatory mechanisms, such as targeting other important signalling molecules or transcription factors (i.e. KLF4), which leads to transcriptional activation of these genes.

Either hsa-miR-206-3p *or* hsa-miR-381-3p *regulates endogenous M2 macrophage-associated markers and chemokines* CD206, TGM2, CCL17, CL22, *and* CCL24 *transcript levels partially through KLF4-mediated transcriptional activation*

To determine whether the observed *hsa-miR-206-3p* and/or *hsa-miR-381-3p*-mediated M2 macrophage-associated marker

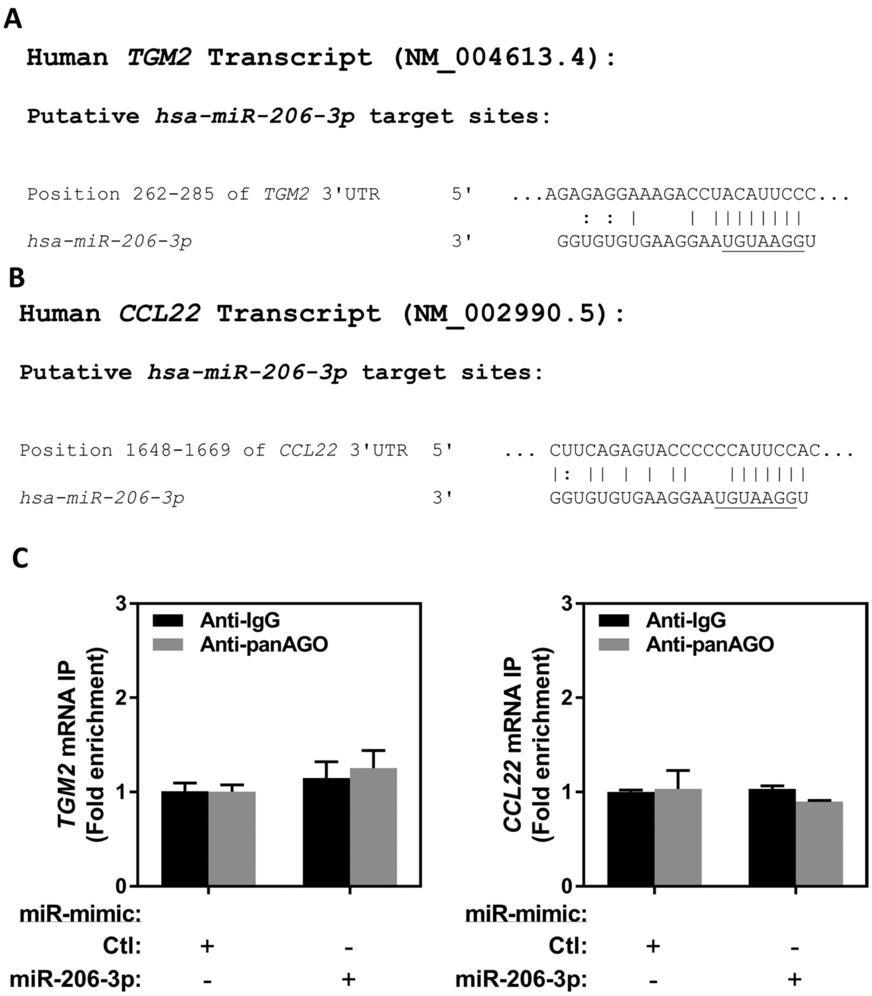

Figure 7. *hsa-miR-206-3p* does not target either human *TGM2* or *CCL22* transcript in THP-1 macrophages. (A) Alignment of the *TGM2*-3'UTR region indicating putative *hsa-miR-206-3p* binding sites. Seed sequence of *hsa-miR-206-3p* is underlined. (B) Alignment of the *CCL22*-3'UTR region indicating putative *hsa-miR-206-3p* binding sites. Seed sequence of *hsa-miR-206-3p* is underlined. (C) Differentiated THP-1 macrophages were transfected with 25 nM of indicated miR-mimics or nontargeting miR-mimic-Ctl for 24 h. The cells were collected and immunoprecipitated using the panAGO or isotype IgG antibody after 24 h transfection. RNA was isolated from the RISC as indicated in either miR-mimic-206-3p or miR-mimic-Ctl transfected THP-1 macrophages by *miRVana*™ miR isolation kit, reverse transcribed, and the fold enrichment of either *TGM2* or *CCL22* transcript was measured by TaqMan RT-qPCR ($N = 3$; bars, SEM).

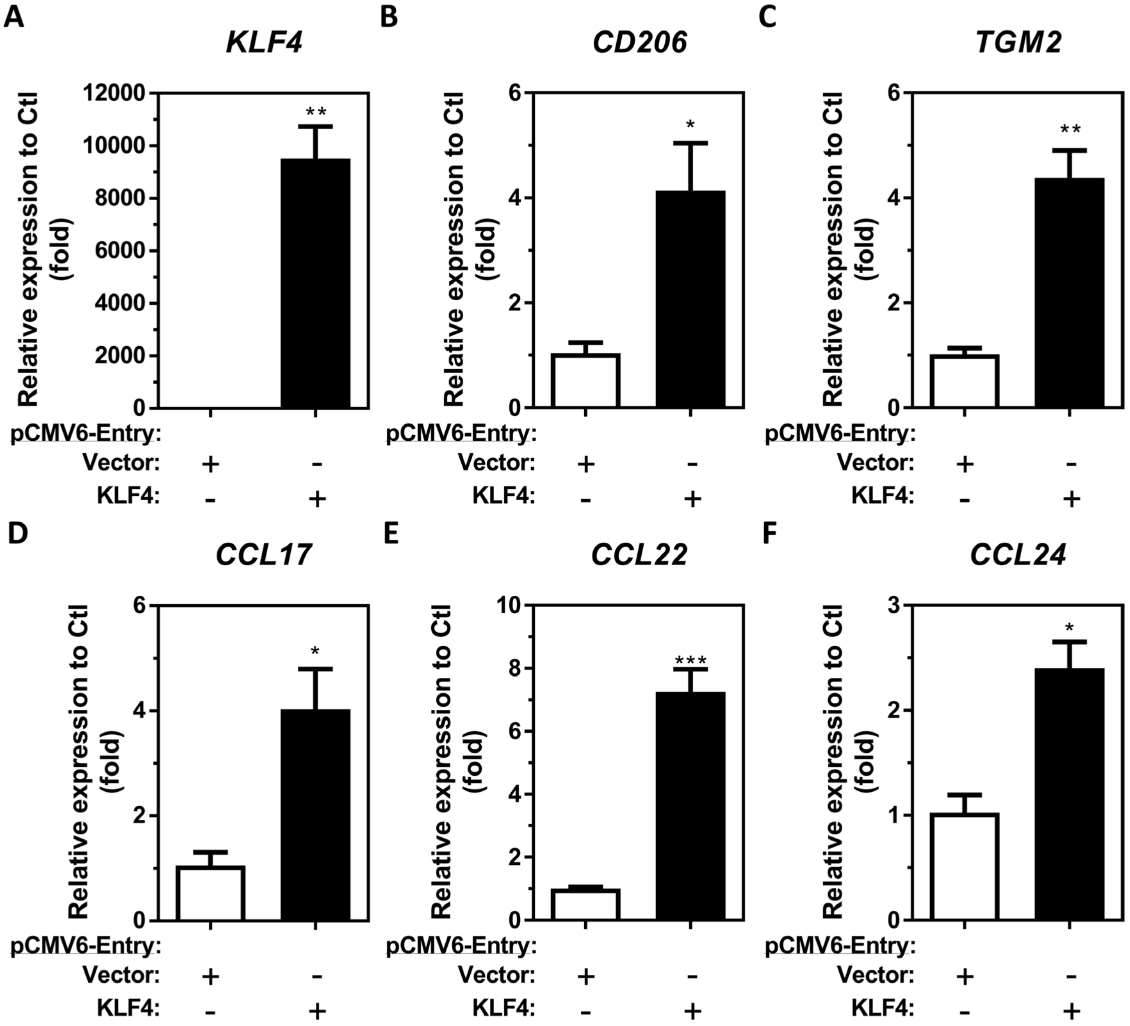

Figure 8. KLF4 overexpression increases M2 macrophage-associated markers and chemokines in differentiated THP-1 macrophages. Differentiated macrophages were transfected with 2.5 µg of either pCMV-Entry-KLF4 or pCMV-Entry vector plasmids for 48 h. Total RNA was isolated from plasmids transfected THP-1 macrophages by *miRVana*™ miR isolation kit, reverse transcribed, and subjected to TaqMan RT-qPCR. Endogenous M2 macrophage-associated markers (A) *KLF4* (B) *CD206*, (C), *TGM2*, (D) *CCL17* (E) *CCL22*, and (F) *CCL24* mRNA levels were determined by RT-qPCR ($N = 3$; bars, SEM). ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

gene upregulation (Figure 3) proceeds *via* KLF4-mediated transcriptional control, we performed both a gain-of-function study by overexpression of KLF4 and a loss-of-function study by using a KLF4 small molecule chemical inhibitor, kenpaullone, to repress KLF4 and KLF4 associated transcriptional activation in miR-inhibitor-206-3p and miR-inhibitor-381-3p transfected THP-1 macrophages. To investigate whether KLF4 can mediate M2 macrophage maker gene transcription as previously reported (Lin et al. 2023), we determined M2 macrophage-associated markers and chemokines *CD206, TGM2, CCL17, CCL22*, and *CCL24* expression using an *in vitro* KLF4 overexpression model. Compared to pCMV-Entry vector control transfected THP-1 macrophages, transfection of KLF4 overexpression plasmid successfully upregulates transgene *KLF4* transcript levels by 9015.2-fold (Figure 8A), whereas the *KLF4* transgene significantly induced endogenous *CD206, TGM2, CCL17, CCL22* and *CCL24* mRNAs by 4.14-, 4.38-, 4.03-, 7.25-, and 2.40-fold, respectively (Figure 8B–F). These results suggest that KLF4 may regulate M2 macrophage-associated markers and chemokines *CD206, TGM2, CCL17, CCL22* and *CCL24* expression.

Consistent with the finding that miR-inhibitors-206-3p and −381-3p upregulate M2 macrophage-associated transcriptional factor *KLF4* as well as M2 macrophage-associated markers and chemokines *CD206, TGM2, CCL17, CCL22*, and *CCL24* mRNA in THP-1 macrophages (Figure 3), independent transfections of miR-inhibitor-206-3p and miR-inhibitor-381-3p upregulate endogenous *KLF4* transcripts by 1.78- and 1.77-fold, respectively (Fig. 9A). Furthermore, transfection of miR-inhibitor-206-3p upregulates *CD206, TGM2, CCL17, CCL22,* and *CCL24* by 5.68-, 10.3-, 8.95-, 3.31- and 3.15-fold, respectively, whereas transfection of miR-inhibitor-381-3p upregulates *CD206, TGM2, CCL17, CCL22* and *CCL24* by 4.55-, 6.68-, 7.14-, 2.53- and 2.87-fold, respectively (Figure 9B–F). Similar to the finding that treatment with the small molecule KLF4 inhibitor kenpaullone downregulates endogenous *KLF4, CD206, CCL17,* and *CCL22* transcripts in THP-1 macrophages (Figure 2A,B,D, and E), treatment of 1 µM kenpaullone significantly downregulated endogenous *KLF4* transcripts by 28.5-fold (Figure 9A), and decreased the endogenous *CD206, CCL17* and *CCL22* transcripts by 2.69-, 3.38-, and 4.05-fold, in miR-inhibitor nontargeting control (miR-inhibitor-Ctl) transfected macrophages, respectively (Figure 9B, D, and E).

Furthermore, treatment of kenpaullone attenuated the induction of M2 macrophage-associated transcription factor as well as the marker and chemokine mRNAs by either miR-inhibitor-206-3p or miR-inhibitor-381-3p transfection in THP-1 macrophages (Figure 9A–F). These results indicate that KLF4-mediated signalling is important for *CD206, TGM2, CCL17, CCL22*, and *CCL24* transcriptional activation by inhibition of *hsa-miR-206-3p* and *hsa-miR-381-3p*.

Discussion

Our previous report showed that the KLF4 transcription factor can be induced by MDI/MDI-GSH exposure both *in vivo* and *in vitro*, and that KLF4 may play an important role as a downstream regulator/effector for MDI exposure-mediated induction of M2 macrophage-associated markers and chemokines in macrophages (Lin et al. 2023); however, the detailed molecular mechanism(s) that regulates the induction of KLF4 after MDI-exposure is currently unknown. In the current report, we have identified a potential *hsa-miR-206-3p/hsa-miR-381-3p*-regulated posttranscriptional mechanism that may be involved in the induction of KLF4 after MDI/MDI-GSH exposure in macrophages. Previous reports found that the endogenous *hsa-miR-206-3p* and *hsa-miR-381-3p* were downregulated in an *in vitro* MDI-GSH conjugate exposure human THP-1 macrophage model (Lin et al. 2020, 2021). The downregulation of endogenous *hsa-miR-206-3p* and *hsa-miR-381-3p* after MDI exposure results in the induction of M2 macrophage-associated markers and chemokines including *CD206, TGM2, CCL17, CCL22*, and *CCL24* transcripts *via* upregulation of KLF4 and KLF4-mediated activation of downstream gene transcriptions (Figure 10). Using an *in vitro* THP-1

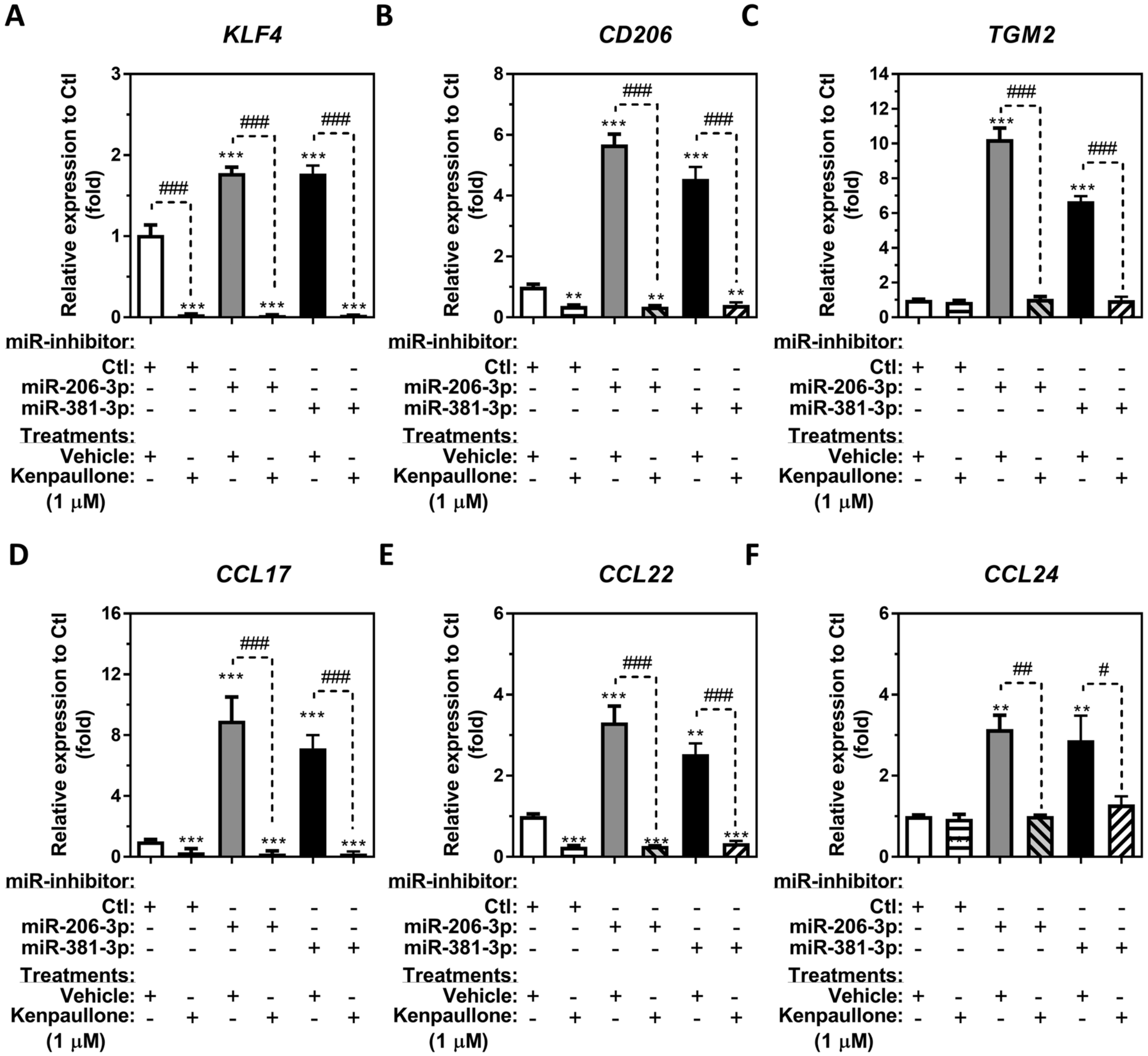

Figure 9. KLF4 inhibition attenuated miR-inhibitor-206-3p and miR-inhibitor-381-3p transfection-mediated induction of M2 macrophage-associated marker and chemokine expression in differentiated THP-1 macrophages. The differentiated THP-1 macrophages were treated either with 1 μM of Kenpaullone or vehicle for 24h followed by transfection of either miR-inhibitor-206-3p, miR-inhibitor-381-3p or nontargeting miR-inhibitor-control (Ctl) for 24h. Total RNA were isolated from THP-1 macrophages with indicated treatments/transfections by *miRVana*™ miR isolation kit, reverse transcribed, and subjected to TaqMan stem-loop RT-qPCR. The endogenous M2 macrophage-associated transcription factor (A) *KLF4*, markers (B) *CD206*, (C), *TGM2*, (D) *CCL17*, (E) *CCL22*, and (F) *CCL24* mRNA levels were determined in total RNA isolated from THP-1 macrophages ($N = 3$; bars, SEM). Vehicle (Ctl): RPMI-1640 media containing 10% FBS and 0.01% DMSO. MDI: 4,4′-methylene diphenyl diisocyanate. GSH: Glutathione. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared to vehicle treated macrophages with transfection of miR-inhibitor-Ctl; #$p < 0.05$, ##$p < 0.01$, ###$p < 0.001$, when compared to macrophages treated with vehicle followed by 10 μM MDI-GSH conjugate exposure as well as with transfection of indicated either miR-inhibitor-206-3p or miR-inhibitor-381-3p).

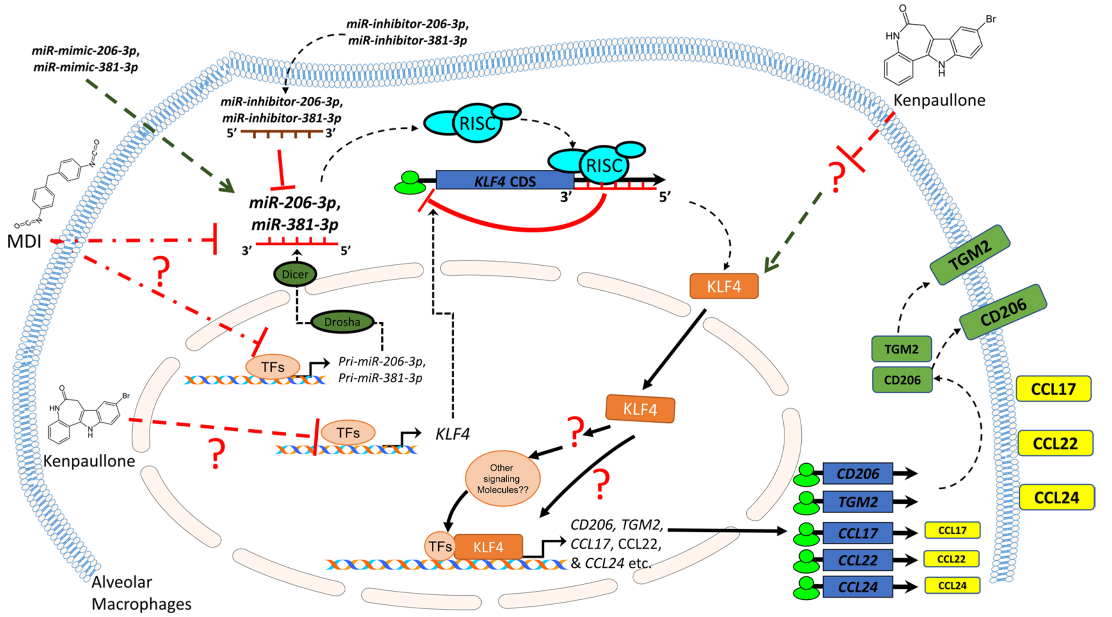

Figure 10. Proposed mechanisms by which MDI exposure induces M2 macrophage-associated markers and chemokine CCL17, CCL22, and CCL24, *via hsa-miR-206-3p/hsa-miR-381-3p* regulated KLF4 activation in macrophages. MDI: 4,4′-methylene diphenyl diisocyanate; TFs: transcription factors; CDS: coding sequences; KLF4: Krüppel-like factor 4. *Note*. Some illustrated schematics were obtained from *motifolio* templates (www.motifolio.com., Accessed 21 January 2024) or openclipart website (www.openclipart.org., Accessed 21 January 2024).

macrophage culture model, we have identified that both *hsa-miR-206-3p* and *hsa-miR-381-3p* target the *KLF4* mRNA transcript which suppresses KLF4 translation and decreases *KLF4* mRNA levels in macrophages (Figure 5). Although the *KLF4* transcript has been reported to be a target of *hsa-miR-206-3p* in many different cell types (Lin et al. 2011; Parasramka et al. 2012; Tang et al. 2013; Sharma et al. 2014; Qinglei et al. 2015; Wang et al. 2021); to our knowledge, this report is the first to confirm that the *KLF4* transcript is a target of *hsa-miR-381-3p*.

Dysfunction of macrophages that are regulated by KLF4-mediated molecular changes in signals, cellular processes, and polarisation status may participate in the pathogenesis of different diseases such as asthma and alcoholic liver disease (ALD). Previous reports have revealed that the role of KLF4 in the monocytes/macrophages is associated with pathophysiological development of allergic asthma by promoting airway inflammation and airway remodelling (Nimpong et al. 2017). KLF4 has been demonstrated to promote monocyte differentiation *in vivo* (Alder et al. 2008). Furthermore, Liao et al. determined that KLF4 is strongly induced in M2 macrophages by IL4/IL13 activated STAT6 signalling whereas its expression is reduced by LPS-mediated NF-κB inhibition in M1 macrophages (Liao et al. 2011). Moreover, KLF4-deficient macrophages display increased pro-inflammatory cytokine expression which is associated with an M1 macrophage phenotype (Liao et al. 2011). These observations indicate a crucial role for KLF4 in promoting resting macrophage (M0) population polarisation towards the M2 phenotype. Saha et al. showed that KLF4 overexpression was associated with elevated $CD163^+/CD206^+$ M2 macrophage populations in an *in vivo* ALD mouse model, and KLF4 has been identified as one important transcriptional regulator for M2 macrophage polarisation. (Saha et al. 2015). We note that the M2 macrophage population has been found to be increased in asthma. Girodet et al. compared the phenotype and function of bronchoalveolar lavage fluid (BALF) macrophages/alveolar macrophages from healthy control subjects to subjects with asthma and found that the alveolar macrophages isolated from BALF of subjects with asthma expressed high levels of M2 macrophage-associated markers CD206 and MHCII, when compared to control subjects (Girodet et al. 2016). *In vitro* functional studies reveal that the M2 macrophages have elevated IL-6, IL-10, and IL-12p40 production and can modulate dendritic and $CD4^+$ T-cell interactions. These reports suggest that the role of KLF4 may be associated with promoting M2 macrophage-associated marker expression in asthma. Ultimately, KLF4 may promote asthma pathogenesis through mediating M2 macrophage polarisation and macrophage induced recruitment of immune cells into the lung microenvironment and sustaining local inflammation within the lung.

Our previous study employed gain- and loss-of-function strategies using KLF4 overexpression plasmid transfection and KLF4 siRNA knockdown, respectively, to demonstrate that KLF4 plays an important role in regulating the M2 macrophage-associated markers CD206 and TGM2 as well as chemokines CCL17, CCL22 and CCL24 (Lin et al. 2023). The current report employs a different strategy to conduct the loss-of-function study by using a KLF4 small molecule inhibitor, kenpaullone, to inhibit KLF4 function (Figure 2). Both siRNA- and kenpaullone-mediated KLF4 inhibition demonstrated similar regulatory effects on M2 macrophage-associated marker and chemokine expression. The primary mechanism by which siRNAs and miRs can interfere with specific gene silencing/RNA degradation/translation inhibition involves the interactions between siRNAs or miRs with the RISC to the siRNA or miR targeted sequences in the gene transcript (Lam et al. 2015). One major aim of the current study was to examine whether miRs can post-transcriptionally regulate KLF4 expression, and the siRNA strategy previously used to knockdown KLF4 (Lin et al. 2023) could introduce artefacts that allow exogenous KLF4 siRNAs to compete with endogenous miRs to bind RISC proteins and KLF4 transcripts; thus, we employed a loss-of function study by treatment with kenpaullone, a specific KLF4 small chemical inhibitor, instead of using specific KLF4 siRNAs to knockdown the endogenous KLF4 level in the THP-1 macrophages (Figures 2 and 9). Consistent with our previous observations, kenpaullone treatment significantly downregulates the endogenous *KLF4* as well as the endogenous *CD206*, *CCL17* and *CCL22* transcripts whereas the expression of *TGM2* and *CCL24* transcripts are unaffected (Figure 2). These results further strengthen our previous observation that *KLF4* may directly regulate the transcription of *CD206*, *CCL17* and *CCL22* whereas the expression of *TGM2* and *CCL24* transcripts are indirectly regulated by other transcription factors that may be activated by KLF4-mediated signalling in the macrophages.

Molecular mediators that regulate KLF4 expression and function in macrophages may play important roles in the pathogenesis of many diseases including asthma development. Among these molecular mediators, miRs have been found to play a crucial role in regulating KLF4 expression. MiRs regulate many cell functions in normal cell homeostasis and disease states by affecting diverse signalling pathways through negative regulation of target genes. For miR-mediated KLF4 signalling regulation, including the two miRs *hsa-miR-206-3p* and *hsa-miR-381-3p* that are observed to regulate *KLF4* in the current study. Currently, the following miRs have been suggested to post-transcriptionally regulate *KLF4* in cells under either normal or disease conditions, *miRs-7, −9-5p, −10a, −10b, -15a, −25, -29a, -34a, −103/107, −137, −143, −145, -146a, −152, −200c, −206, −429, −200,* and *−2909* (Xu et al. 2009; Davis-Dusenbery et al. 2011; Lin et al. 2011; Chen et al. 2012; Jiang et al. 2013; Sureban et al. 2013; Ma et al. 2014; Malik et al. 2014; Meza-Sosa et al. 2014; Tang et al. 2014; Xiao et al. 2014; Tabrizi et al. 2015; Dong et al. 2019; Zhang et al. 2023). In asthma, Xu et al. revealed that the level of *hsa-miR-206-3p* from peripheral blood mononuclear cells (PBMCs) of asthmatic children in the attack stage were significantly lower than those in healthy children, whereas the KLF4 levels from PBMCs of asthmatic children were significantly higher than control (Qinglei et al. 2015). Our previous studies have shown that endogenous levels of *hsa-miR-206-3p* and *hsa-miR-381-3p* were downregulated in both BALCs isolated from mice exposed to MDI dust and differentiated THP-1 macrophages exposed to MDI-GSH conjugates, resulting in induced endogenous *iNOS, CCL2, CCL3, CCL5,* and *CXCL8* transcription partially through calcineurin mediated signalling activation (Lin et al. 2020, 2021). Through *in silico* miR targeting analysis, we identified that the human *KLF4* 3′UTR contains both *hsa-miR-206-3p* and *hsa-miR-381-3p* binding sites (Figure 5A). We further confirmed that human KLF4 can be regulated by either endogenous *hsa-miR-206-3p* or *hsa-miR-381-3p* in macrophages through loss- or gain-of-functional analysis (Figures 5 and 6). Given that we identified that *KLF4* transcripts can be post-transcriptionally regulated by either *hsa-miR-206-3p* or *hsa-miR-381-3p*, and that many other miRs have been shown to regulate KLF4, and that KLF4 plays an important role in regulating macrophage polarisation (Lawrence and Natoli 2011; Tugal et al. 2013); it will be interesting to investigate whether MDI-exposure mediates other miR responses that may play major regulatory roles in MDI-OA pathogenesis.

Mechanistic studies to understand how MDI/MDI-GSH conjugate exposure downregulates endogenous *hsa-miR-206-3p* and *hsa-miR-381-3p* in macrophages to initiate MDI/MDI-GSH mediated induction of *KLF4* and downstream KLF4-mediated M2 macrophage-associated marker responses are not currently available. The endogenous miR levels can be regulated *via* biosynthesis and degradation (Bartel 2018). At biosynthesis regulation of either *hsa-miR-206-3p* or *hsa-miR-381-3p,* recent reports suggest that the transcription factor Myocardin Related Transcription Factor A (MRTF-A) promotes the biosynthesis of *hsa-miR-206-3p* through binding to the promoter region of *MIR206* gene and activating its transcription in breast cancer cells (Xiang et al. 2017), whereas the transcription factor B cell receptor-associated protein 31 (BAP31) has been reported to inhibit *hsa-miR-206-3p* expression in colorectal cancer (Zhang et al. 2023). Zhou and his colleagues revealed that the histone methyltransferase, enhancer of zeste homolog 2 (EZH2), repressed *hsa-miR-381-3p* by promoting H3K27me3 signature on histone H3, deactivating *MIR381* promoter activities through histone methylation in hepatocellular cancer (Zhou et al. 2022). Furthermore, the degradation/inhibition of *hsa-miR-206-3p* and *hsa-miR-381-3p* was found to be regulated by various linear long non-coding RNAs (lncRNAs) or circular RNAs (circRNAs) which bind the miRs through miR response elements (MRE) that exist in the lncRNA/circRNA sequences (Salmena et al. 2011; Chiu et al. 2018). The linear lncRNAs that can bind to endogenous *hsa-miR-206-3p* in many different cell types include *lncRNA_HOTAIR* (Shengnan et al. 2020), *lncRNA_UCA1* (Li et al. 2019), *lncRNA_SNHG14* (Zhao et al. 2020), *lncRNA_MALAT1* (Tang et al. 2018), and *lncRNA_ROR* (Fei et al. 2019), etc., while *hsa-miR-381-3p* binding linear lncRNAs include *lncRNA_CAT104* (Xia et al. 2018; Yuan et al. 2018), *lncRNA_DLEU1* (Gao et al. 2019), *lncRNA_TUG1* (Zhang et al. 2017), etc. The endogenous circular RNAs that bind *hsa-miR-206-3p* includes *hsa_circ_0000199* (Li et al. 2021), *hsa_circ_0008726* (Han et al. 2022; Zhang et al. 2022), *hsa_circ_0056618* (Li et al. 2018; Zheng et al. 2020), *hsa_circ_0057558* (Ding et al. 2021), etc., while *hsa-miR-381-3p* sponging circRNAs including *hsa_circ_0000284* (Bi et al. 2021), *hsa_circ_0084003* (Zhang et al. 2019) and *hsa_circ_0099188* (Sun et al. 2022). In addition, the expression of *hsa-miR-381-3p* can be regulated by several molecules including Periostin (Hu et al. 2017), EZH2 (Dou et al. 2019), and WISP-1 (Tsai et al. 2017). Whether or not MDI/MDI-GSH exposure in macrophages can activate or inhibit regulators involved in the biosynthesis or degradation of *hsa-miR-206-3p* and *hsa-miR-381-3p* in macrophages will need to be the subject of future study.

Strengths of the current *in vitro* THP-1 macrophage MDI/MDI-GSH exposure model include the ability to perform gain- or loss-of-function experiments with miR mimics/inhibitors, *KLF4* transgenes, and *KLF4*-3′UTR luciferase reporter assays with identical genetics from a single cell type to investigate the underlying molecular mechanism that regulates MDI/MDI-GSH mediated effects. However, one inherent limitation of the model is that it leaves uncertain whether or not the identified miR-mediated KLF4 and KLF4-mediated M2 macrophage-associated marker and chemokine mechanism reflects similar lung immune responses, that may participate in the early steps in asthma pathogenesis in the real-world workers with MDI-OA. Future studies will be needed to better elucidate potential connections of diisocyanate exposure with the identified molecular miR regulatory mechanisms in real-world MDI workers.

Conclusion

In conclusion, this report implicates *hsa-miR-206-3p* and *hsa-miR-381-3p* as important post-transcriptional regulators of *KLF4* transcripts and KLF4-mediated signalling, ultimately targeting M2 macrophage-associated markers and chemokine transcription in macrophages after MDI/MDI-GSH conjugate exposure. Decreased expression of *hsa-miR-206-3p* and *hsa-miR-381-3p* was observed following MDI-GSH conjugate exposure in an *in vitro* human THP-1 macrophage *in vitro* model. This miR-regulated mechanism may contribute to the upregulation of M2 macrophage-associated markers and chemokines including *CD206, TGM2, CCL17, CCL22*, and *CCL24* transcription in the macrophages following MDI/MDI-GSH exposure through upregulation of M2 macrophage-associated transcriptional factor, KLF4.

Disclosure statement

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Data availability statement

Data available within the article or its supplementary tables.

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