HHS Public Access

Author manuscript

Xenobiotica. Author manuscript; available in PMC 2024 August 14.

Published in final edited form as:

Xenobiotica. 2023 December ; 53(12): 653–669. doi:10.1080/00498254.2023.2284867.

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

Chen-Chung Lin,
Brandon F. Law,
Justin M. Hettick

Allergy and Clinical Immunology Branch, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, WV 26505

Abstract

1. Occupational exposure to the most widely used monomeric diisocyanate (dNCO), 4,4'-methylene diphenyl diisocyanate (MDI), may lead to the development of occupational asthma (OA). Alveolar macrophages with alternatively activated (M2) phenotype have been implicated in allergic airway responses and the pathogenesis of asthma. Recent *in vivo* studies demonstrate that M2 macrophage-associated markers and chemokines are induced by MDI-exposure, , however, the underlying molecular mechanism(s) by which this proceeds is unclear.

2. Following MDI exposure (*in vivo* and *in vitro*) M2 macrophage-associated transcription factors (TFs), markers, and chemokines were determined by RT-qPCR, western blots, and ELISA.

3. Expression of M2 macrophage-associated TFs and markers including *Klf4*/KLF4, *Cd206*/CD206, *Tgm2*/TGM2, *Ccl17*/CCL17, *Ccl22*/CCL22, and CCL24 were induced by MDI/MDI-GSH exposure in bronchoalveolar lavage cells (BALCs)/THP-1 macrophages. The expression of *CD206, TGM2, CCL17, CCL22, and CCL24* are upregulated by 3.83-, 7.69-, 6.22-, 6.08-, and 1.90-fold in KLF4-overexpressed macrophages, respectively. Endogenous *CD206* and *TGM2* were downregulated by 1.65–5.17-fold, and 1.15–1.78-fold, whereas *CCL17, CCL22,* and *CCL24* remain unchanged in KLF4-knockdown macrophages. Finally, MDI-glutathione (GSH) conjugate-treated macrophages show increased chemotactic ability to T-cells and eosinophils, which may be attenuated by KLF4 knockdown.

Contact: Chen-Chung Lin, Ph.D., NIOSH/HELD/ACIB, 1000 Frederick Ln, MS L4020, Morgantown, WV 26505, (304) 285-6126 (fax), mjy2@cdc.gov.

Disclosure statement

The authors declare that they have no conflicting financial interests. The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

4. Our data suggest that MDI exposure may induce M2 macrophage-associated markers partially through induction of KLF4.

Keywords

Occupational Asthma (OA); Diisocyanates (dNCOs); 4,4'-methylene diphenyl diisocyanate (MDI); Krüppel-Like Factor 4 (KLF4); M2 Macrophage Polarization

INTRODUCTION

4,4'-Methylene diphenyl diisocyanate (MDI), a highly reactive chemical that is essential for manufacturing polyurethane products, is the most widely used diisocyanate (dNCO) globally (Allport et al. 2003). MDI is utilized in many different applications including spray foam insulation, truck bed liners, paints, adhesives, elastomers, and coatings (Munn SJ 2005). Occupational MDI exposure has been identified as one of the leading causes for occupational asthma (OA) and may result in severe outcomes, up to and including death (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). The detailed pathophysiological mechanism(s) by which MDI may cause asthma remains an active research area.

In the lung, alveolar macrophages participate in many aspects of asthma pathogenesis (Erle and Sheppard 2014; Fricker and Gibson 2017). When macrophages encounter outside stimuli, they can acquire two major distinct functional phenotypes: classically (M1) and alternatively activated (M2) macrophages. M1 macrophages, usually stimulated by ligand binding to toll-like receptors (TLRs) and interferon (IFN)-γ, produce proinflammatory cytokines, nitric oxide, or reactive oxygen intermediates to protect against bacteria and virus. M2 macrophages, usually stimulated by interleukins (ILs) IL4 and IL13, induce cell proliferation or collagen production, and play major roles in wound healing and repair (Biswas and Mantovani 2010; Shapouri-Moghaddam et al. 2018). In the asthmatic airway, M2 macrophage populations have been found to be elevated (Melgert et al. 2011; Girodet et al. 2016), suggesting a role during asthma pathogenesis. Using gene microarray expression methodology and mass spectrometry, previous reports have identified that MDI can induce myeloid/innate immune responses and M2 macrophage-associated gene signatures (Wisnewski et al. 2015; Wisnewski et al. 2020). Secretory murine M2 macrophage-associated marker protein levels including Chitinase YM-1 and IL-12/IL-23β subunit were increased in the cell-free airway lavage fluids obtained from MDI-GSH conjugate-exposed mice (Wisnewski et al. 2015). Furthermore, endogenous murine M2 macrophage-associated marker mRNAs including *Chil3 (Ym1), Chil4 (Ym2), Retnla (Fizz1), Clec7a (Dectin1), Clec10a (Mgl2), and Cd209e (DC-SIGN)* were induced in RNA isolated from lungs of mice subjected to MDI/MDI-GSH conjugate dermal sensitization/respiratory challenge (Wisnewski et al. 2020), suggesting MDI exposure induces M2 macrophage polarization gene signatures. However, whether these M2 macrophage-associated markers are indeed induced in BALCs/alveolar macrophages after MDI exposure and the detailed molecular mechanism(s) by which MDI induces these M2 macrophage-associated markers has not been determined. The current study aims to investigate the molecular mechanisms that

mediate the induction of M2 signature genes in BALCs/alveolar macrophages after MDI exposure.

Several transcription factors including Signal Transducer and Activator of Transcription 6 (STAT6), Interferon Regulatory Factor 4 (IRF4), Spi-1 Proto-Oncogene (SPI1), Peroxisome Proliferator Activated Receptor Gamma (PPARγ), CCAAT Enhancer Binding Protein Beta (CEBPB), and Krüppel-Like Factor 4 (KLF4) have been reported to mediate M2 polarization (Tugal et al. 2013; Li H et al. 2018; Gharib et al. 2019). STAT6 plays an important role in regulating many mouse M2 marker genes, including arginase 1 (Arg1), macrophage mannose receptor 1 (Mrc1/Cd206), resistin-like-α (Retnla/Fizz1) and chitinase 3-like 3 (Chi3l3/Ym1) that are induced by IL4 and IL13-mediated M2 macrophage polarization signaling (Martinez et al. 2009). IRF4 can be induced by IL4 through Jumonji domain-containing protein (JMJD) 3, which is important for the induction of M2 macrophage polarization when macrophages are exposed to chitin, a fungal and parasitic cell wall component (El Chartouni et al. 2010; Satoh et al. 2010). SPI1 has been found to promote M2 macrophage polarization in asthmatic airways (Qian et al. 2015). PPARγ-deficient macrophages were not able to undergo M2 polarization after M2 macrophage stimulation (Odegaard et al. 2007), suggesting the key role of PPARγ in M2 macrophage polarization. CEBPB binds to the promoter region of M2 macrophage-associated markers such as *Arg1*, therefore promoting macrophages toward M2 phenotypes (El Kasmi et al. 2008). KLF4 may cooperate with STAT6 to induce M2 macrophage polarization and can sequester the cofactors of NF-kB, the major M1 macrophage-associated transcription factor, to inhibit M1 polarization and promote M2 polarization (Liao et al. 2011). Whether these M2 macrophage-associated transcription factors play any role in MDI-mediated M2 macrophage-associated marker expression has not been investigated. We hypothesize that MDI may induce M2 macrophage-associated markers by regulating these M2 macrophage-associated transcription factors in macrophages.

The current report is focused on characterizing possible MDI-mediated M2 macrophage polarization responses by measuring M2 macrophage-associated marker gene expression after MDI exposure and then characterizing the involvement of MDI-mediated M2 macrophage-associated transcription factors as one of the downstream molecular mechanisms to regulate expression of M2 macrophage-associated markers. We determine transcriptional changes in M2 macrophage-associated markers following MDI exposure using an *in vivo* murine MDI dermal sensitization/respiratory challenge model, as well as an *in vitro* THP-1 macrophage culture model. Using an *in vitro* MDI-GSH conjugate cell culture model, as well as gain- and loss-of-function experiments, we determine that MDI-mediated upregulation/activation of endogenous KLF4 may participate in M2 macrophage-associated markers and chemokines upregulation. This report provides a putative KLF4-mediated mechanism to describe how MDI may induce M2 macrophage-associated markers and chemokines in macrophages.

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, radioimmunoprecipitation assay (RIPA) buffer, 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 h 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 a prior study (Lin et al. 2019). Briefly, female BALB/c mice, 6–8 weeks old, were purchased from Taconic (Germantown, New York). Mice were acclimated for five days before being randomly assigned into different treatment groups. Treatment groups of five mice were housed in ventilated plastic cages with hardwood chip bedding enriched with a section of polyvinyl chloride (PVC) pipe (1.5" O.D. × 6") to acclimate animals to the dimensions of the nose-only restraint device. Further acclimation to the restraint device was performed by three stays of increasing duration (15 minutes, 30 minutes, 60 minutes) on three consecutive days and was well-tolerated. An NIH-31 modified 6% irradiated rodent diet (Harlan Teklad) and tap water were administered ad libitum. Housing facilities were maintained at 20–22 °C and 36–57% relative humidity with a 12-hour light-dark cycle. During MDI exposure, mice were dermal exposed 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/m^3 MDI aerosol or house air control for 1 h using the in-house constructed nose-only inhalation exposure system (NOIES) as previous described (Hettick et al. 2018). 24 hours post-exposure, euthanasia was performed by intraperitoneal injection of sodium pentobarbital. Following a non-reflexive response to a toe pinch, euthanasia was further confirmed by exsanguination via cardiac puncture. Blood/sera were collected and stored at −80°C for circulating microRNA(miR) analysis (Lin et al. 2019). Bronchoalveolar lavage fluid (BALF) was collected via 3 × 1ml ice-cold PBS lavages following 10-ml ice cold PBS perfusion. Bronchoalveolar lavage cells (BALCs) were collected from BALF by centrifugation at 300 × g for 10 min at 4°C and stored at −80°C 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 (Protocol # 16-JH-M-009).

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 × 10^6/ml in RPMI-1640 media supplement with 10% FBS, and 1× PSG (Complete RPMI media) at 37°C in a humidified atmosphere with 5% CO_2 as previous described (Lin et al. 2021). THP-1 cells (2 × 10^6 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 72 h. 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 × 10^5 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 a 0.2 μm syringe filter. Reaction products containing MDI-GSH conjugates were immediately added into enhanced differentiated THP-1 macrophages at either 0, 1, 10 μM or at 10 μM as the indicated concentrations shown in the figure legends.

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 × 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 *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 *mir*Vana™ miR Isolation Kit (Thermo Fisher Scientific) according to manufacturer's instructions for RT-qPCR expression analyses. For KLF4 overexpression associated protein analysis, 5 × 10^6 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 *Trans*IT®−2020 transfection reagent in 10-cm dish for 48 h. After 48 h, cell lysates were prepared for western blotting.

For KLF4 siRNA knockdown studies, two commercially available *Silencer*® Select siRNAs specifically targeted 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 24 h 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 24 h, 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 $^{\Delta\Delta}C_T$ 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 radioimmunoprecipitation assay (RIPA) buffer as previously described (Lin et al. 2020). Following electrophoresis, proteins were transferred onto nitrocellulose membranes and probed with diluted antibodies in Tris-buffered saline with 0.1% Tween-20 Detergent (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 Santa Cruz Biotechnology (Dallas, TX). Bound antibodies were detected using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific).

Chemokine ELISA

Conditioned media was collected 24 h after THP-1 macrophages were treated with MDI-GSH conjugates or 24 h 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). Briefly, the cell chemotaxis/migration assays were performed using a 24-well plate format with Transwell™ inserts containing 3 μm pore polycarbonate membrane (Corning™ Transwell™ plates, ThermoFisher Scientific). A total of 1×10^6 naive T-cells (Jurkat_E6–1 T cells) or eosinophils (butyric acid differentiated HL-60_C15 cells) in 100 μl serum-free RPMI 1640 media were added to each upper chamber and placed on the lower chamber containing chemoattractant. Five hundred microliters of cell-free conditioned media from either KLF4 overexpression plasmid or KLF4 knockdown siRNA transfected THP-1 macrophages were placed in the lower chamber as a chemoattractant, and the immune cells were 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 was 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.

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 $P<0.05$.

RESULTS

MDI exposure upregulates endogenous M2 macrophage-associated marker and chemokine transcripts in BALCs isolated from MDI exposed mice

MDI-OA patients' asthma attacks are often associated with previous dermal MDI exposure history followed by acute MDI-aerosol inhalation in occupational settings (Petsonk et al. 2000). Recent gene transcript microarray and mass spectrometry data reveal that M2 macrophage-associated markers and chemokines are induced in murine lung tissue after combined dermal/respiratory MDI/MDI-GSH exposure (Wisnewski et al. 2015; Wisnewski et al. 2020) indicating that MDI exposure in sensitized animals may induce alveolar macrophages to undergo M2 macrophage polarization. To investigate whether combined dermal/respiratory MDI exposure could upregulate the levels of M2 macrophage-associated markers and chemokines in the BALCs, we analyzed endogenous M2 macrophage-associated marker and chemokine transcript levels using total RNAs isolated from stored BALCs (Fig.1 A) (Lin et al. 2019). We determined the endogenous mRNA expression of M2 macrophage-associated genes including *Chil3, Chil4, Fizz1, Clec7a, Clec10a, Ccl17, Ccl22,* and *Ccl24,* as well as common M2 macrophage-associated genes *Cd163,* and *Cd206,* and newly identified M2 macrophage-associated genes *Cd273* and *Tgm2* from BALCs of MDI-aerosol exposed or house-air exposed control mice (Fig. 1). M2 macrophage-associated markers from BALCs in dermally exposed to MDI compared to control (D+/M− vs. D−/M−) mice exhibited no significant change in *Chil3, Chil4, Retnlb/Fizz1, Clec10a, Ccl17, Ccl22, Cd206*, and *Tgm2* levels (Fig. 1B–D, F–I, and K) whereas dermal exposure to MDI significantly upregulates endogenous *Clec7a* and *Cd273* transcripts (Fig. 1E and J). In contrast, endogenous M2 macrophage-associated markers *Chil3, Chil4, Retnlb, Clec7a, Clec10a, Ccl17, Ccl22, Cd206, Cd273,* and *Tgm2* transcript levels were significant upregulated 2.13-, 1.92-, 2.57-, 1.51-, 2.24-, 4.17-, 2.80-, 1.90-, 15.3-, and 2.29-fold at 24 h following 1h MDI-aerosol inhalation (D+/M+24h), respectively (Fig. 1B–K). The endogenous mRNA transcripts of *Cd163,* and *Ccl24* were not detected in BALCs isolated from any groups of mice (data not shown). These data indicate that respiratory exposure to MDIincreases M2 macrophage-associated markers and chemokines in BALCs from mice previously exposed to MDI dermally.

Upregulation of endogenous *Klf4* and *Pparg* in the BALCs isolated from mice with combined dermal/respiratory MDI exposure

Macrophage polarization toward either M1 or M2 phenotypes can be regulated by many different environmental stimuli and downstream signaling pathways that lead to transcriptional activation of either M1 or M2 macrophage-associated genes. Several transcription factors have been found to play major roles in regulation of macrophage polarization by promoting gene expression which dictates the functional polarization of macrophages. Among these transcription factors, STAT6, IRF4, SPI1, CEBPB, PPARγ, and KLF4 have been found to mediate M2 macrophage polarization (Lawrence and Natoli 2011; Tugal et al. 2013). We, therefore, hypothesize that some of these M2 macrophage-

associated transcription factors can be induced by MDI exposure and the induction of the specific M2 macrophage-associated transcription factor(s) may be responsible for the induction of M2 macrophage-associated genes in MDI exposed macrophages. To determine which candidate M2 macrophage-associated transcription factor(s) are induced by MDI exposure, we examined the endogenous mRNA levels of *Stat6, Irf4, Spi1, Cebpb, Pparg,* and *Klf4* in BALCs after exposure to MDI. When comparing the endogenous M2 macrophage-associated transcription factor mRNA levels in BALCs isolated from [D+/M− vs. D−/M−] animals, no significant differences are observed (Fig. 2 A–F). However, addition of MDI aerosol exposure significantly induced *Pparg* and *Klf4* transcription factor expression when compared to the house-air exposed only control (D+/M+24h vs. D−/M−) (Fig. 2E and F). These results indicate that MDI exposure induces M2 macrophage marker expression through upregulation of either KLF4 or PPARγ transcription factors in BALCs.

MDI-GSH conjugate exposure upregulates endogenous M2 macrophage-associated markers and chemokines in differentiated THP-1 macrophages

Given that macrophages make up greater than 80 percent of the BALCs population (Heron et al. 2012) and previous reports have shown that *in vivo* MDI-glutathione (GSH) conjugate exposure upregulates many M2 macrophage marker genes in lung tissue (Wisnewski et al. 2015; Wisnewski et al. 2020), we used differentiated/enhanced human THP-1 macrophages as an *in vitro* cell model to investigate the macrophage polarization response upon exposure to MDI-GSH conjugate. Since many of the M2 macrophage-associated markers in Fig.1 are described as M2 macrophage-associated markers in mice but not in humans, we elected to determine the expression of M2 macrophage-associated markers including CD206 and TGM2 as well as secretory chemokines CCL17, CCL22 and CCL24 that have been identified as both mouse and human M2 macrophage-associated markers (Huber et al. 2010; Martinez Fernando O. et al. 2013; Martinez and Gordon 2014). Differentiated THP-1 macrophages were treated with MDI-GSH conjugates at 0, 1, and 10 μM for 24 h. The endogenous M2 macrophage markers *CD206* and *TGM2* transcripts were significantly upregulated by MDI-GSH conjugate treatment from 1.42-fold to 6.99-fold for *CD206* (Fig. 3A), and from 1.67-fold to 2.22-fold for *TGM2* (Fig. 3B), respectively. Furthermore, the endogenous protein levels of CD206 and TGM2 markers were also upregulated by treatment of MDI-GSH conjugate (Fig. 3C). MDI-GSH conjugate treatment induced endogenous M2 macrophage-associated chemokines *CCL17, CCL22*, and *CCL24* mRNAs (Fig. 3 D–F). Furthermore, the secreted CCL17, CCL22, and CCL24 proteins were increased by MDI-GSH conjugate (10 μM) treatment (Fig. 3 G–I). These results indicated that exposure to MDI-GSH conjugates can upregulate M2 macrophage-associated markers and chemokines in human macrophages.

MDI-GSH conjugate exposure induced endogenous KLF4 in differentiated THP-1 macrophages

From *in vivo* experiments, *Klf4* and *Pparg* transcripts were the only two M2 macrophage-associated transcription factors tested that were induced by MDI-aerosol in BALCs isolated from the MDI dermal exposed animals (Fig. 2E and F), indicating that *KLF4* and *PPARG* may be upregulated by MDI-GSH conjugate exposure in alveolar macrophages. To investigate whether MDI-GSH conjugate exposure regulates candidate M2 transcription

factors including KLF4 and PPARγ in human macrophages, the differentiated THP-1 macrophages were treated with MDI-GSH conjugates at concentrations of 0, 1, and 10 μM for 24 h and the endogenous M2 macrophage-associated transcription factors mRNAs were determined by RT-qPCR. The results are presented in Fig. 4. MDI-GSH conjugate treatment failed to induce the endogenous M2 macrophage-associated transcription factor mRNAs *STAT6, IRF4, SPI1, CEBPB* and *PPARG* (Fig. 4A–E); however, treatment with MDI-GSH upregulates endogenous KLF4 in macrophages (Fig. 4 F&G). Endogenous *KLF4* mRNA was significantly upregulated by MDI-GSH conjugate exposure from 1.42-fold to 2.12-fold (Fig. 4F). In addition, the endogenous KLF4 protein level was also upregulated in response to MDI-GSH conjugate treatment (Fig. 4G). These results confirm that the exposure to MDI in the form of MDI-GSH conjugate can upregulate endogenous KLF4 but not PPARγ expression in differentiated human macrophages. These results suggest that the transcription factor KLF4 may play an important role in regulating M2 macrophage-associated markers expression after MDI exposure in alveolar macrophages.

Overexpression of KLF4 induced M2 macrophage-associated markers and chemokines

The transcription factor KLF4 plays an important role in macrophage differentiation and M2 macrophage polarization (Park et al. 2016). To investigate whether KLF4 can mediate M2 macrophage-associated marker and chemokine expression, we determined expression of M2 macrophage-associated markers and chemokines *CD206, TGM2*, *CCL17, CCL22*, and *CCL24* using an *in vitro* KLF4 overexpression model in THP-1 macrophages. Compared to vector-transfected differentiated THP-1 macrophages, transfection of KLF4 overexpression plasmids for 48 h significantly induced endogenous *CD206,* and *TGM2* mRNAs by 3.83-, and 7.69-fold, respectively (Fig. 5A–B). In addition, the protein expression levels of CD206 and TGM2 were also upregulated by transfection of KLF4 overexpression plasmid (Fig. 5C). Furthermore, KLF4 overexpression upregulated the endogenous chemokines *CCL17, CCL22* and *CCL24* mRNAs by 6.22-, 6.08-, and 1.90-fold, respectively (Fig. 5D–F). These results indicated that induction of KLF4 transcription factors after MDI-GSH conjugate exposure may induce M2 markers *CD206,* and *TGM2* as well as chemokines *CCL17, CCL22*, and *CCL24* transcription and expression in human macrophages.

KLF4 plays an important role for MDI-GSH conjugate-mediated induction of M2 macrophage-associated markers and chemokines

To study the endogenous roles of KLF4 in regulation of MDI-GSH conjugate-mediated induction of M2 macrophage-associated gene transcripts, we performed a loss-of-function experiment using two different siRNAs to knockdown endogenous KLF4 in macrophages. Differentiated THP-1 macrophages were transfected with either siKLF4–793 or siKLF4–794 siRNAs designed to target two specific sites in the coding region of the human KLF4 transcript (Fig. 6A). Transfection of either siKLF4–793 or siKLF4–794 significantly inhibited the endogenous expression of KLF4 mRNA and protein (Fig. 6A). Consistent with the finding that exposure to MDI-GSH conjugate upregulates endogenous KLF4 transcription factor as well as M2 macrophage-associated markers and chemokines mRNA in THP-1 macrophages (Figs. 3 & 4), independent treatment of 10 mM MDI-GSH conjugates to the nontargeting siRNA control (siCtl) transfected THP-1 macrophages upregulated endogenous *KLF4, CD206, TGM2, CCL17, CCL22,* and *CCL24* mRNAs by

4.49-, 3.61-, 2.35-, 3.13-, 1.78-, and 2.34-fold, respectively, when compared to control mock-treated nontargeting siCtl transfected THP-1 macrophages (Fig. 6B–G). In addition, MDI-GSH conjugate treatments failed to induce endogenous levels of *KLF4, CD206, TGM2, CCL17, CCL22,* and *CCL24* mRNAs in either siKLF4–793 or siKLF4–794 siRNAs transfected THP-1 macrophages. (Fig. 6 B–G). These results suggest that KLF4 may play an important role in regulating the expression of M2 macrophage-associated markers *CD206* and *TGM2* as well as chemokines *CCL17, CCL22*, and *CCL24* after macrophage exposure to MDI-GSH conjugate.

MDI-GSH conjugate induces naïve T-cell and eosinophil chemoattraction through KLF4-mediated pathways

When macrophages undergo M2 polarization, mediators and chemokines including CCL17, CCL22 and CCL24 are secreted to attract T-cells and other immune cell types (Mantovani et al. 2004). In addition to T-cells, one report demonstrated that eosinophils can be chemoattracted by CCL24 (White et al. 1997). Furthermore, our previous report revealed induced naïve T-cell and eosinophil chemotactic activities in MDI-GSH conjugate exposed macrophages (Lin et al. 2021). Given that KLF4 transcription factor plays important roles in regulation of M2 macrophage-associated chemokine *CCL17, CCL22*, and *CCL24* expression after MDI-GSH exposure in macrophages (Figs. 5&6) and these chemokines contribute to T-cell, eosinophil, and other immune cell chemoattraction, we hypothesized that endogenous KLF4 transcription factor is involved in regulation of T-cell and/or eosinophil chemotaxis by MDI-GSH exposure in macrophages by secreting CCL17, CCL22, and CCL24. To test this hypothesis, we first examined the protein levels of secreted CCL17, CCL22, and CCL24 in conditioned media collected from KLF4 overexpressed macrophages, KLF4 overexpression induced chemokine CCL17, CCL22 and CCL24 protein levels when compared to vector transfected THP-1 macrophages (Fig. 7 A–C). In addition, we performed chemotaxis/migration assays using conditioned media collected from KLF4 overexpression plasmid transfected THP-1 macrophages. Compared to the conditioned media collected from vector-transfected differentiated THP-1 macrophages, media collected from KLF4 overexpressed macrophages induced T-cell (Jurkat_E6–1 cells) chemotaxis/migration by 1.55-fold, whereas it induced eosinophil (butyric acid differentiated HL-60_C15 cells) chemotaxis/migration by 1.48-fold (Fig. 7D), indicating that KLF4 plays a role in regulation of macrophage-mediated T-cell and eosinophil chemoattraction/migration. Furthermore, we determined the secreted protein levels of CCL17, CCL22, and CCL24 in conditioned media collected from siRNA-mediated KLF4 knockdown macrophages treated with or without MDI-GSH conjugates (Fig. 7 E–G). Similar to the findings that treatment of MDI-GSH conjugates upregulated endogenous CCL17, CCL22, and CCL24 in THP-1 macrophages (Fig. 3 D–I), the secreted chemokine CCL17, CCL22, and CCL24 protein levels were induced by MDI-GSH conjugate treatment in THP-1 macrophages transfected with nontargeting control siRNA, whereas the MDI-GSH conjugate-induced secreted chemokine protein levels in the media were decreased by KLF4 knockdown via siKLF4 transfection (Fig. 7 E–G). Conditioned media collected from MDI-GSH conjugate treated siCtl-transfected THP-1 macrophages induced T-cell (Jurkat_E6–1 cells) and eosinophil (butyric acid differentiated HL-60_C15 cells) chemotaxis/migration, whereas the MDI-GSH conjugate-induced T-cell (Jurkat_E6–1 cells) and eosinophil (butyric acid differentiated

HL-60_C15 cells) chemotaxis/migration activities were decreased by KLF4 knockdown (Fig. 7H). These results indicate that KLF4-mediated induction of chemokines CCL17, CCL22, and CCL24 may be important for T-cell and eosinophil migration induced by MDI/MDI-GSH exposed alveolar macrophages.

DISCUSSION

Alternatively activated macrophages (M2 macrophages) play an important role in asthma pathogenesis. These polarized macrophages can secrete mediators and chemokines to recruit immune cells, including T-cells, eosinophils, and other immune cell types into the lung microenvironment to initiate immune response and inflammation. This report identifies a potential regulatory role of KLF4 transcription factor as a downstream regulator/effector for MDI exposure-mediated induction of M2 macrophage-associated markers and chemokines as well as T-cell and eosinophil recruitment response in macrophages (Fig. 8). Furthermore, this KLF4-mediated mechanism may provide a potential mechanistic answer for the observation that MDI exposure induces M2 macrophage-associated marker expression in animal models. (Wisnewski et al. 2015; Wisnewski et al. 2020).

Pulmonary macrophages can be found in the airways/alveoli as well as in lung tissue (Byrne et al. 2016). Pulmonary alveolar macrophages are among the first immune cell types to respond to inhaled substances; whereas pulmonary interstitial macrophages in the tissue interstitium/parenchyma can be chemoattracted to alveoli and become alveolar macrophages to enforce the immune response at a later stage (Byrne et al. 2015; Byrne et al. 2016). By using both mRNA microarray and proteomic methods, Wisnewski and colleagues observed that many M2 macrophage-associated markers are induced in the lung tissues from animals exposed to MDI, which therefore suggests that MDI exposure may potentially promote M2 macrophage polarization in the lung (Wisnewski et al. 2015; Wisnewski et al. 2020). However, these studies analyzed RNA and protein from cell free lavage fluid or whole lung tissue following lavage, rather than from BALCs isolated from the lower airway/alveoli, which may result in exclusion of the alveolar macrophage population of the BALCs. The observation of induced M2 macrophage-associated marker expression in that dataset therefore may not apply to the alveolar macrophage population but rather represents the M2 macrophage-associated marker expression patterns for either the residential interstitial macrophages or other cell types in the lung such as epithelial cells, basal cells, stroma cells, etc. The current study directly measures the mRNA expression of M2 macrophage-associated marker and chemokine expression using BALCs (>80% of BALCs are alveolar macrophages) isolated from combined dermal/respiratory MDI exposure models and observed similar induction of M2 macrophage-associated markers and chemokine transcripts in these cells after MDI exposure (Fig.1). This data further suggests that MDI exposure has the potential to activate M2 macrophage polarization through upregulation of M2 markers and chemokines expression in alveolar macrophages.

One major mechanism that induces macrophages to undergo M2 macrophage polarization involves IL4/IL13 cytokines and their downstream signaling leading to STAT6 activation; however, these two cytokines are generally not associated with human diisocyanate asthma (Bernstein et al. 2002; Redlich and Karol 2002; Jones et al. 2006; Wisnewski and Jones

2010). Given data suggesting IL4/IL13 are not the driving factor for MDI asthma, our finding that identifies KLF4 transcription factor as a major regulator responsible for MDI-mediated induction of M2 macrophage-associated markers and chemokines in macrophages may explain why MDI exposure induces M2 macrophage-associated markers expression. Liao *et al.* demonstrated that IL4-induced M2 macrophage polarization proceeds through induction of STAT6 and KLF4 expression in macrophages, and these two transcription factors can induce each other and promote M2 macrophage polarization (Liao et al. 2011). Although we do not observe induction of *Stat6/STAT6* transcripts by MDI exposure (Figs. 2A and 4A), nor did we examine whether the STAT6 transcription factor is activated by MDI exposure; the current report demonstrates that KLF4 plays an important role in regulation of M2 macrophage-associated markers and chemokines expression (Fig. 5 and 6). Previously, Kapoor *et al.* revealed that MCP-1–induced protein (MCPIP) is one of the downstream regulators for KLF4-mediated M2 polarization (Kapoor et al. 2015). MCPIP, which is induced by KLF4, inhibits macrophages from undergoing M1 polarization via MCPIP-mediated NFkB inhibition, while promoting M2 macrophage polarization through processes including ROS production, ER stress, and autophagy (Kapoor et al. 2015). The current report identifies KLF4, but not other M2 macrophage-associated transcription factors, as induced by MDI/MDI-GSH conjugate exposure both *in vivo* and *in vitro* (Fig. 2F and 4F). We hypothesize that the induction of KLF4 by MDI exposure may account for one of many potential mechanisms that lead to the induction of M2 macrophage-associated markers and chemokines in the alveolar macrophages after MDI exposure.

The mechanism by which MDI exposure upregulates KLF4 in macrophages is currently unknown; therefore, further mechanistic studies are needed. Given that the expression of KLF4 transcription factor is tightly controlled at both the transcriptional, posttranscriptional, and posttranslational levels, multiple pathways involving epigenetic factors or miRs-mediated regulation may participate in MDI/MDI-GSH exposure-mediated induction of KLF4 in macrophages. Studies shown that the expression and activity of KLF4 can be post-translationally regulated by such modifications as ubiquitination (Hao et al. 2017) and SUMOylation (Wang et al. 2017). At the transcription level, KLF4 can be transactivated by many different signaling pathways such as TGFβ, IFNγ, IL-1β, IL10, and IL17 signaling (Chen et al. 2000; King et al. 2003; Liu et al. 2007; An et al. 2011; Liu J et al. 2012). In cancers and stem cells, several reports have found that the CpG islands of KLF4 promoter were hypermethylated and modulation of histone methylation status can regulate the KLF4 promoter activities (Cho et al. 2007; Nakahara et al. 2010; Yang and Zheng 2014). During monocyte/macrophage differentiation, the KLF4 promoter undergoes active demethylation and the expression of KLF4 is induced (Karpurapu et al. 2014). Multiple transcription factors also play regulatory roles in KLF4 expression in the cells including SP1, KLF4, KLF5, ELF4, SLUG, CDX2, LIF and others (Dang et al. 2001; Dang et al. 2002; Deaton et al. 2009; Yamada et al. 2009; Li X et al. 2012; Liu YN et al. 2012). In addition to transcriptional control, epigenetic regulation by miRs has been identified as one of the important mechanisms for modulation of KLF4 expression in many different cell types in both normal and abnormal disease conditions (Ghaleb and Yang 2017; Li ZY et al. 2023). Whether or not MDI exposure can activate these transcription factors or cause a histone methylation status change on the KLF4 promoter region to induce KLF4 expression, as well

as cause a miR-mediated KLF4 regulation in macrophages will need to be the subject of future study.

Infiltration of T-cells, eosinophils, and other immune cell types into the lung microenvironment following exposure to inhaled allergens or irritants in the airways has been identified as an important pathophysiological step in early development of asthma (Barnes 2008). In dNCO-OA, activated T-cells have been demonstrated to be infiltrated into airways of dNCO-OA patients and they may play important roles in dNCO-OA pathogenesis (Bentley et al. 1992; Redlich et al. 1996; Hur et al. 2008). Here, MDI exposure induced M2 macrophage-associated chemokines *Ccl17*/CCL17, *Ccl22*/CCL22 (Figs. 1 G&H and Fig. 3 D, E, G & H), and CCL24 expression (Fig. 3 F & I). Chemokines CCL17 and CCL22 have been previously determined to be elevated in asthmatic patients and these two chemokines can induce naïve T-cell activation into Th2-type cells via binding to CCR4 on the $CD4^+$ naïve T-cells (Hirata et al. 2019). In addition, CCL24 mRNAs and proteins were elevated in asthmatic patients (Ying et al. 1997; Ying et al. 1999; Zeibecoglou et al. 1999) where the function of CCL24 contributes to naïve T-cell chemoattraction. Besides T-cell chemoattraction, CCL24 can attract eosinophils through binding to CCR3 on the eosinophils (Pope et al. 2005). Wisnewski and colleagues observed that chemokines *Ccl17* and *Ccl22* isolated from MDI-sensitized animals were induced by MDI-GSH inhalation/challenge (Wisnewski et al. 2020) indicating that MDI-GSH exposure may regulate chemokine expression and contribute to T-cells chemotaxis. Our previous report indicated that T-cells were one of the most responsive immune cell types that can be attracted by MDI-GSH conjugate exposed macrophages due to elevated levels of chemokines CCL2, CCL3, CCL5, and IL8, which were induced by miR-mediated calcineurin signaling in the macrophages (Lin et al. 2021). In this report, the M2 macrophage-associated chemokines *Ccl17*/CCL17, *Ccl22*/CCL22 and CCL24 are also induced by MDI/MDI-GSH conjugate exposure in BALCs/macrophages (Fig. 1 and 3) through MDI-mediated KLF4 signaling (Fig. 5 and 6). This may provide further mechanistic explanation of how MDI exposure in the lung can potentially attract T-cells into the airway microenvironments.

Additionally, TGM2, one of the recently identified M2 macrophage-associated polarization markers in both human and mouse (Martinez F. O. et al. 2013), was induced by MDI exposure in BALCs/differentiated macrophages (Fig.1 and Fig. 3). TGM2 is a multifunctional enzyme that is associated with a variety of either physiological or pathological functions, such as regulating cell growth, differentiation, development, adhesion and morphology changes, rearrangement of cell cytoskeleton, and extracellular matrix, participating in inflammatory processes, receptor-mediated endocytosis, and apoptosis; dysregulation of this enzyme is associated with many human diseases (Iismaa et al. 2009; Odii and Coussons 2014; Chrobok et al. 2017). In asthma, the expression of TGM2 is elevated in the asthmatic airways, and the induced TGM2 levels in the airway was found to be associated with increased production of eicosanoids and eosinophilic inflammation in the airways (Hallstrand et al. 2010). In asthmatic airways, the enzymatic activity of the secreted phospholipase A2 (PLA2) group X (sPLA2-X) were increased by TGM2, causing airway mast cells and eosinophils to upregulate the production of inflammatory cysteinyl leukotrienes (CysLT; eicosanoids), including CysLT E4, to induce eosinophil and basophil recruitment to the airway, further increasing airway hyperresponsiveness and vascular

permeability (Gauvreau et al. 2001; Hallstrand et al. 2007; Hallstrand et al. 2010). As airway eosinophilia is a common observation for diisocyanate-induced occupational asthma including MDI-OA (Saetta, Di Stefano, et al. 1992; Saetta, Maestrelli, et al. 1992; De Vooght et al. 2013; Wisnewski et al. 2015; Wisnewski et al. 2020), induced levels of TGM2 in macrophages after MDI/MDI-GSH exposure may account for a potential mechanism by which MDI exposure may cause airway eosinophilia during MDI-OA pathogenesis.

Conclusion

In conclusion, this report demonstrates that many M2 macrophage-associated markers, transcription factors and chemokines including *Cd206*/CD206, *Tgm2*/TGM2, *Klf4*/KLF4, *Ccl17*/CCL17, *Ccl22*/CCL22, and CCL24 were upregulated after MDI aerosol or MDI-GSH conjugate exposure in BALCs/macrophages. The KLF4-mediated signaling pathways were identified as an important pathway to upregulate M2 macrophage-associated markers *CD206* and *TGM2* as well as chemokines *CCL17, CCL22,* and *CCL24* transcription in macrophages after MDI exposure. The chemotaxis/cell migration of naïve T-cells and eosinophils may be partially attributed to increased expression of M2 macrophage-associated chemokines CCL17, CCL22, and CCL24 as well as TGM2-mediated activities during MDI-induced responses of immune sensitized mice. Thus KLF4-mediated induction of M2 macrophage-associated markers and chemokines expression may play important roles for the pathogenesis of MDI-OA.

Funding

This work was supported by intramural funds (CAN#19390BN8 and #39390KK5) from the National Institute for Occupational Safety and Health, Centers for Disease Control and Prevention.

REFERENCES

Allport DC, Gilbert DS, Outterside SM. 2003. MDI and TDI : a safety, health and the environment : a source book and practical guide. New York: J. Wiley.

An J, Golech S, Klaewsongkram J, Zhang Y, Subedi K, Huston GE, Wood WH 3rd, Wersto RP, Becker KG, Swain SL, Weng N. 2011. Krüppel-like factor 4 (KLF4) directly regulates proliferation in thymocyte development and IL-17 expression during Th17 differentiation. Faseb j. 25(10):3634–3645. eng. [PubMed: 21685331]

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. [PubMed: 12192108]

Barnes PJ. 2008. Immunology of asthma and chronic obstructive pulmonary disease. Nat Rev Immunol. 8(3):183–192. [PubMed: 18274560]

Baxter EW, Graham AE, Re NA, Carr IM, Robinson JI, Mackie SL, Morgan AW. 2020. Standardized protocols for differentiation of THP-1 cells to macrophages with distinct M(IFNγ+LPS), M(IL-4) and M(IL-10) phenotypes. Journal of Immunological Methods. 478:112721. [PubMed: 32033786]

Bentley AM, Maestrelli P, Saetta M, Fabbri LM, Robinson DS, Bradley BL, Jeffery PK, Durham SR, Kay AB. 1992. Activated T-lymphocytes and eosinophils in the bronchial mucosa in isocyanate-induced asthma. J Allergy Clin Immunol. 89(4):821–829. [PubMed: 1532807]

Bernstein DI, Cartier A, Cote J, Malo JL, Boulet LP, Wanner M, Milot J, L'Archeveque J, Trudeau C, Lummus Z. 2002. Diisocyanate antigen-stimulated monocyte chemoattractant protein-1 synthesis has greater test efficiency than specific antibodies for identification of diisocyanate asthma. Am J Respir Crit Care Med. 166(4):445–450. [PubMed: 12186818]

Bernstein DI, Korbee L, Stauder T, Bernstein JA, Scinto J, Herd ZL, Bernstein IL. 1993. The low prevalence of occupational asthma and antibody-dependent sensitization to diphenylmethane diisocyanate in a plant engineered for minimal exposure to diisocyanates. J Allergy Clin Immunol. 92(3):387–396. [PubMed: 8360389]

Biswas SK, Mantovani A. 2010. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol. 11(10):889–896. [PubMed: 20856220]

Byrne AJ, Maher TM, Lloyd CM. 2016. Pulmonary Macrophages: A New Therapeutic Pathway in Fibrosing Lung Disease? Trends Mol Med. 22(4):303–316. eng. [PubMed: 26979628]

Byrne AJ, Mathie SA, Gregory LG, Lloyd CM. 2015. Pulmonary macrophages: key players in the innate defence of the airways. Thorax. 70(12):1189–1196. [PubMed: 26286722]

Chen ZY, Shie J, Tseng C. 2000. Up-regulation of gut-enriched krüppel-like factor by interferon-gamma in human colon carcinoma cells. FEBS Lett. 477(1–2):67–72. eng. [PubMed: 10899312]

Cho YG, Song JH, Kim CJ, Nam SW, Yoo NJ, Lee JY, Park WS. 2007. Genetic and epigenetic analysis of the KLF4 gene in gastric cancer. Apmis. 115(7):802–808. eng. [PubMed: 17614846]

Chrobok NL, Sestito C, Wilhelmus MM, Drukarch B, van Dam AM. 2017. Is monocyte- and macrophage-derived tissue transglutaminase involved in inflammatory processes? Amino Acids. 49(3):441–452. eng. [PubMed: 27659795]

Dang DT, Mahatan CS, Dang LH, Agboola IA, Yang VW. 2001. Expression of the gut-enriched Krüppel-like factor (Krüppel-like factor 4) gene in the human colon cancer cell line RKO is dependent on CDX2. Oncogene. 20(35):4884–4890. eng. [PubMed: 11521200]

Dang DT, Zhao W, Mahatan CS, Geiman DE, Yang VW. 2002. Opposing effects of Krüppel-like factor 4 (gut-enriched Krüppel-like factor) and Krüppel-like factor 5 (intestinal-enriched Krüppel-like factor) on the promoter of the Krüppel-like factor 4 gene. Nucleic Acids Res. 30(13):2736–2741. eng. [PubMed: 12087155]

De Vooght V, Smulders S, Haenen S, Belmans J, Opdenakker G, Verbeken E, Nemery B, Hoet PH, Vanoirbeek JA. 2013. Neutrophil and eosinophil granulocytes as key players in a mouse model of chemical-induced asthma. Toxicol Sci. 131(2):406–418. eng. [PubMed: 23091170]

Deaton RA, Gan Q, Owens GK. 2009. Sp1-dependent activation of KLF4 is required for PDGF-BB-induced phenotypic modulation of smooth muscle. Am J Physiol Heart Circ Physiol. 296(4):H1027–1037. eng. [PubMed: 19168719]

El Chartouni C, Schwarzfischer L, Rehli M. 2010. Interleukin-4 induced interferon regulatory factor (Irf) 4 participates in the regulation of alternative macrophage priming. Immunobiology. 215(9–10):821–825. eng. [PubMed: 20580461]

El Kasmi KC, Qualls JE, Pesce JT, Smith AM, Thompson RW, Henao-Tamayo M, Basaraba RJ, König T, Schleicher U, Koo MS et al. 2008. Toll-like receptor-induced arginase 1 in macrophages thwarts effective immunity against intracellular pathogens. Nat Immunol. 9(12):1399–1406. eng. [PubMed: 18978793]

Engfeldt M, Isaksson M, Zimerson E, Bruze M. 2013. Several cases of work-related allergic contact dermatitis caused by isocyanates at a company manufacturing heat exchangers. Contact Dermatitis. 68(3):175–180. [PubMed: 23046053]

Erle DJ, Sheppard D. 2014. The cell biology of asthma. J Cell Biol. 205(5):621–631. [PubMed: 24914235]

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. [PubMed: 3184985]

Fricker M, Gibson PG. 2017. Macrophage dysfunction in the pathogenesis and treatment of asthma. Eur Respir J. 50(3).

Gauvreau GM, Parameswaran KN, Watson RM, O'Byrne PM. 2001. Inhaled leukotriene E(4), but not leukotriene D(4), increased airway inflammatory cells in subjects with atopic asthma. Am J Respir Crit Care Med. 164(8 Pt 1):1495–1500. eng. [PubMed: 11704602]

Ghaleb AM, Yang VW. 2017. Kruppel-like factor 4 (KLF4): What we currently know. Gene. 611:27–37. [PubMed: 28237823]

Gharib SA, McMahan RS, Eddy WE, Long ME, Parks WC, Aitken ML, Manicone AM. 2019. Transcriptional and functional diversity of human macrophage repolarization. J Allergy Clin Immunol. 143(4):1536–1548. [PubMed: 30445062]

Girodet PO, Nguyen D, Mancini JD, Hundal M, Zhou X, Israel E, Cernadas M. 2016. Alternative Macrophage Activation Is Increased in Asthma. Am J Respir Cell Mol Biol. 55(4):467–475. [PubMed: 27248771]

Hallstrand TS, Chi EY, Singer AG, Gelb MH, Henderson WR Jr. 2007. Secreted phospholipase A2 group X overexpression in asthma and bronchial hyperresponsiveness. Am J Respir Crit Care Med. 176(11):1072–1078. eng. [PubMed: 17901411]

Hallstrand TS, Wurfel MM, Lai Y, Ni Z, Gelb MH, Altemeier WA, Beyer RP, Aitken ML, Henderson WR. 2010. Transglutaminase 2, a novel regulator of eicosanoid production in asthma revealed by genome-wide expression profiling of distinct asthma phenotypes. PLoS One. 5(1):e8583. eng. [PubMed: 20052409]

Hao Z, Sheng Y, Duncan GS, Li WY, Dominguez C, Sylvester J, Su Y-W, Lin GHY, Snow BE, Brenner D et al. 2017. K48-linked KLF4 ubiquitination by E3 ligase Mule controls T-cell proliferation and cell cycle progression. Nature Communications. 8(1):14003.

Heron M, Grutters JC, ten Dam-Molenkamp KM, Hijdra D, van Heugten-Roeling A, Claessen AM, Ruven HJ, van den Bosch JM, van Velzen-Blad H. 2012. Bronchoalveolar lavage cell pattern from healthy human lung. Clin Exp Immunol. 167(3):523–531. [PubMed: 22288596]

Hettick JM, Law BF, Lin CC, Wisnewski AV, Siegel PD. 2018. Mass spectrometry-based analysis of murine bronchoalveolar lavage fluid following respiratory exposure to 4,4'-methylene diphenyl diisocyanate aerosol. Xenobiotica. 48(6):626–636. [PubMed: 28629263]

Hirata H, Yukawa T, Tanaka A, Miyao T, Fukuda T, Fukushima Y, Kurasawa K, Arima M. 2019. Th2 cell differentiation from naive CD4+ T cells is enhanced by autocrine CC chemokines in atopic diseases. Clinical & Experimental Allergy. 49(4):474–483. [PubMed: 30431203]

Huber S, Hoffmann R, Muskens F, Voehringer D. 2010. Alternatively activated macrophages inhibit T-cell proliferation by Stat6-dependent expression of PD-L2. Blood. 116(17):3311–3320. eng. [PubMed: 20625006]

Hur GY, Sheen SS, Kang YM, Koh DH, Park HJ, Ye YM, Yim HE, Kim KS, Park HS. 2008. Histamine release and inflammatory cell infiltration in airway Mucosa in methylene diphenyl diisocyanate (MDI)-induced occupational asthma. J Clin Immunol. 28(5):571–580. [PubMed: 18484168]

Iismaa SE, Mearns BM, Lorand L, Graham RM. 2009. Transglutaminases and disease: lessons from genetically engineered mouse models and inherited disorders. Physiol Rev. 89(3):991–1023. eng. [PubMed: 19584319]

Jan RL, Chen SH, Chang HY, Yeh HJ, Shieh CC, Wang JY. 2008. Asthma-like syndrome in school children after accidental exposure to xylene and methylene diphenyl diisocyanate. J Microbiol Immunol Infect. 41(4):337–341. [PubMed: 18787742]

Jones MG, Floyd A, Nouri-Aria KT, Jacobson MR, Durham SR, Taylor AN, Cullinan P. 2006. Is occupational asthma to diisocyanates a non-IgE-mediated disease? J Allergy Clin Immunol. 117(3):663–669. [PubMed: 16522468]

Kapoor N, Niu J, Saad Y, Kumar S, Sirakova T, Becerra E, Li X, Kolattukudy PE. 2015. Transcription factors STAT6 and KLF4 implement macrophage polarization via the dual catalytic powers of MCPIP. J Immunol. 194(12):6011–6023. [PubMed: 25934862]

Karpurapu M, Ranjan R, Deng J, Chung S, Lee YG, Xiao L, Nirujogi TS, Jacobson JR, Park GY, Christman JW. 2014. Krüppel like factor 4 promoter undergoes active demethylation during monocyte/macrophage differentiation. PLoS One. 9(4):e93362. eng. [PubMed: 24695324]

King KE, Iyemere VP, Weissberg PL, Shanahan CM. 2003. Krüppel-like Factor 4 (KLF4/GKLF) Is a Target of Bone Morphogenetic Proteins and Transforming Growth Factor β1 in the Regulation of Vascular Smooth Muscle Cell Phenotype*. Journal of Biological Chemistry. 278(13):11661–11669. [PubMed: 12538588]

Lawrence T, Natoli G. 2011. Transcriptional regulation of macrophage polarization: enabling diversity with identity. Nat Rev Immunol. 11(11):750–761. [PubMed: 22025054]

Li H, Jiang T, Li MQ, Zheng XL, Zhao GJ. 2018. Transcriptional Regulation of Macrophages Polarization by MicroRNAs. Front Immunol. 9:1175. [PubMed: 29892301]

Li X, Song Y, Wang D, Fu C, Zhu Z, Han Y, Li C, Wang N, Zhu Y. 2012. LIF maintains progenitor phenotype of endothelial progenitor cells via Krüppel-like factor 4. Microvasc Res. 84(3):270–277. eng. [PubMed: 22835519]

Li ZY, Zhu YX, Chen JR, Chang X, Xie ZZ. 2023. The role of KLF transcription factor in the regulation of cancer progression. Biomed Pharmacother. 162:114661. eng. [PubMed: 37068333]

Liao X, Sharma N, Kapadia F, Zhou G, Lu Y, Hong H, Paruchuri K, Mahabeleshwar GH, Dalmas E, Venteclef N et al. 2011. Kruppel-like factor 4 regulates macrophage polarization. J Clin Invest. 121(7):2736–2749. [PubMed: 21670502]

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. [PubMed: 31609387]

Lin CC, Law BF, Hettick JM. 2021. MicroRNA-mediated calcineurin signaling activation induces CCL2, CCL3, CCL5, IL8, and chemotactic activities in 4,4'-methylene diphenyl diisocyanate exposed macrophages. Xenobiotica. 51(12):1436–1452. eng. [PubMed: 34775880]

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. [PubMed: 30074411]

Liu J, Yang T, Liu Y, Zhang H, Wang K, Liu M, Chen G, Xiao X. 2012. Krüppel-like factor 4 inhibits the expression of interleukin-1 beta in lipopolysaccharide-induced RAW264.7 macrophages. FEBS Lett. 586(6):834–840. eng. [PubMed: 22449968]

Liu J, Zhang H, Liu Y, Wang K, Feng Y, Liu M, Xiao X. 2007. KLF4 regulates the expression of interleukin-10 in RAW264.7 macrophages. Biochem Biophys Res Commun. 362(3):575–581. [PubMed: 17719562]

Liu YN, Abou-Kheir W, Yin JJ, Fang L, Hynes P, Casey O, Hu D, Wan Y, Seng V, Sheppard-Tillman H et al. 2012. Critical and reciprocal regulation of KLF4 and SLUG in transforming growth factor β-initiated prostate cancer epithelial-mesenchymal transition. Mol Cell Biol. 32(5):941–953. eng. [PubMed: 22203039]

Lofgren DJ, Walley TL, Peters PM, Weis ML. 2003. MDI Exposure for Spray-On Truck Bed Lining. Appl Occup Environ Hyg. 18(10):772–779. [PubMed: 12959888]

Maeß MB, Wittig B, Cignarella A, Lorkowski S. 2014. Reduced PMA enhances the responsiveness of transfected THP-1 macrophages to polarizing stimuli. J Immunol Methods. 402(1–2):76–81. eng. [PubMed: 24269601]

Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. 2004. The chemokine system in diverse forms of macrophage activation and polarization. Trends Immunol. 25(12):677–686. [PubMed: 15530839]

Martinez FO, Gordon S. 2014. The M1 and M2 paradigm of macrophage activation: time for reassessment. F1000Prime Rep. 6:13. [PubMed: 24669294]

Martinez FO, Helming L, Gordon S. 2009. Alternative activation of macrophages: an immunologic functional perspective. Annu Rev Immunol. 27:451–483. eng. [PubMed: 19105661]

Martinez FO, Helming L, Milde R, Varin A, Melgert BN, Draijer C, Thomas B, Fabbri M, Crawshaw A, Ho LP et al. 2013. Genetic programs expressed in resting and IL-4 alternatively activated mouse and human macrophages: similarities and differences. Blood. 121(9):e57–69. [PubMed: 23293084]

Martinez FO, Helming L, Milde R, Varin A, Melgert BN, Draijer C, Thomas B, Fabbri M, Crawshaw A, Ho LP et al. 2013. Genetic programs expressed in resting and IL-4 alternatively activated mouse and human macrophages: similarities and differences. Blood. 121(9):e57–e69. [PubMed: 23293084]

Melgert BN, ten Hacken NH, Rutgers B, Timens W, Postma DS, Hylkema MN. 2011. More alternative activation of macrophages in lungs of asthmatic patients. J Allergy Clin Immunol. 127(3):831–833. [PubMed: 21167569]

mMunn SJ AR, Aschberger K, Cosgrove O, Pakalin S, PayaPerez A, Pellegrini G, Schwarz-Schulz B, Vegro S. 2005. European Union Risk Assessment Report: methylenediphenyl diisocyanate (MDI). Luxembourg: European Commission, Office for Official Publications of the European Communities.

Nakahara Y, Northcott PA, Li M, Kongkham PN, Smith C, Yan H, Croul S, Ra Y-S, Eberhart C, Huang A et al. 2010. Genetic and Epigenetic Inactivation of Kruppel-like Factor 4 in Medulloblastoma. Neoplasia. 12(1):20–27. [PubMed: 20072650]

mNIOSH. 1994a. Letter from NIOSH to Distinctive Designs International Inc. with a study report. Cincinnati, OH: US Department of Health and Human Services, Public Health Service, Centers for Disease Control, National Institute for Occupational Safety and Health.

mNIOSH. 1994b. Letter from NIOSH to Jim Walter Resources, Inc. with a study report. Cincinnati, OH: US Department of Health and Human Services, Public Health Service, Centers for Disease Control, National Institute for Occupational Safety and Health.

mNIOSH. 2004. A Summary of Health Hazard Evaluations: Issues Related to Occupational Exposure to Isocyanates, 1989 to 2002.

Odegaard JI, Ricardo-Gonzalez RR, Goforth MH, Morel CR, Subramanian V, Mukundan L, Red Eagle A, Vats D, Brombacher F, Ferrante AW, Chawla A. 2007. Macrophage-specific PPARgamma controls alternative activation and improves insulin resistance. Nature. 447(7148):1116–1120. eng. [PubMed: 17515919]

Odii BO, Coussons P. 2014. Biological functionalities of transglutaminase 2 and the possibility of its compensation by other members of the transglutaminase family. ScientificWorldJournal. 2014:714561. eng. [PubMed: 24778599]

Park CS, Shen Y, Lewis A, Lacorazza HD. 2016. Role of the reprogramming factor KLF4 in blood formation. J Leukoc Biol. 99(5):673–685. [PubMed: 26908828]

Petsonk EL, Wang ML, Lewis DM, Siegel PD, Husberg BJ. 2000. Asthma-like symptoms in wood product plant workers exposed to methylene diphenyl diisocyanate. Chest. 118(4):1183–1193. [PubMed: 11035694]

Pope SM, Zimmermann N, Stringer KF, Karow ML, Rothenberg ME. 2005. The eotaxin chemokines and CCR3 are fundamental regulators of allergen-induced pulmonary eosinophilia. J Immunol. 175(8):5341–5350. eng. [PubMed: 16210640]

Qian F, Deng J, Lee YG, Zhu J, Karpurapu M, Chung S, Zheng JN, Xiao L, Park GY, Christman JW. 2015. The transcription factor PU.1 promotes alternative macrophage polarization and asthmatic airway inflammation. J Mol Cell Biol. 7(6):557–567. [PubMed: 26101328]

Redlich CA, Homer RJ, Smith BR, Wirth JA, Cullen MR. 1996. Immunologic responses to isocyanates in sensitized asthmatic subjects. Chest. 109(3 Suppl):6S–8S. [PubMed: 8598159]

Redlich CA, Karol MH. 2002. Diisocyanate asthma: clinical aspects and immunopathogenesis. Int Immunopharmacol. 2(2–3):213–224. [PubMed: 11811926]

Saetta M, Di Stefano A, Maestrelli P, De Marzo N, Milani GF, Pivirotto F, Mapp CE, Fabbri LM. 1992. Airway mucosal inflammation in occupational asthma induced by toluene diisocyanate. Am Rev Respir Dis. 145(1):160–168. eng. [PubMed: 1309963]

Saetta M, Maestrelli P, Di Stefano A, De Marzo N, Milani GF, Pivirotto F, Mapp CE, Fabbri LM. 1992. Effect of cessation of exposure to toluene diisocyanate (TDI) on bronchial mucosa of subjects with TDI-induced asthma. Am Rev Respir Dis. 145(1):169–174. eng. [PubMed: 1309964]

Satoh T, Takeuchi O, Vandenbon A, Yasuda K, Tanaka Y, Kumagai Y, Miyake T, Matsushita K, Okazaki T, Saitoh T et al. 2010. The Jmjd3-Irf4 axis regulates M2 macrophage polarization and host responses against helminth infection. Nat Immunol. 11(10):936–944. eng. [PubMed: 20729857]

Shapouri-Moghaddam A, Mohammadian S, Vazini H, Taghadosi M, Esmaeili SA, Mardani F, Seifi B, Mohammadi A, Afshari JT, Sahebkar A. 2018. Macrophage plasticity, polarization, and function in health and disease. J Cell Physiol. 233(9):6425–6440.

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. [PubMed: 7616105]

Tugal D, Liao X, Jain MK. 2013. Transcriptional control of macrophage polarization. Arterioscler Thromb Vasc Biol. 33(6):1135–1144. [PubMed: 23640482]

Wang K, Zhou W, Cai Q, Cheng J, Cai R, Xing R. 2017. SUMOylation of KLF4 promotes IL-4 induced macrophage M2 polarization. Cell Cycle. 16(4):374–381. eng. [PubMed: 28059602]

White JR, Imburgia C, Dul E, Appelbaum E, O'Donnell K, O'Shannessy DJ, Brawner M, Fornwald J, Adamou J, Elshourbagy NA et al. 1997. Cloning and functional characterization of a novel human CC chemokine that binds to the CCR3 receptor and activates human eosinophils. J Leukoc Biol. 62(5):667–675. eng. [PubMed: 9365122]

Wisnewski AV, Cooney R, Hodgson M, Giese K, Liu J, Redlich CA. 2022. Severe asthma and death in a worker using methylene diphenyl diisocyanate MDI asthma death. Am J Ind Med. eng.

Wisnewski AV, Jones M. 2010. Pro/Con debate: Is occupational asthma induced by isocyanates an immunoglobulin E-mediated disease? Clin Exp Allergy. 40(8):1155–1162. [PubMed: 20545707]

Wisnewski AV, Liu J, Colangelo CM. 2015. Glutathione reaction products with a chemical allergen, methylenediphenyl diisocyanate, stimulate alternative macrophage activation and eosinophilic airway inflammation. Chem Res Toxicol. 28(4):729–737. [PubMed: 25635619]

Wisnewski AV, Liu J, Redlich CA. 2020. Analysis of Lung Gene Expression Reveals a Role for Cl(−) Channels in Diisocyanate-induced Airway Eosinophilia in a Mouse Model of Asthma Pathology. Am J Respir Cell Mol Biol. 63(1):25–35. [PubMed: 32101465]

Yamada T, Park CS, Mamonkin M, Lacorazza HD. 2009. Transcription factor ELF4 controls the proliferation and homing of CD8+ T cells via the Krüppel-like factors KLF4 and KLF2. Nat Immunol. 10(6):618–626. eng. [PubMed: 19412182]

Yang WT, Zheng PS. 2014. Promoter hypermethylation of KLF4 inactivates its tumor suppressor function in cervical carcinogenesis. PLoS One. 9(2):e88827. eng. [PubMed: 24551169]

Ying S, Meng Q, Zeibecoglou K, Robinson DS, Macfarlane A, Humbert M, Kay AB. 1999. Eosinophil chemotactic chemokines (eotaxin, eotaxin-2, RANTES, monocyte chemoattractant protein-3 (MCP-3), and MCP-4), and C-C chemokine receptor 3 expression in bronchial biopsies from atopic and nonatopic (Intrinsic) asthmatics. J Immunol. 163(11):6321–6329. eng. [PubMed: 10570327]

Ying S, Robinson DS, Meng Q, Rottman J, Kennedy R, Ringler DJ, Mackay CR, Daugherty BL, Springer MS, Durham SR et al. 1997. Enhanced expression of eotaxin and CCR3 mRNA and protein in atopic asthma. Association with airway hyperresponsiveness and predominant co-localization of eotaxin mRNA to bronchial epithelial and endothelial cells. Eur J Immunol. 27(12):3507–3516. [PubMed: 9464841]

Zeibecoglou K, Macfarlane AJ, Ying S, Meng Q, Pavord I, Barnes NC, Robinson DS, Kay AB. 1999. Increases in eotaxin-positive cells in induced sputum from atopic asthmatic subjects after inhalational allergen challenge. Allergy. 54(7):730–735. eng. [PubMed: 10442529]

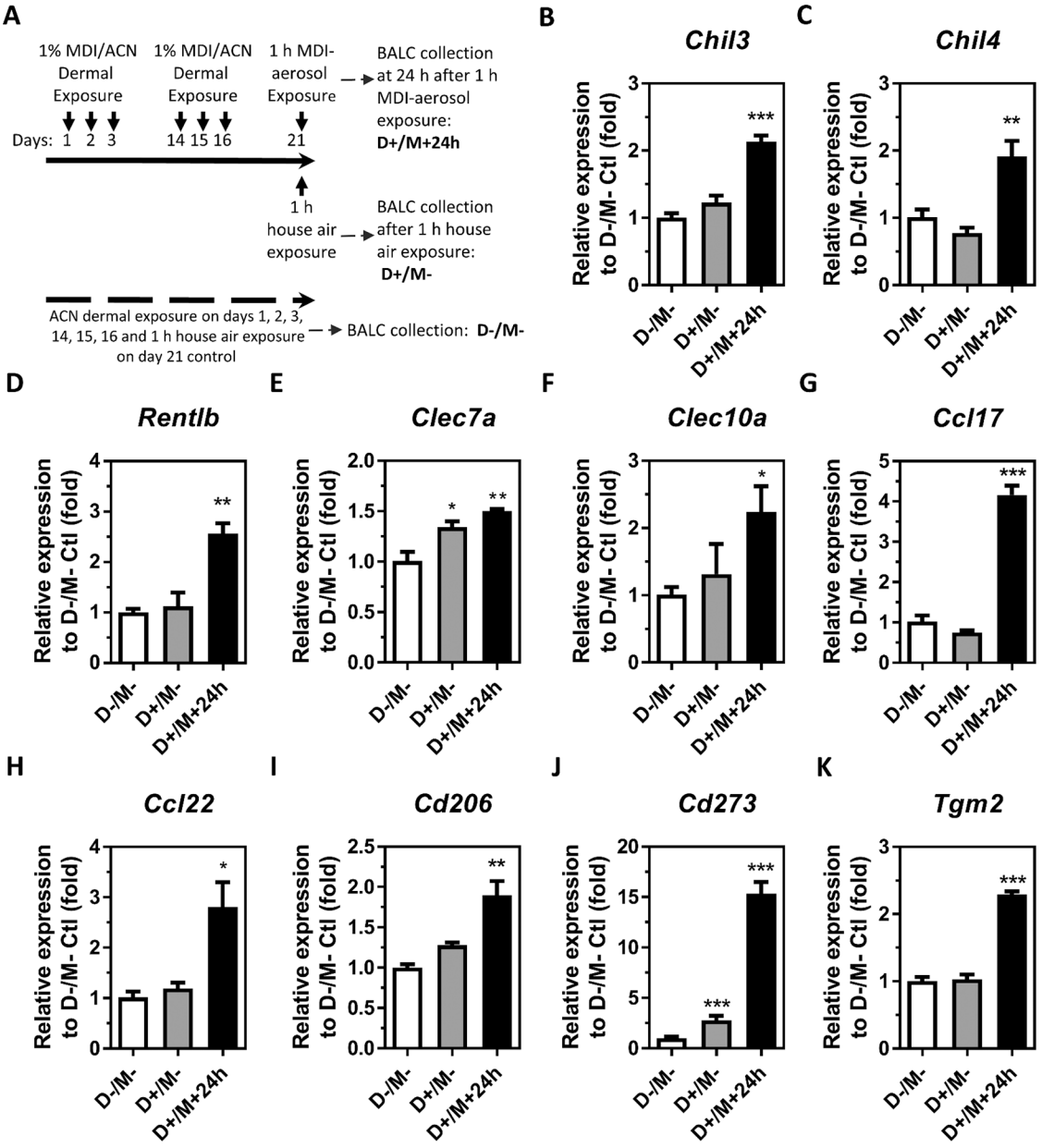

Figure 1. ***In vivo*** **MDI aerosol exposure upregulates M2 macrophage-associated markers and chemokine mRNA in BALCs isolated from mice with combined dermal/aerosol MDI exposure.**

(A) Experimental timelines showing MDI exposure time points, routes. Total RNA was isolated from BALCs from either dermal exposed or dermal/aerosol exposed mice by *miRVana*™ miR isolation kit, reverse transcribed, and subjected to TaqMan RT-qPCR. M2 macrophage-associated markers and chemokine mRNA expression of (B) *Chil3,* (C) *Chil4* (D) *Rentlb*, (E) *Clec7a*, (F) *Clec10a*, (G) *Ccl17*, (H) *Ccl22*, (I) *Cd206,* (J) *Cd273* and (K) *Tgm2* were determined. (N=3; bars, s.e.m) (*P<0.05, **P<0.01, ***P<0.001). Ctl: Control; ACN: Acetone; MDI: 4,4'-methylene diphenyl diisocyanate. D−/M−: BALC RNA isolated from animals with neither MDI dermal exposure nor MDI-aerosol exposure, D+/M-: BALC RNA isolated from animals with MDI dermal exposure on days 1, 2, 3, 14, 15, 16 followed by 1 h house-air control exposure on day 21. D+/M+24h: Total RNA isolated from BALCs

collected 24h post-exposure to 1h MDI-aerosol exposure on day 21 with prior MDI dermal exposure on days 1, 2, 3, 14, 15, 16.

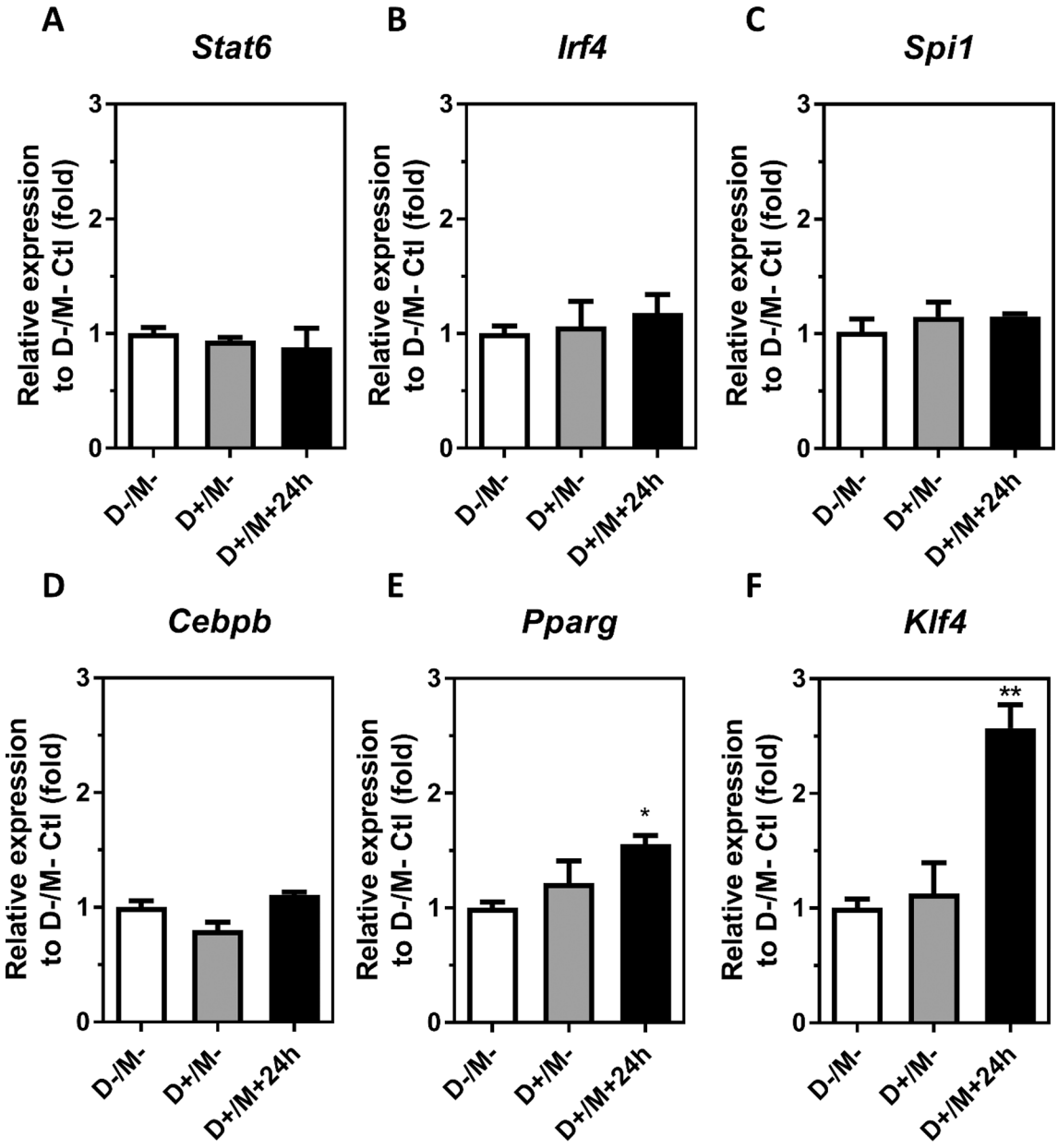

Figure 2. *In vivo* MDI-aerosol exposure upregulates *Klf4* and *Pparg* transcription factor mRNA in BALCs isolated from mice with combined dermal/aerosol MDI exposure.

Total RNA was isolated from BALCs from either dermal exposed or dermal/aerosol exposed mice by *miRVana*™ miR isolation kit, reverse transcribed, and subjected to TaqMan RT-qPCR. Candidate M2 macrophage-associated transcriptional factor expression of (A) *Stat6,* (B) *Irf4*, (C) *Spi1,* (D) *Cebpb,* (E) *Pparg*, and (F) *Klf4* were determined. (N=3; bars, s.e.m). MDI: 4,4'-methylene diphenyl diisocyanate. (*P<0.05, **P<0.01)

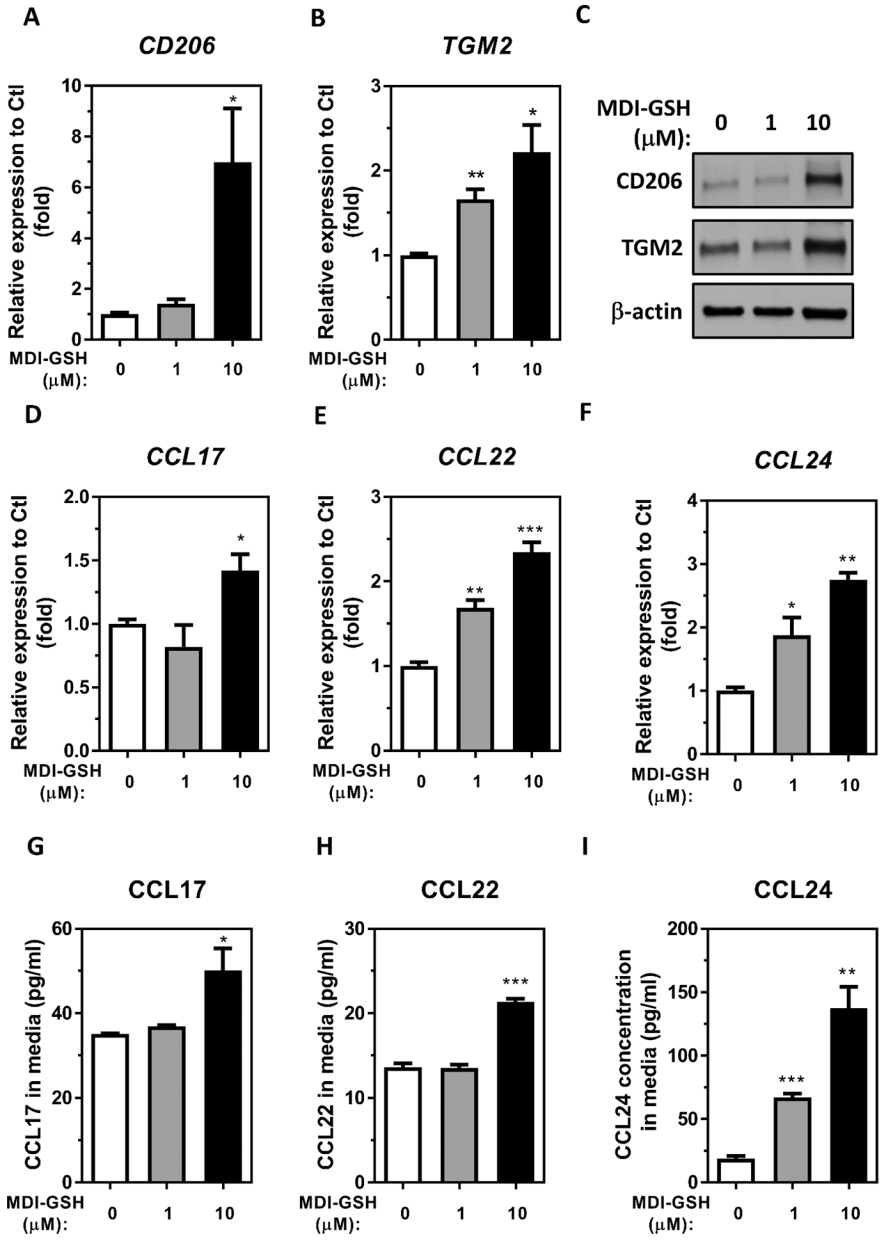

Figure 3. *In vitro* MDI-GSH conjugate treatment induces M2 macrophage markers and chemokines in differentiated THP-1 macrophages.

Total RNA was isolated from MDI-GSH conjugate treated THP-1 macrophages by *miRVana*™ miR isolation kit, reverse transcribed, and subjected to TaqMan RT-qPCR. Endogenous mRNA expressions of (A) *CD206* (B) *TGM2*, (D) *CCL17*, (E) *CCL22*, and (F) *CCL24* were determined at 24 h post exposure (N=3; bars, s.e.m). (C) Endogenous M2 markers CD206 and TGM2 protein expression of THP-1 macrophages treated with MDI-GSH conjugate were determined by immunoblot analysis. β-actin served as a loading control. The secreted chemokine levels of (G) CCL17, (H) CCL22 and (I) CCL24 in conditioned media collected from MDI-GSH conjugate treated THP-1 macrophages were determined by ELISA according to manufacturer's instructions. MDI: 4,4'-methylene diphenyl diisocyanate. GSH: Glutathione. (*P<0.05, **P<0.01, ***P<0.001).

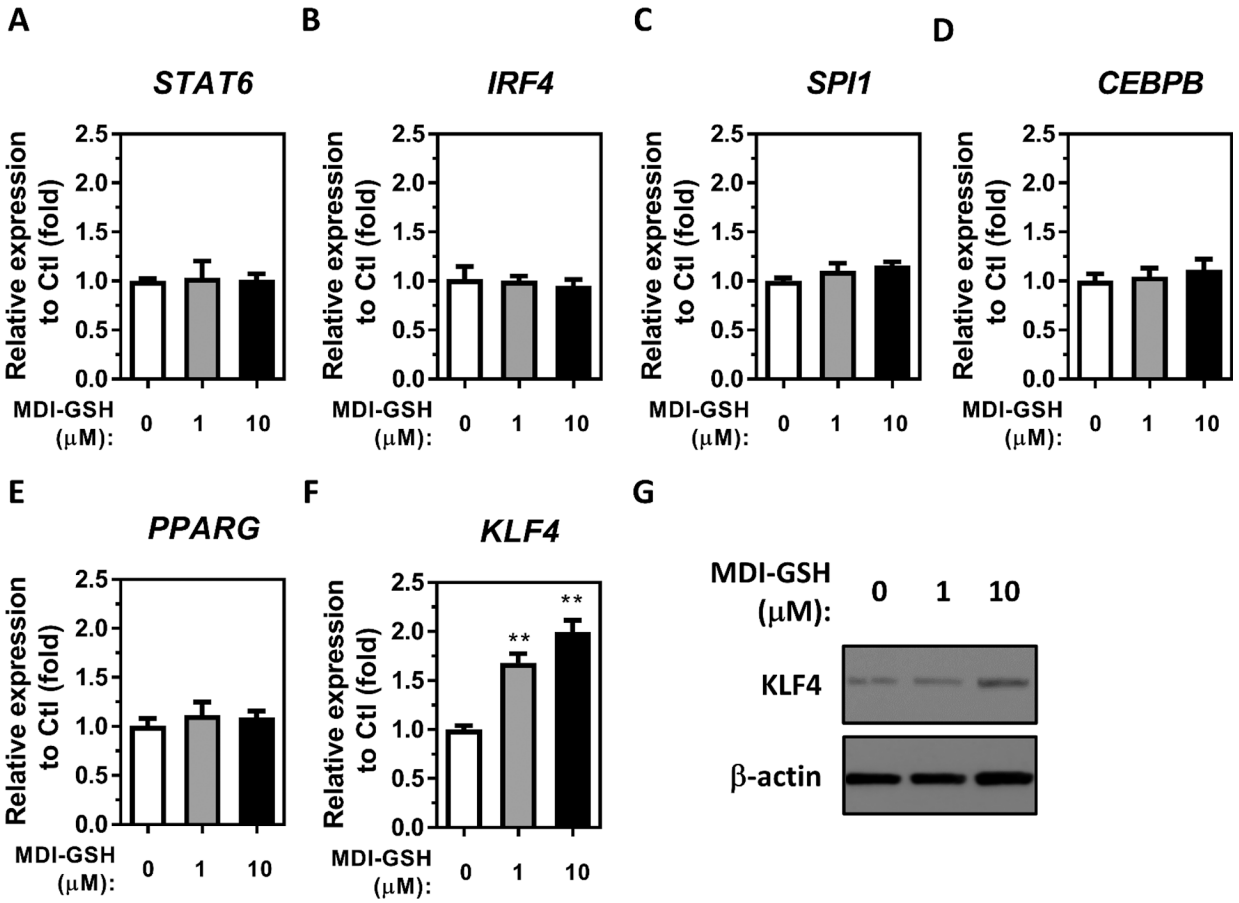

Figure 4. MDI-GSH conjugate treatment induces KLF4 but not *PPARG* in differentiated THP-1 macrophages.

Total RNA was isolated from MDI-GSH conjugate treated THP-1 macrophages by *miRVana*™ miR isolation kit, reverse transcribed, and subjected to TaqMan stem-loop RT-qPCR. mRNA expressions of (A) *STAT6*, (B) *IRF4*, (C) *SPI1*, (D) *CEBPB*, (E) *PPARG*, and (F) *KLF4* were determined (N=3; bars, s.e.m). (G) The endogenous KLF4 protein of differentiated THP-1 macrophages treated with indicated MDI-GSH conjugates was analyzed by immunoblot. β-actin served as a loading control. MDI: 4,4'-methylene diphenyl diisocyanate. GSH: Glutathione. (**P<0.01)

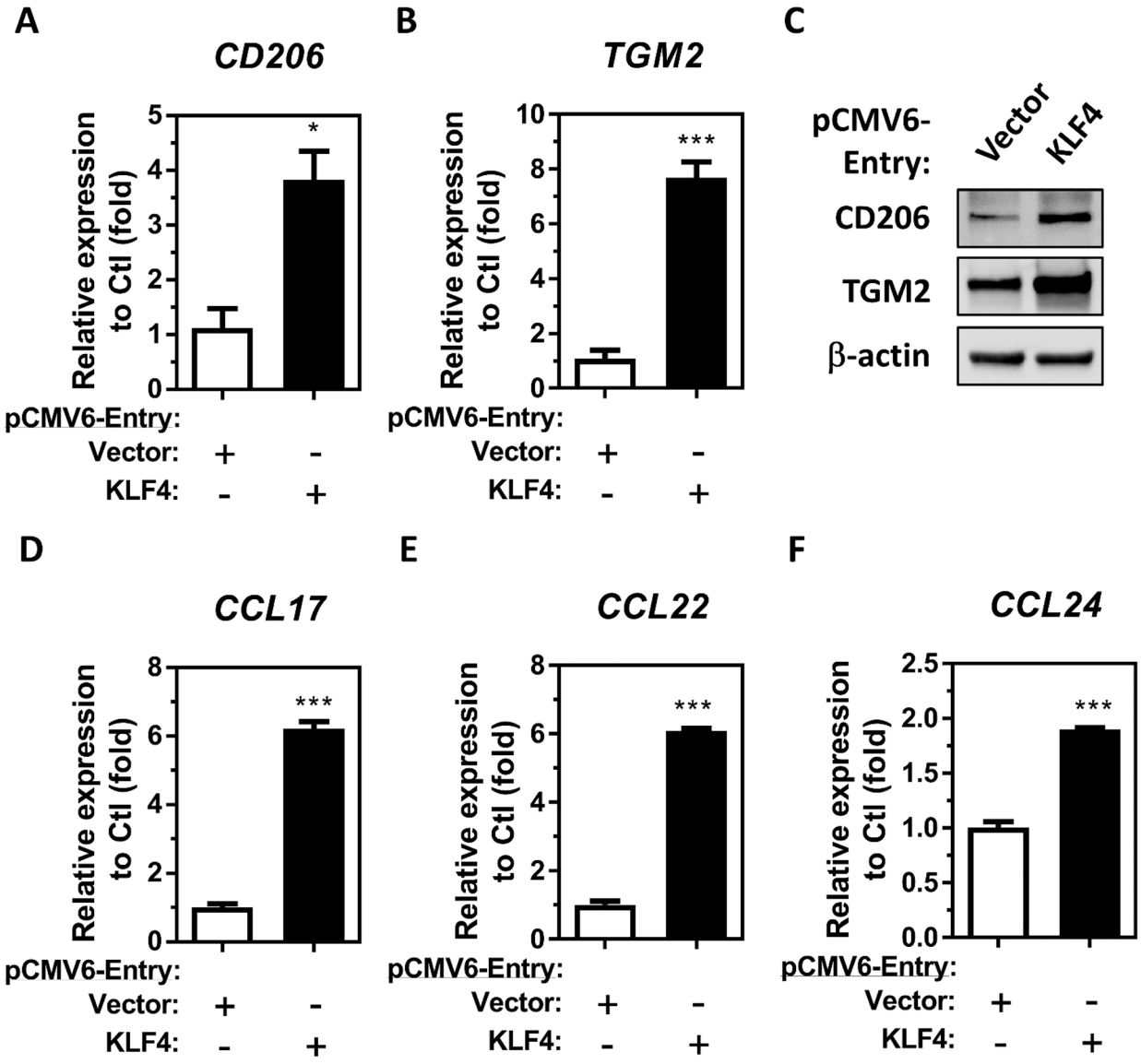

Figure 5. KLF4 overexpression increases M2 macrophage-associated markers and chemokines in differentiated THP-1 macrophages.

Macrophages were transfected with 2.5 mg of either pCMV6-Entry-KLF4 or pCMV6-Entry vector plasmids for 48 h. Total RNA was isolated from plasmid transfected THP-1 macrophages by *miRVana*™ miR isolation kit, reverse transcribed, and subjected to TaqMan stem-loop RT-qPCR. Endogenous M2 markers of (A) *CD206*, (B), *TGM2*, (D) *CCL17* (E) *CCL22*, and (F) *CCL24* mRNA levels were determined (N=3; bars, s.e.m). (D) The endogenous CD206 and TGM2 proteins of THP-1 macrophages transfected with either pCMV6-Entry-KLF4 or pCMV6-Entry vector plasmids was analyzed by immunoblot. β-actin served as a loading control. MDI: 4,4'-methylene diphenyl diisocyanate. GSH: Glutathione. (*P<0.05, ***P<0.001)

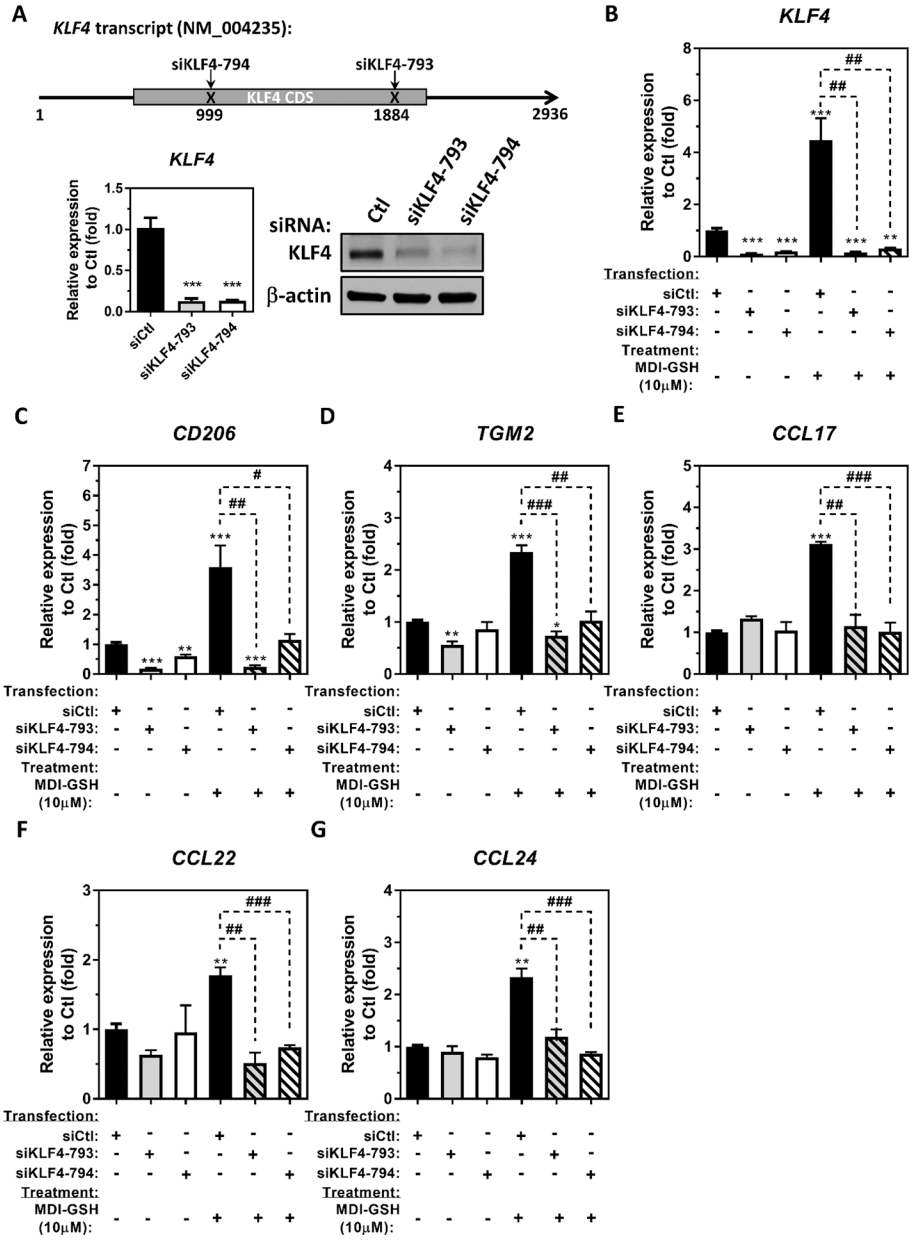

Figure 6. KLF4 knockdown attenuates MDI mediated induction of M2 macrophage-associated markers and chemokine mRNA in differentiated THP-1 macrophages.

(A) Illustration of two siRNA target sites in KLF4 coding sequences (CDS). Endogenous *KLF4* transcripts were determined using total RNA isolated from differentiated macrophages transfected with 25 nM of either two different KLF4 siRNAs or nontargeting siRNA controls for 48 h (N=3; bars, s.e.m). The endogenous KLF4 protein expression of THP-1 macrophages treated with MDI-GSH conjugate was analyzed by immunoblot. β-actin served as a loading control. Endogenous M2 macrophage-associated transcription factor of (B) *KLF4*, markers of (C) *CD206*, (D), *TGM2*, and chemokines (E) *CCL17* (F) *CCL22*, and (G) *CCL24* mRNA levels were determined in total RNA isolated from differentiated macrophages transfected with KLF4 siRNAs for 48h following treatments with or without 10 mM MDI-GSH conjugates for 24h (N=3; bars, s.e.m). Total RNA

was isolated from siRNA transfected THP-1 macrophages by *miRVana*™ miR isolation kit, reverse transcribed, and subjected to TaqMan stem-loop RT-qPCR. MDI: 4,4'-methylene diphenyl diisocyanate. GSH: Glutathione. CDS: coding sequences (*P<0.05, ***P<0.001, when compared to nontargeting siRNA control (siCtl); #p < 0.05, ##p < 0.01, ###p < 0.001, when compared to macrophages transfected with siCtl followed by 10 mM MDI-GSH conjugate exposure.)

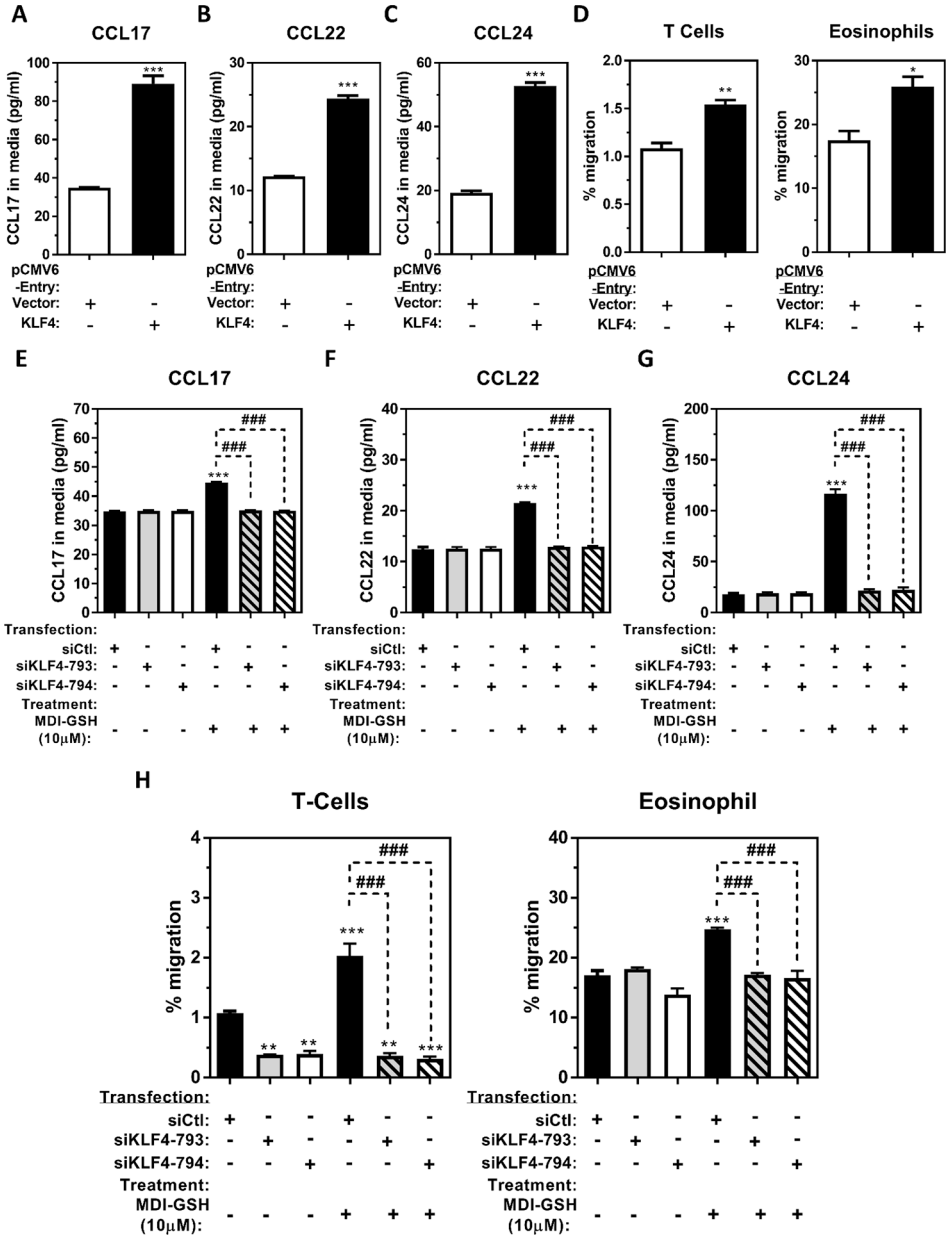

Figure 7. KLF4 plays an important role in the regulation of MDI mediated T-cell and eosinophil chemotaxis/migration in macrophages.

Cell-free conditioned media were obtained from THP-1 macrophages transfected with either KLF4 overexpression plasmid or empty vector for 48 h or else collected 24 h after differentiated THP-1 macrophages subjected with KLF4 siRNAs or nontargeting siRNA control transfection as indicated where the siRNA transfected macrophages were treated with or without 10 mM MDI-GSH conjugates. The secreted protein levels of (A&E) CCL17 (B&F) CCL22, and (C&G) CCL24 in conditioned media from either KLF4 overexpressed THP-1 macrophages or KLF4-knockdown macrophages treated with MDI-GSH conjugates were determined by ELISA according to manufacturer's instructions. The isolated conditioned media were used as chemoattractant to attract (D&H) Jurkat T-cells clone E6–1 or differentiated HL-60 C_15 eosinophils. T-cells and eosinophils migration

responding to the conditioned media from either (D) KLF4 overexpression or (H) KLF4-knockdown macrophages was measured after 6 h. Percent of cells migrated towards the bottom chamber are shown. MDI: 4,4'-methylene diphenyl diisocyanate. GSH: Glutathione. (*P<0.05, **P<0.01, ***P<0.001, when compared to nontargeting siRNA control (siCtl); #p < 0.05, ##p < 0.01, ###p < 0.001, when compared to macrophages transfected with siCtl followed by 10 mM MDI-GSH conjugate exposure.)

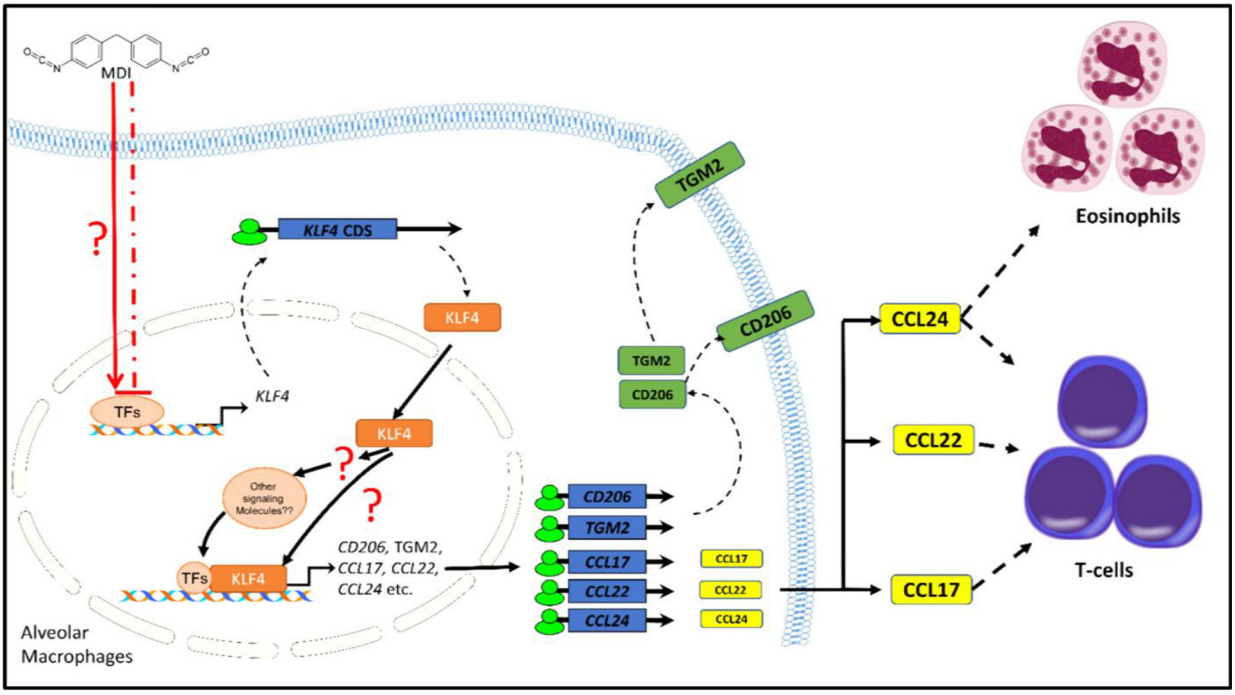

Figure 8. Proposed mechanisms by which MDI exposure induces M2 macrophage-associated markers and chemokines as well as chemotactic activity in T-cells and eosinophils via KLF4-mediated signaling pathway activation in macrophages.

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 29 August 2023) or *openclipart* website (www.openclipart.org, Accessed 29 August 2023).