



Published in final edited form as:

Toxicol Sci. 2025 November 01; 208(1): 122–135. doi:10.1093/toxsci/kfaf114.

Circular RNA *hsa_circ_0099188* Regulates Inducible Nitric Oxide Synthase and Chemokine Transcription in Macrophages by Targeting the *hsa-miR-381-3p/PPP3CA* and *hsa-miR-381-3p/KLF4* Pathways in Response to 4,4'-Methylene Diphenyl Diisocyanate-Glutathione Conjugate Exposure

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Abstract

Workplace exposure to 4,4'-methylene diphenyl diisocyanate (MDI), the most used monomeric diisocyanate, can lead to the development of occupational asthma (OA). However, the molecular mechanisms by which MDI induces OA remain poorly understood. Previous studies have shown that exposure to MDI or MDI-glutathione (GSH) conjugate reduces the levels of endogenous human (*hsa*)/murine (*mmu*)-microRNA(*miR*)-206/381-3p, triggering the activation of calcineurin/nuclear factor of activated T cells (NFAT)/inducible nitric oxide synthase (*NOS2*) regulatory axis and Krüppel-Like Factor 4 (KLF4)/chemokine pathways in macrophages. Circular RNAs (circRNAs) play important roles on miR and miR-mediated functions in the cells. CircRNA *hsa_circ_0008726* is induced by MDI-glutathione (GSH) to downregulate endogenous *hsa-miR-206-3p* in macrophages; however, the MDI-GSH mediated circRNA response to downregulate *hsa-miR-381-3p* is currently unknown. The expression of previously identified candidate circRNAs that bind *hsa-miR-381-3p* were analyzed in differentiated/enhanced THP-1 macrophages treated with MDI-GSH conjugates using RT-qPCR. MDI-GSH exposure induces endogenous *hsa_circ_0099188* and its host gene thyrotropin-releasing hormone-degrading ectoenzyme (*TRHDE*); however, other candidate circRNAs were neither detected nor altered. RNA immunoprecipitation (RIP) experiments confirmed the binding of *hsa-miR-381-3p* to *hsa_circ_0099188*. Further experiments demonstrate that modulating *hsa_circ_0099188* expression through siRNAs or overexpression plasmids alter the levels of endogenous *hsa-miR-381-3p*, *PPP3CA*, and *KLF4*, as well as *NOS2* and M2 macrophage-associated markers and chemokine transcripts. These findings suggest that MDI/MDI-GSH exposure leads to the

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DECLARATION OF CONFLICTING INTERESTS

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.

downregulation of *hsa-miR-381-3p* by inducing the expression of *hsa_circ_0099188/TRHDE*, thereby enhancing the regulatory effects of *hsa-miR-381-3p* in macrophages.

Keywords

4,4'-methylene diphenyl diisocyanate (MDI); Circular RNAs (circRNAs); *hsa-miR-381-3p*; Occupational Asthma (OA); Krüppel-Like Factor 4 (KLF4); protein phosphatase 3 catalytic subunit alpha (PPP3CA)

INTRODUCTION

Diisocyanates (dNCOs) are highly reactive crosslinking agents that are essential to the manufacture of polyurethane (PU) products (Allport et al. 2003). Among these, 4,4'-methylene diphenyl diisocyanate (MDI) is the most widely produced and utilized globally, with demand anticipated to continue to increase (Statista 2024). MDI exposure during manufacture and utilization of PU products is a leading cause of occupational asthma (OA) development (Engfeldt et al. 2013; Jan et al. 2008; NIOSH 1994; Wisnewski et al. 2022); however the underlying molecular mechanisms by which MDI exposure triggers OA are still being actively investigated.

In the occupational clinic, physicians diagnose dNCO-induced occupational asthma (dNCO-OA) by reviewing patient histories of dNCO exposure, changes in lung function, and in some cases, isocyanate-specific IgG or IgE. The gold standard for confirming the OA diagnosis is a dNCO-specific inhalation challenge (SIC) (Vandenplas et al. 2014); however, this test is only available at a few specialized institutions worldwide (Wisnewski 2007). In the context of OA, elevated fractional exhaled nitric oxide (FeNO) levels have been observed in MDI-OA subjects following MDI-SIC (Allmers et al. 2000; Baur and Barbinova 2005; Ferrazzoni et al. 2009). Nitric oxide (NO) production in the airways primarily originates from the induction of inducible nitric oxide synthase (iNOS; encoded by *NOS2*) in airway cells (Brindicci et al. 2007; Lane et al. 2004). Our previous study demonstrated that *NOS2* expression is induced via downregulation of either *hsa-miR-206-3p* or *hsa-miR-381-3p*, resulting in the upregulation of protein phosphatase 3 catalytic subunit alpha (*PPP3CA*), and activation of calcineurin/NFAT signaling after exposure to MDI/MDI-GSH in macrophages (Lin et al. 2020).

Immune cells infiltrate into the lung microenvironment following inhalation of irritants/allergens and their interaction with lung airway cells plays a crucial role in the development of asthma (Barnes 2008; Boonpiyathad et al. 2019; Holgate 2008). Following inhalation into the distal lung microenvironment, irritants/allergens first encounter alveolar macrophages, and this is linked to asthma pathogenesis (Fricker and Gibson 2017). Cell populations of alternatively activated (M2) macrophages are elevated in the airways of patients with asthma (Girodet et al. 2016). Both *in vitro/vivo* studies have shown that exposure to MDI/MDI-GSH induces M2 macrophage-associated gene signatures (Wisnewski et al. 2015; Wisnewski et al. 2020) which are potentially mediated through the transcriptional activation of Krüppel-Like Factor 4 (KLF4) (Lin et al. 2023). Previous study has suggested that endogenous microRNAs (miRs) may play a role in the regulation of MDI/MDI-GSH-induced KLF4

activation in macrophages (Lin et al. 2024b); yet the detailed mechanisms through which MDI/MDI-GSH exposure alters miR levels in macrophages remain largely unknown.

Circular RNAs (circRNAs) are a class of noncoding RNA (ncRNA) characterized by their circular structure. They have been implicated in the pathogenesis of various diseases, including asthma (Huang et al. 2019; Zeng et al. 2021) mainly through regulation of endogenous miR function and downstream targeting of biological processes (Salmena et al. 2011). Previous studies identified that MDI/MDI-GSH exposure results in the downregulation of two endogenous miRs, *hsa-miR-206-3p*/*hsa-miR-381-3p*, and the upregulation of endogenous KLF4 and KLF4-mediated M2 macrophage-associated genes in macrophages (Lin et al. 2020; 2023; Lin et al. 2019). We discovered a putative mechanism for circRNA *hsa_circ_0008726*-mediated downregulation of endogenous *hsa-miR-206-3p* following MDI/MDI-GSH exposure in macrophages (Lin et al. 2024a); however, the mechanism(s) by which MDI downregulates endogenous *hsa-miR-381-3p* in macrophages remains to be elucidated. Given that one of the key functions of endogenous circRNA is to degrade or downregulate endogenous miRs through binding (Patop et al. 2019), we hypothesize that circRNA may respond to MDI/MDI-GSH exposure to regulate endogenous *hsa-miR-381-3p* in macrophages.

This report continues our prior investigation of how MDI/MDI-GSH exposure downregulates endogenous *hsa-miRs-206-3p/-381-3p* in macrophages. We have identified a potential circRNA-mediated regulatory mechanism of endogenous *hsa-miR-206-3p* downregulation (Lin et al. 2024a), and the current report focuses on characterizing the MDI-mediated responses of candidate circRNAs that sponge/target *hsa-miR-381-3p* and verifying the interactions between *hsa-miR-381-3p* and these candidate circRNAs. Additionally, this report explores the downstream molecular effects of MDI/MDI-GSH-induced circRNA changes on the regulation of calcineurin/NFAT signaling mediated *NOS2* transcripts as well as *KLF4* transcripts and KLF4-mediated regulation of M2 macrophage-associated markers and chemokine responses in *in vitro* human THP-1 macrophage culture. Exposure to MDI-GSH conjugates *in vitro* results in the downregulation of endogenous *hsa-miR-381-3p* and upregulation of *hsa_circ_0099188* and its host gene, thyrotropin-releasing hormone-degrading enzyme (*TRHDE*) transcript. This, in turn, leads to the induction of *PPP3CA* and *NOS2* transcription and activation of KLF4 signaling and the induction of M2 macrophage-associated markers and chemokines. This report proposes a circRNA-regulated mechanism to explain how MDI/MDI-GSH exposure downregulates endogenous *hsa-miR-381-3p* in macrophages.

MATERIALS AND METHODS

Chemicals and Reagents

Acetone (HPLC grade; CAS No. 67-64-1), 4,4'-methylene diphenyl diisocyanate (MDI, 98%; CAS No. 101-68-8), 3Å molecular sieve (4-8 mesh; CAS No. 308080-99-1), Tween-20 (CAS No. 9005-64-5), dimethyl sulfoxide (DMSO; CAS No. 67-68-5), phorbol 12-myristate 13-acetate (PMA; CAS No. 16561-29-8), butyric acid (CAS No. 107-92-6), bovine serum albumin (BSA; CAS No. 9048-46-8), tris-buffered saline (TBS), and reduced-glutathione (GSH; CAS No. 70-18-8) were acquired from MilliporeSigma (St. Louis,

MO). Roswell Park Memorial Institute (RPMI)-1640 culture medium, Phosphate buffered saline (PBS), and Penicillin-Streptomycin-Glutamine (PSG; 100×) were acquired from ThermoFisher Scientific (Waltham, MA). Hyclone™ fetal bovine serum (FBS) was obtained from Cytiva Life Sciences (Marlborough, MA). Dry acetone was prepared by incubating HPLC grade acetone with 3Å molecular sieves for a minimum of 24 h to adsorb water.

Cell culture and differentiation

Cell culture and differentiation were carried out as previously described (Lin et al. 2021; 2024a). Briefly, 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 $0.5 - 1 \times 10^6$ /ml in complete RPMI media (RPMI-1640 media supplement, 10% FBS, 1 × PSG) at 37°C in a humidified atmosphere with 5% CO₂ (Lin et al. 2021). THP-1 cells (2×10^6 cells) were differentiated using 10 ng/ml PMA in 10-cm culture dishes for 72 h. The PMA-differentiated THP-1 macrophages were enhanced by washing twice with PBS following removal of PMA and incubation of the cells in PMA-free fresh complete media for another 72 h. A concentration of 10 ng/ml PMA has been shown to enhance the macrophage response to polarizing stimuli (Baxter et al. 2020; Maess et al. 2014). All *in vitro* cell experiments described in this study used differentiated/enhanced THP-1 macrophages. For eosinophil differentiation 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 previously described (Badewa et al. 2002; Fischkoff 1988; Tiffany et al. 1995).

MDI-GSH conjugation reactions

MDI-GSH conjugate was prepared as previously described (Lin et al. 2020; 2021; 2024a; Wisniewski et al. 2013). Briefly, 10 mM GSH in 200 mM sodium phosphate buffer (pH = 7.4) was prepared. Dry acetone was freshly prepared and 50 µl 10% MDI (w/v) in dry acetone were added to 25 mL of GSH solution dropwise with stirring. In our early studies, treatment of THP-1 macrophages directly with 10 µM of MDI in dry acetone induced extensive cytotoxicity (data not shown), however, the THP-1 macrophages tolerate MDI-GSH reaction products with little observed cytotoxicity. MDI-GSH conjugate reactions were performed at 25 °C with end-over-end mixing for 1 h and then centrifuged 5 min at 10,000 × g and filtered with a 0.2 µm syringe filter to remove any precipitate that may have formed during the conjugation procedure. Of note, this reaction generates a mixture of products including mono and bis-MDI-GSH and methylenedianiline (MDA) (Wisniewski et al. 2013), however the relative amounts of each of these components has not been determined. Freshly prepared MDI-GSH conjugate was immediately added into differentiated/enhanced THP-1 macrophages at 0, 1, and 10 µM nominal MDI concentration as indicated.

Plasmid construction

Plasmids were constructed similar to previous reports (Lin et al. 2020; 2024a; 2024b). The circRNA overexpression vector, pcDNA3.1⁽⁺⁾_CircRNA_Mini Vector, was obtained from Addgene (plasmid id#60648; Watertown, MA), deposited by Dr. Jeremy Wilusz at Baylor College of Medicine (Liang and Wilusz 2014). To generate a wildtype (WT) *hsa_circ_0099188* overexpression plasmid, a 0.34-kb cDNA fragment representing

the full length of human circRNA *hsa_circ_0099188* was amplified by PCR using forward and reverse primers containing *PacI* and *HpaI* restriction sites, respectively. The forward and reverse primers are: *PacI* restriction site containing forward primer (cccttaattaaTACCTAAAAATAACTGAGATACAG) and *HpaI* restriction site containing reverse primer (cccgttaacCTTAATTCAACTTTCCTTTATGTA). The PCR product was treated with *PacI* and *HpaI*. This fragment was inserted into pcDNA3.1⁽⁺⁾_CircRNA_Mini Vector that was prepared by sequential enzyme treatments with *PacI*, *SacII*, and calf intestinal alkaline phosphatase (CIP) to generate pcDNA3.1⁽⁺⁾_Circ_Mini-*hsa_circ_0099188* overexpression plasmid.

Plasmid, microRNA mimics/inhibitors and siRNA transfection

Nucleic acid transfections were performed as previously described (Lin et al. 2020; 2021; 2023; 2024a). Briefly, for the circRNA overexpression study, 1×10^6 differentiated/enhanced THP-1 macrophages were reverse transfected with 2.5 µg of either pcDNA3.1⁽⁺⁾_CircRNA_Mini-*hsa_circ_0099188*, or pcDNA3.1⁽⁺⁾_CircRNA_Mini Vector using Mirus TransIT[®]-2020 transfection reagent for 48 h. Total RNA was then isolated using the *mirVana*[™] miR Isolation Kit (ThermoFisher Scientific) according to the manufacturer's instructions. For functional analyses on binding and regulation with *hsa_circ_0099188*, the following *mirVana*[™] miRNA inhibitors (MH; Cat#4464084) and miR-mimics (MC; Cat#4464066) 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), *mirVana*[™] miRNA Inhibitor, negative control #1 (Cat#4464076), and *mirVana*[™] miRNA Mimic, negative control #1 (Cat#4464058). For circRNA *hsa_circ_0099188* knockdown studies, two Dharmacon custom siRNAs targeting *hsa_circ_0099188* were synthesized and the ON-TARGETplus Non-targeting Control Pool (Cat# D-001810-10-05) were purchased from Horizon Discovery (Lafayette, CO). The custom-designed siRNA sequences are: si-*hsa_circ_0099188*#1 (Sense): 5'-GGAAAGUUGAAUUAAGUACCUUU-3'; si-*hsa_circ_0099188*#1 (Antisense): 5'-AGGUACUUAUUAACUUUCCUU-3'; si-*hsa_circ_0099188*#2 (Sense): 5'-AUUAAGUACCUAAAAUAACUUU-3'; si-*hsa_circ_0099188*#2 (Antisense): 5'-AGUUAUUUUUAGGUACUUAUUU-3'. As previously described, differentiated/enhanced THP-1 macrophages were reverse transfected and forward transfected after 24 h (Lin et al. 2011). Total RNA was prepared for RT-qPCR expression analyses 24 h following forward transfection.

Expression analyses

To assess the expression of endogenous circRNA *hsa_circ_0099188* in differentiated/enhanced THP-1 macrophages, total RNA was extracted using *mirVana*[™] miR Isolation Kit (ThermoFisher Scientific). First, linear RNA species were depleted by treating 3 µg of purified THP-1 total RNA with 20 U Ribonuclease R (RNase R; LGC Biosearch Technologies, Teddington, UK). 3 µg total RNA, 20 U of RNase inhibitor (Cat# N8080119; ThermoFisher Scientific), 1 × RNase R buffer and 20 U of RNase R in a total volume of 20 µl was incubated at 37°C for 30 min. Control reactions were performed without RNase R. After RNase R treatment, the RNA was further purified using *mirVana*[™] miR Isolation Kit (ThermoFisher Scientific) according to

manufacturer's instructions. RNase R protein was removed during the second RNA purification step using the *mirVana*[™] miR Isolation Kit to ensure the sample was free of proteins. Total RNA, with or without RNase R treatment, was reverse transcribed to cDNA using a High-Capacity cDNA Synthesis Kit (Thermo Fisher Scientific) following the manufacturer's protocol. PCR reactions were carried out using either *TRHDE* convergent primers (*TRHDE*-F: AGAGCACCACAGAATAACTTATTTG; *TRHDE*-R: GGTTCATAGTTGACTCTAAAATAGCC) or circRNA *hsa_circ_0099188* divergent primers (di-*hsa_circ_0099188*-F: TGA CTAGAATGAGAAGCTGGGT; di-*hsa_circ_0099188*-R: CCCTTCCCAGGAGGTCTGTA).

The mRNA, circRNA and miR levels in THP-1 total RNA were analyzed using RT-qPCR as previously described (Lin et al. 2020; 2021; 2023; 2024a; 2024b; Lin et al. 2019; Lin et al. 2011). Briefly, all RT-qPCR reactions were normalized to human beta-2 microglobulin (*B2M*) for mRNA analysis, or to *U6* snRNA for circRNA and miR analysis. TaqMan gene expression and miR specific assays were purchased from ThermoFisher Scientific and include human *PPP3CA* (Cat#4331182; Assay ID: Hs00174223_m1), *KLF4* (Hs00358836_m1), *TRHDE* (Hs01072025_m1), *NOS2* (Hs01075529_m1), *CD206* (Hs00267207_m1), *TGM2* (Hs01096681_m1), *CCL17* (Hs00171074_m1), *CCL22* (Hs01574247_m1), *CCL24* (Hs00171082_m1), and *B2M* (Hs00187842_m1), *hsa-miR-381-3p* (Cat# 4427975; Assay ID No. 000571; *hsa/Homo sapiens*), and *U6* snRNA (No. 001973). For circRNA assays, SYBR Green-based qPCR was performed using *PowerTrack*[™] SYBR Green Master Mix from ThermoFisher Scientific (Cat#A46110) with divergent primer sets listed in Supplemental Table 1 and normalized using *U6* snRNA with following primer sequences: *U6* snRNA-F: CTCGCTTCGGCAGCACA; *U6* snRNA-R: AACGCTTCACGAATTTGCGT. PCR reactions were conducted on an ABI 7500 Real-Time PCR System (ThermoFisher Scientific, Waltham, MA, USA). Expression of circRNA, mRNAs and miRs were determined using the $\Delta\Delta CT$ method (Livak and Schmittgen 2001) and presented as fold change.

Validation of miR-circRNA interaction by RNA-immunoprecipitation (RIP)

The miR-containing RNA inducing silencing complex (miR/RISC) and its targeting circRNA(s) were immunoprecipitated (IP) using the miRNA target IP kit (Active Motif, Carlsbad, CA) as previously described (Lin et al. 2020; 2021; 2023; 2024a). Briefly, differentiated/enhanced THP-1 macrophages were trypsinized and seeded at 8×10^6 cells in a 10-cm dish. The macrophages were transfected with 25 nM of either miR-mimic-206-3p, miR-mimic-381-3p or miR-mimic negative control #1 (Cat# 4464058) for 24 h. For each IP reaction, two 10-cm dishes containing an equal number of 1.6×10^7 cells were used. The cells were lysed, and the lysates were divided into two equal aliquots. Each aliquot underwent IP using either a pan-AGO antibody (to precipitate the RISC containing AGOs/miRs/mRNAs/circRNAs) or an isotype IgG antibody control. The precipitate was collected, and RNA was purified from the precipitate using the *mirVana*[™] miR Isolation Kit (ThermoFisher Scientific). The RNA was then reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) and *hsa_circ_0099188* divergent primer was combined with *PowerTrack*[™] SYBR Green Master Mix for RT-qPCR

reactions. Data was analyzed by comparing the cells transfected with miR-mimics or non-target miR-mimic-control and the fold enrichment of *hsa_circ_0099188* was calculated from the anti-panAGO and the IgG isotype antibody IP preparations as per manufacturer's instructions.

Chemokine ELISA

Secreted protein concentrations of CCL17, CCL22 and CCL24 in conditioned media were determined as previously described (Lin et al. 2023; 2024a). Briefly, conditioned media was collected 48 h after transfecting differentiated/enhanced THP-1 macrophages with 2.5 µg of either pcDNA3.1⁽⁺⁾_CircRNA_Mini-*hsa_circ_0099188* overexpression plasmid or empty pcDNA3.1⁽⁺⁾_CircRNA_Mini vector. The following enzyme-linked immunosorbent assay (ELISA) kits were obtained from R&D systems (Minneapolis, MN): Human CCL17/TARC ELISA kit (Cat#DY364); Human CCL22/MDC (Cat#DY336), and human CCL24/Eotaxin-2 (#DY343). The sensitivity of each chemokine is as follows: CCL17 (7.8 pg/ml), CCL22 (7.8 pg/ml), and CCL24 (31.2 pg/ml). The levels of human CCL17, CCL22, and CCL24 chemokines released into the conditioned media from plasmid-transfected THP-1 macrophages were determined by ELISA according to manufacturer's directions.

Chemotaxis assays and quantification of migrated cells

Chemotaxis/cell migration in response to conditioned media collected from THP-1 macrophages treated with pcDNA3.1⁽⁺⁾_CircRNA_Mini-*hsa_circ_0099188* overexpression plasmid or pcDNA3.1⁽⁺⁾_CircRNA_Mini vector were performed as previously described (Lin et al. 2021; 2023; 2024a). Briefly, chemotaxis/cell migration assays were conducted in a 24-well plate with Transwell™ inserts (3 µm pore, Corning™ Transwell™ plates, ThermoFisher Scientific). Naive T-cells (Jurkat_E6-1 T cells) or eosinophils (butyric acid differentiated HL-60_C15 cells) were seeded at 1×10^6 cells in 100 µl serum-free RPMI 1640 media in the upper chamber while 500 µL of cell-free conditioned media from THP-1 macrophages was transfected with either pcDNA3.1⁽⁺⁾_CircRNA_Mini-*hsa_circ_0099188* overexpression plasmid or the empty vector was added into the lower chamber as chemoattractant. The cells were incubated for 6 h at 37 °C in a 5% CO₂ humidified incubator. Cells that migrated into the lower chamber media were collected and stored on ice. Cells that failed to migrate from the upper chamber were aspirated and discarded. The Transwell inserts were washed twice with PBS, and 500 µl cell detaching media (0.25% Trypsin-EDTA, Cat#25200056, ThermoFisher Scientific) was added to the lower chambers followed by reinstallation of the upper chamber. The whole plate was further incubated for 30 min at 37 °C to detach the remaining migrated cells. After incubation, detached cells were combined with the previously collected migrated cells in conditioned media, centrifuged at $300 \times g$ for 5 min, washed twice with PBS, and stored at -80 °C. The total number of migrated cells was quantified using CyQUANT® Cell proliferation assay (ThermoFisher Scientific) according to manufacturer's instructions.

Statistical analysis

Statistical analysis was performed as previously described (Lin et al. 2020; 2021; 2023; 2024a). Briefly, 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 in GraphPad Prism 10.3.0 (GraphPad Software, La Jolla, CA, USA.). $P < 0.05$ was used to assign significance.

RESULTS

MDI/MDI-GSH exposure upregulates endogenous *hsa_circ_0099188* in differentiated/enhanced THP-1 human macrophages.

Our previous reports demonstrated that exposure to MDI/MDI-GSH conjugates, both *in vivo* and *in vitro*, leads to the downregulation of endogenous *mmu/hsa-miR-381-3p* in bronchoalveolar lavage cells (BALCs) from an MDI-exposed mouse model and in THP-1 macrophages treated with acute MDI-GSH conjugates (Lin et al. 2020; 2021). These findings suggest that MDI/MDI-GSH conjugates downregulate endogenous *hsa-miR-381-3p* through an unknown mechanism in alveolar macrophages. Through a PubMed literature search (09/2024), we identified four endogenous circRNAs including *hsa_circ_0021593* (Gu et al. 2020), *hsa_circ_0038467* (Yang et al. 2024), *hsa_circ_0084003* (Zhang et al. 2019), and *hsa_circ_0099188* (Sun et al. 2022) that have been reported to bind/sponge and regulate the levels and function of *hsa-miR-381-3p* in various cell types. To investigate whether MDI/MDI-GSH conjugate exposure alters the expression of these candidate circRNAs, we used differentiated/enhanced human THP-1 macrophages as an *in vitro* model. The circRNA responses to MDI-GSH conjugate exposure and subsequent cellular signaling pathways were examined in the following experiments. Differentiated/enhanced THP-1 macrophages were treated with MDI-GSH conjugates at concentrations of 0, 1, and 10 μM for 24 hours. Endogenous *hsa-miR-381-3p* and candidate circRNA levels were quantified by RT-qPCR using TaqMan assays for *hsa-miR-381-3p* and specific divergent primers for the candidate circRNAs (Supplemental Table 1). Consistent with our previous findings, MDI-GSH conjugate treatments significantly downregulated endogenous *hsa-miR-381-3p* levels, with reductions ranging from 16.3% to 6.1% of control (Fig. 1A). Among the four identified candidate circRNAs, MDI-GSH treatment upregulated the levels of *hsa_circ_0099188* by 1.42- to 3.04-fold (Fig. 1E), while the levels of *hsa_circ_0021593* remained unchanged (Fig. 1B). We were unable to detect the other candidate circRNAs, *hsa_circ_0038467* and *hsa_circ_0084003*, in THP-1 macrophages with or without MDI-GSH treatment (Fig. 1C and D). These results indicate that exposure to MDI-GSH conjugates can upregulate endogenous *hsa_circ_0099188* while downregulating *hsa-miR-381-3p* in macrophages.

MDI-GSH conjugates upregulate the endogenous *hsa_circ_0099188* host gene *TRHDE* RNA transcripts in macrophages.

In silico queries (09/2024) of the circBase (Glažar et al. 2014) and Circular RNA Interactome database (Dudekula et al. 2016) showed that the mature *hsa_circ_0099188* consists of 341 nucleotides and is backspliced from intron 2 of the host gene *TRHDE*, located on chromosome 12 (Fig. 2A). The *TRHDE* mRNA transcript (NM_013381) includes 19 exons after splicing and spans 11,047 nucleotides (Fig. 2B). CircRNA *hsa_circ_0099188* is derived from intron 2 of the *TRHDE* RNA transcript via a backsplicing mechanism during *TRHDE* mRNA maturation in the nucleus (Fig. 2B). To confirm the expression of *hsa_circ_0099188* in THP-1 macrophages, total RNA isolated from differentiated/enhanced

THP-1 macrophages was treated with RNase R to degrade linear RNA species, and RT-PCR was performed using *TRHDE* convergent primers (designed to detect mature *TRHDE* mRNA) and *hsa_circ_0099188* divergent primers (designed to detect mature *hsa_circ_0099188*) (Fig. 2B). We successfully detected *hsa_circ_0099188* in THP-1 RNA, whether treated with or without RNase R, using the *hsa_circ_0099188* divergent primers. However, we could not detect *TRHDE* mRNA expression in RNase R-treated THP-1 RNA using the *TRHDE* convergent primers (Fig. 2C). This result confirms that *hsa_circ_0099188* is expressed in THP-1 macrophages. To determine whether MDI-GSH conjugate exposure regulates *hsa_circ_0099188* by upregulating its host gene *TRHDE*, differentiated/enhanced THP-1 macrophages were treated with MDI-GSH conjugates at concentrations of 0, 1, and 10 μ M for 24 hours. The results showed that *TRHDE* transcript levels were significantly upregulated by MDI-GSH treatment, with increases ranging from 1.22- to 1.68-fold (Fig. 2D). These findings suggest that *hsa_circ_0099188* is expressed in THP-1 macrophages, and exposure to MDI-GSH conjugates upregulates *hsa_circ_0099188* through induction of *TRHDE* RNA transcription in macrophages.

Circular RNA *hsa_circ_0099188* binds with *hsa-miR-381-3p* in THP-1 macrophages.

Previous studies have shown that *hsa_circ_0099188* can target and sponge *hsa-miR-381-3p* in human lung fibroblast WI-38 cells (Sun et al. 2022). However, it is not certain whether *hsa_circ_0099188* regulates *hsa-miR-381-3p* in macrophages. To explore the molecular mechanism by which *hsa_circ_0099188* may regulate *hsa-miR-381-3p* through sponging or targeting, we analyzed the potential binding between *hsa_circ_0099188* and *hsa-miR-381-3p* using the *in silico* tool Circbank (<http://www.circbank.cn/>) (Liu et al. 2019). We identified one potential miR response element (MRE) site for *hsa-miR-381-3p* located at position 158–181 of *hsa_circ_0099188* (Fig. 3A), but no MRE site for *hsa-miR-206-3p* was found (data not shown).

CircRNAs are usually stable with long half-lives due to their closed circular structure (lacking the 5'- or 3'-ends of linear RNA species that can be targeted for degradation by exonucleases). However, studies have shown that circRNA degradation can occur when miRs bind to them. This binding facilitates the recruitment of Argonaute (AGO) proteins from RNA-induced silencing complexes (RISC) to circRNAs through the MRE sites, leading to direct RNA cleavage by AGO proteins. This cleavage generates free ends on the circRNA, exposing it to exonucleases for degradation (Zhou et al. 2021). To investigate whether *hsa_circ_0099188* is directly regulated by *hsa-miR-206-3p* or *hsa-miR-381-3p*, we first confirmed the binding of *hsa-miR-206-3p* or *hsa-miR-381-3p* to *hsa_circ_0099188* by performing an AGO antibody pulldown assay. This method precipitates RISC containing AGOs, miRs, and circRNAs (RISC-IP; RIP), followed by analysis of the regulatory roles of these miRs on *hsa_circ_0099188* using gain- or loss-of-function studies with miR mimics or inhibitors. Consistent with the *in-silico* prediction, which suggested that *hsa_circ_0099188* contains an MRE site for *hsa-miR-381-3p* but not for *hsa-miR-206-3p*, transfection of miR-mimic-381-3p resulted in a 5.16-fold increase in the precipitation of *hsa_circ_0099188* compared to a non-targeting miR-mimic control. In contrast, transfection of miR-mimic-206-3p did not increase the precipitation of *hsa_circ_0099188* compared to

the control (Fig. 3B). These results confirm that *hsa_circ_0099188* binds to *hsa-miR-381-3p* but not to *hsa-miR-206-3p* within RISC complexes.

To further investigate whether *hsa-miR-381-3p* regulates endogenous *hsa_circ_0099188* through sponging or miR-mediated degradation in differentiated/enhanced THP-1 macrophages, we conducted gain- and loss-of-function experiments by transfecting miR mimics or inhibitors of *hsa-miR-206-3p* and *hsa-miR-381-3p* into the macrophages. Transfection of miR-mimic-381-3p caused a 51.9% reduction of endogenous *hsa_circ_0099188* (Fig. 3C), whereas transfection of miR-inhibitor-381-3p upregulated *hsa_circ_0099188* by 4.96-fold (Fig. 3D), compared with the non-targeting controls. To further examine the binding/sponging/degradation mechanism, we transfected THP-1 macrophages with miR mimics or inhibitors for *hsa-miR-206-3p*. Because *hsa_circ_0099188* does not contain an MRE site for *hsa-miR-206-3p*, neither miR-mimic-206-3p nor miR-inhibitor-206-3p altered the levels of *hsa_circ_0099188* (Fig. 3C and D). These findings provide further evidence that *hsa_circ_0099188* interacts with *hsa-miR-381-3p* through the MRE site on *hsa_circ_0099188* and can be regulated by endogenous *hsa-miR-381-3p* in macrophages.

MDI-GSH conjugates upregulate the endogenous *hsa_circ_0099188* host gene *TRHDE* RNA transcripts in macrophages.

To investigate the endogenous roles of *hsa_circ_0099188* in regulating *hsa-miR-381-3p*, we designed two distinct siRNAs targeting *hsa_circ_0099188* and performed a loss-of-function experiment. Differentiated/enhanced THP-1 macrophages were transfected with either si-*hsa_circ_0099188*#1 or si-*hsa_circ_0099188*#2, which target different regions of the circRNA *hsa_circ_0099188*. Transfection of si-*hsa_circ_0099188*#1 resulted in a significant reduction of endogenous *hsa_circ_0099188* levels by 64.3%, while si-*hsa_circ_0099188*#2 reduced *hsa_circ_0099188* levels by 32.9% (Fig. 4A). In line with our previous findings that *hsa_circ_0099188* acts as a sponge/target for *hsa-miR-381-3p* in macrophages (Fig. 3), silencing of *hsa_circ_0099188* with si-*hsa_circ_0099188*#1 significantly increased endogenous *hsa-miR-381-3p* expression by 3.53-fold, whereas transfection with si-*hsa_circ_0099188*#2 resulted in a 1.56-fold increase in *hsa-miR-381-3p* levels (Fig. 4B). These results suggest that endogenous *hsa_circ_0099188* plays a role in regulating the levels of *hsa-miR-381-3p* in macrophages.

Circular RNA *hsa_circ_0099188* regulates endogenous *PPP3CA*, *NOS2*, *KLF4* and M2 macrophage-associated markers *CD206*, *TGM2*, *CCL17*, *CCL22*, and *CCL24* transcript levels.

Our previous studies demonstrated that MDI/MDI-GSH exposure upregulates calcineurin/NFAT signaling activation, leading to *NOS2* upregulation through *hsa-miR-206/381-3p*-mediated *PPP3CA* upregulation (Lin et al. 2020). Furthermore, MDI/MDI-GSH exposure upregulates the transcription factor *KLF4*, as well as M2 macrophage-associated markers and chemokines, including *CCL17*, *CCL22*, and *CCL24*, through *KLF4*-mediated activation in macrophages (Lin et al. 2023). Further investigation revealed that *KLF4* transcription factor can be regulated by endogenous *hsa-miR-381-3p* in macrophages (Lin et al. 2024b). To examine the role of *hsa_circ_0099188* in regulating the MDI-GSH conjugate-

induced activation of calcineurin/NFAT signaling mediated *NOS2* transcription, and M2 macrophage-related gene expression, we performed a loss-of-function experiment using si-*hsa_circ_0099188* siRNA to knock down endogenous *hsa_circ_0099188* in macrophages. Since si-*hsa_circ_0099188*#1 exhibited stronger knockdown of *hsa_circ_0099188* compared to si-*hsa_circ_0099188*#2 (Fig. 4A), we focused on the use of si-*hsa_circ_0099188*#1 for subsequent experiments. Transfection with si-*hsa_circ_0099188*#1 reduced the expression of endogenous *hsa_circ_0099188*, *PPP3CA*, *KLF4*, and *TGM2* mRNAs by 59.0%, 45.6%, 65.9%, and 40.8%, respectively (Fig. 5 A, C, D, and G), and increased *hsa-miR-381-3p* expression by 1.61-fold (Fig. 5 B) compared to control nontargeting siRNA (siCtl)-treated THP-1 macrophages. Consistent with our previous findings that MDI-GSH conjugate exposure upregulates *PPP3CA* and *NOS2* (Lin et al. 2020), *KLF4* and M2 macrophage-related markers and chemokines in THP-1 macrophages (Lin et al. 2023), as well as *hsa_circ_0099188* (Fig. 1), treatment with 10 μ M MDI-GSH conjugates in siCtl-transfected macrophages increased the expression of *hsa_circ_0099188*, *PPP3CA*, *KLF4*, *NOS2*, *CD206*, *TGM2*, *CCL17*, *CCL22*, and *CCL24* mRNAs by 6.09-, 2.80-, 1.96-, 7.02, 1.97-, 1.52-, 2.14-, 4.04-, and 2.75-fold, respectively (Fig. 5 A, C–J), and downregulated *hsa-miR-381-3p* by 37.9% (Fig. 5 B) compared to untreated siCtl-transfected THP-1 macrophages. However, when THP-1 macrophages were transfected with si-*hsa_circ_0099188*#1, MDI-GSH treatment did not induce the expression of *hsa_circ_0099188*, *PPP3CA*, *KLF4*, *NOS2*, *CD206*, *TGM2*, *CCL17*, *CCL22*, or *CCL24* mRNAs (Fig. 5A, C–J). These results suggest that *hsa_circ_0099188* plays a role in regulating *NOS2* expression through calcineurin/NFAT signaling pathways activation via *PPP3CA* and the expression of the M2 macrophage-associated transcription factor *KLF4*, as well as the markers *CD206*, *TGM2*, and the chemokines *CCL17*, *CCL22*, and *CCL24*, following exposure of macrophages to MDI-GSH conjugates.

Overexpression of *hsa_circ_0099188* induced endogenous *PPP3CA*, *NOS2*, *KLF4* as well as M2 macrophage-associated markers and chemokines transcripts.

To explore whether *hsa_circ_0099188* regulates the expression of calcineurin-NFAT signaling mediated expression of *NOS2* via *PPP3CA* and M2 macrophage-associated markers and chemokines via *KLF4*, we assessed the expression of *PPP3CA* and *NOS2* as well as the markers and chemokines *KLF4*, *CD206*, *TGM2*, *CCL17*, *CCL22*, and *CCL24* transcripts in an *in vitro* overexpression model of *hsa_circ_0099188* in THP-1 macrophages. Compared to the vector-transfected differentiated/enhanced THP-1 macrophages, transfection with *hsa_circ_0099188* overexpression plasmids for 48 hours resulted in a 7.01-fold increase in *hsa_circ_0099188* expression (Fig. 6A) and a 2.41- and 6.33-fold increase in the mRNA levels of *PPP3CA* and *NOS2* (Fig. 6C, E), and 2.02-, 5.26-, and 2.30-fold increase in the mRNA levels of *KLF4*, *CD206*, and *TGM2*, respectively (Fig. 6 D, F–G). In addition, *hsa_circ_0099188* overexpression plasmid transfection led to a 69.2% reduction of endogenous *hsa-miR-381-3p* levels in macrophages (Fig. 6 B). Moreover, overexpression of *hsa_circ_0099188* significantly upregulated the mRNA expression of the chemokines *CCL17*, *CCL22*, and *CCL24* by 3.81-, 1.75-, and 1.62-fold, respectively (Figure 6 H–J). These findings suggest that exposure to MDI-GSH conjugates induces *hsa_circ_0099188*, which may reduce endogenous *hsa-miR-381-3p* levels and simultaneously enhance the transcription and expression of calcineurin-NFAT signaling

mediated induction of *NOS2* via *PPP3CA* upregulation. In addition, the M2 macrophage-associated transcription factor *KLF4*, as well as the markers and chemokines *CD206*, *TGM2*, *CCL17*, *CCL22*, and *CCL24* transcripts can be induced by MDI-GSH conjugates induced *hsa_circ_0099188* in human macrophages.

Circular RNA *hsa_circ_0099188* induces naïve T-cell and eosinophil chemoattraction by induced secretion of chemokine CCL17, CCL22, and CCL24 in macrophages.

Previous studies demonstrate that exposure to MDI-GSH conjugates induces chemotactic activity in both naïve T-cells and eosinophils (Lin et al. 2021; 2023). In addition, we found that the *KLF4* transcription factor plays a critical role in regulating the expression of M2 macrophage-associated chemokines (*CCL17*, *CCL22*, and *CCL24*) following MDI-GSH exposure, and that MDI-GSH exposure increases the levels of *hsa_circ_0099188*, *KLF4*, *CCL17*, *CCL22*, and *CCL24* transcripts (Figure 5). Since these chemokines are involved in the recruitment of T-cells, eosinophils, and other immune cells, we hypothesize that endogenous *hsa_circ_0099188* may regulate T-cell and/or eosinophil chemotaxis in macrophages exposed to MDI-GSH by enhancing the secretion of *CCL17*, *CCL22*, and *CCL24* proteins -- similar to previous findings that *hsa_circ_0008726* regulates *CCL17*, *CCL22*, and *CCL24* transcripts through targeting the *hsa-miR-206-3p*/*KLF4* regulatory axis (Lin et al. 2024b). To test this hypothesis, we first assessed the protein levels of *CCL17*, *CCL22*, and *CCL24* in the conditioned media from macrophages transfected with the *hsa_circ_0099188* overexpression plasmid. The overexpression of *hsa_circ_0099188* significantly increased the secretion of *CCL17*, *CCL22*, and *CCL24* proteins by 10.01-, 5.03-, and 2.26-fold, respectively, compared to the vector-transfected THP-1 macrophages (Fig. 7 A–C). Next, we performed chemotaxis/migration assays using conditioned media from these macrophages. Compared to the conditioned media from vector-transfected THP-1 macrophages, media from *hsa_circ_0099188* overexpressing macrophages promoted T-cell (Jurkat_E6-1 cells) migration by 1.57-fold (Fig. 7 D) and eosinophil (butyric acid-differentiated HL-60_C15 cells) migration by 2.04-fold (Fig. 7 E). These results suggest that MDI-GSH-induced *hsa_circ_0099188* plays a key role in regulating macrophage-mediated chemotaxis and migration of T-cells and eosinophils.

DISCUSSION

Our previous studies have shown that MDI/MDI-GSH exposure leads to the downregulation of endogenous *hsa-miR-206-3p* and *hsa-miR-381-3p* levels (Lin et al. 2019; 2020; 2021), while upregulating *PPP3CA*/Calcineurin/NFAT signaling activation mediated *NOS2* expression as well as *KLF4* transcription factor-mediated M2 macrophage-associated markers and chemokines *in vivo* and *in vitro*. However, the precise molecular mechanisms behind the downregulation of *hsa-miR-206-3p* and *hsa-miR-381-3p* following MDI-GSH-exposure remain unclear. Our prior work identified circRNA *hsa_circ_0008726* as a potential regulator of *hsa-miR-206-3p* downregulation in response to MDI-GSH exposure (Lin et al. 2024a). In this study, we explore the role of another circRNA, *hsa_circ_0099188*, in regulating *hsa-miR-381-3p*. In our *in vitro* MDI-GSH exposure model using human THP-1 macrophages, we observed that MDI-GSH exposure upregulates both *hsa_circ_0099188* and its host gene *TRHDE* transcript (Figs. 1 and 2), while

hsa_circ_0099188 contains a *hsa-miR-381-3p* MRE site, enabling it to sponge and downregulate *hsa-miR-381-3p*. This downregulation of *hsa-miR-381-3p* leads to the upregulation of *PPP3CA* and *KLF4* expression, which in turn activates downstream gene transcription, enhancing *NOS2* as well as M2 macrophage associated markers and chemokines including *CD206*, *TGM2*, *CCL17*, *CCL22*, and *CCL24* transcripts. (Fig. 8). Additionally, we demonstrated that *hsa_circ_0099188* sponges *hsa-miR-381-3p* in macrophages, resulting in its degradation and subsequent *PPP3CA* and *KLF4* upregulation. Although *hsa_circ_0099188* has been reported to target *hsa-miR-381-3p* in other cell types, including WI-38 human fetal lung cells (Sun et al. 2022), to our knowledge, the current report is the first to identify and verify that *hsa_circ_0099188* is expressed in human macrophages and can regulate the expression of endogenous *hsa-miR-381-3p* and *hsa-miR-381-3p*-mediated posttranscriptional regulation of endogenous gene function in macrophages.

Exposure of macrophages to MDI-GSH conjugates leads to the induction of endogenous *hsa_circ_0099188* through upregulation of its host gene, *TRHDE*, primary transcript production (Fig. 2). While the precise mechanism by which MDI-GSH conjugates induce *TRHDE* remains unclear, this induction could represent a protective response in macrophages to MDI/MDI-GSH conjugate exposure. The *TRHDE* gene encodes the thyrotropin-releasing hormone-degrading enzyme, a member of the peptidase M1 family that degrades and inactivates the neuropeptide thyrotropin-releasing hormone (TRH) (Schomburg et al. 1999). TRH, produced by the hypothalamus, stimulates the release of thyroid-stimulating hormone (TSH) from the pituitary gland into the bloodstream. Additionally, *TRHDE* mRNA is expressed in various human organs, including the brain, pancreas, connective tissue, bone marrow, lymphoid tissue, respiratory system, and gastrointestinal tract based on the Human Protein Atlas database V.24 (<https://www.proteinatlas.org/>) (Uhlen et al. 2015). TRH, regulated/degraded by TRHDE, is crucial for maintaining thyroid hormone (TH) homeostasis by controlling TSH release from the pituitary gland, which in turn stimulates the thyroid gland to produce and release TH into bloodstream (Nikrodhanond et al. 2006). For several decades, studies have shown a positive correlation between worsening of asthma and thyrotoxicosis, a condition where the patient's thyroid gland produces too much TH (Bonneh and Lowenthal 1988; Gomez Huelgas et al. 1989; Parshad et al. 1985; Settipane et al. 1972). More recently, Gau et al. found an increased risk of hyperthyroidism in individuals with asthma (Gau et al. 2022), suggesting a link between elevated TH levels and asthma pathogenesis. Since TRHDE is released into the bloodstream and can travel to the hypothalamus to inactivate TRH, thereby inhibiting TSH release from the pituitary gland, decreased TSH levels in the bloodstream would affect TH production and secretion from the thyroid gland. Thus, the observed induction of *TRHDE* transcripts in MDI/MDI-GSH exposed macrophages may act as a protective mechanism to reduce TH levels systematically and within the lung microenvironment. Further research is needed to explore the potential roles of TRHDE in alveolar macrophages and the potential protective roles of the secreted TRHDE as well as the molecular mechanisms behind the MDI/MDI-GSH conjugate-induced *TRHDE* transcription to be better understand its role in the pathogenesis of MDI-OA.

Alveolar macrophage dysfunction has been identified as a critical factor in the pathogenesis and treatment of asthma (Fricker and Gibson 2017). Disruptions in molecular gene regulation and the maintenance of gene expression homeostasis can significantly impact the function of alveolar macrophages, including their polarization and production/secretion of key mediators. In recent years, circRNAs have emerged as a key class of endogenous regulatory molecules contributing to alveolar macrophage dysfunction in asthma. Several circRNAs have been implicated in regulating macrophage dysfunction in asthma, including circRNA *mmu_circ_0001359* (Shang et al. 2020), *mmu_circ_S100A11* (Liang et al. 2023), *hsa_circ_0001326* (Gong et al. 2022), *hsa_circ_0008726* (Lin et al. 2024a) and others. The current study suggests endogenous circRNA *hsa_circ_0099188* in macrophages may participate in asthma pathogenesis by modulating M2 macrophage polarization by targeting the *hsa-miR-381-3p* involved. Given that MDI exposure, in the form of MDI-GSH conjugates, promotes M2 macrophage-associated markers and chemokines indicative of M2 polarization, future studies will explore whether MDI-GSH conjugate exposure influences the human homologs of murine circRNAs, such as *mmu_circ_0001359*, and *mmu_circ_S100A11*, as well as the human circRNAs *hsa_circ_0001326* or other M2 macrophage promoting circRNA(s).

Because a single miR can target and regulate multiple different mRNAs through imperfect binding to MRE sites on its target transcripts (Bartel 2018), *hsa-miR-381-3p* may target other important immune regulatory genes beyond *PPP3CA* and *KLF4* in macrophages exposed to MDI/MDI-GSH, as discussed here. Wen et al. showed that *hsa-miR-381-3p* was induced in the blood of *Mycobacterium tuberculosis*-infected patients, where it targeted *CD1C* transcripts and downregulated antigen-presenting capability of the dendritic cells (DCs) (Wen et al. 2016). The allergen-induced upregulation of CD1C in airway DCs plays a key role in presenting inhaled allergens to other immune cells and triggering inflammatory responses in the small airways, which is crucial for the development of atopic asthma (McCarthy et al. 2007). Furthermore, Jiang et al. found that elevated levels of *hsa-miR-381-3p* in keratinocyte-derived small vesicles promoted Th1 and Th17 polarization in T cells in psoriasis by targeting the *FOXO1* transcription factor mRNA and inhibiting FOXO1 protein expression. (Jiang et al. 2021). FOXO1 has been identified as a key regulator of asthmatic inflammation and asthma pathogenesis in macrophages (Chung et al. 2019; Chung et al. 2016; Rong et al. 2022). Here, we found that MDI-GSH conjugate exposure upregulated endogenous *hsa_circ_0099188*, which downregulated *hsa-miR-381-3p* while increasing the expression of *PPP3CA*/calcineurin/NFAT signaling and the *KLF4* transcription factor and its downstream chemokines. Whether the endogenous CD1C and FOXO1 are induced through MDI-GSH-mediated activation of the *hsa_circ_0099188*/*hsa-miR-381-3p* regulatory axis remains to be investigated in the context of MDI-OA pathogenesis.

The current *in vitro* model using THP-1 macrophages exposed to MDI-GSH conjugates offers several strengths, including the ability to conduct gain- and loss-of-function experiments with miR mimics/inhibitors, overexpression plasmid transfections of circRNA *hsa_circ_0099188*, and siRNA transfections to knock down endogenous *hsa_circ_0099188*. These experiments allow for the investigation of molecular regulatory mechanisms mediated by circRNA in macrophages exposed to MDI-GSH conjugates within a single immune cell

type. However, THP-1 cells are derived from human leukemia monocytes, making these cells transformed and tumorigenic rather than normal. Although these cells are widely used in research to study the function of monocytes and macrophages; a key limitation of this *in vitro* differentiated/enhanced THP-1 macrophage model is that it may not exhibit the same physiological properties as the primary human lung alveolar macrophages. Other limitations of this THP-1 macrophage model include uncertainty regarding whether the observed induction of *KLF4* via *hsa_circ_0099188* mediated-downregulation of *hsa-miR-381-3p* and the resulting *hsa_circ_0099188/hsa-miR-381-3p/KLF4* regulatory axis, which promotes M2 macrophage-associated markers and chemokines, as well as the *NOS2* induction via *hsa_circ_0099188/hsa-miR-381-3p/PPP3CA* regulatory axis reflects immune responses similar to those in the early stages of asthma pathogenesis and elevated FeNO in MDI-OA. Additionally, the current study primarily relies on the identification of potential *hsa-miR-381-3p*-targeting circRNAs from existing literature, which excludes the possibility of other circRNAs that may be regulated by MDI-GSH conjugates in macrophages. The current experimental design omits other potential *hsa-miR-381-3p* targeting circRNAs that may be regulated by MDI-GSH conjugate exposure in macrophages that contribute to MDI-OA pathogenesis. Future research using *in vivo* MDI-OA animal models and/or alveolar macrophages collected from MDI-OA patients is needed to further explore the connection between diisocyanate exposure and circRNA/miR/miR-target gene regulatory mechanisms in the context of MDI exposure among workers. Finally, the MDI-GSH conjugation reaction produces a small amount of methylenedianiline (MDA, a suspected carcinogen) due to hydrolysis of MDI. Future studies are needed to elucidate what effect, if any, MDA contributes to this model system.

In conclusion, this study identifies *hsa_circ_0099188* as a potential regulator of *hsa-miR-381-3p*, *PPP3CA*, *KLF4* transcripts, and calcineurin/NFAT signaling and *KLF4*-mediated signaling, ultimately influencing *NOS2* and macrophage markers and associated chemokine transcription following MDI-GSH conjugate exposure. In an *in vitro* THP-1 macrophage model, MDI-GSH exposure induces *hsa_circ_0099188* and downregulates *hsa-miR-381-3p*. This *hsa_circ_0099188/hsa-miR-381-3p/KLF4* regulatory axis may contribute to the upregulation of M2-associated markers and chemokines, such as *CD206*, *TGM2*, *CCL17*, *CCL22*, and *CCL24*, in macrophages. In addition, *hsa_circ_0099188/hsa-miR-381-3p/PPP3CA* regulatory axis may contribute to the upregulation of *NOS2* expression. The circRNA *hsa_circ_0099188* may play a significant role in MDI-OA pathogenesis by regulating macrophage-mediated chemokine and *NOS2* expression and promoting immune cell chemotaxis to the lung microenvironment and elevated FeNO in MDI-OA patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

FUNDING

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

REFERENCES

- Allmers H, Chen Z, Barbinova L, Marczyński B, Kirschmann V, Baur X. 2000. Challenge from methacholine, natural rubber latex, or 4,4-diphenylmethane diisocyanate in workers with suspected sensitization affects exhaled nitric oxide [change in exhaled no levels after allergen challenges]. *Int Arch Occup Environ Health*. 73(3):181–186. [PubMed: 10787133]
- 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.
- 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]
- Bartel DP. 2018. Metazoan micrnas. *Cell*. 173(1):20–51. [PubMed: 29570994]
- Baur X, Barbinova L. 2005. Increase of exhaled nitric oxide (eno) after methylene diphenyl diisocyanate (mdi) exposure in isocyanate workers with bronchial hyperresponsiveness. *Allergol Int*. 54:151–158.
- Baxter EW, Graham AE, Re NA, Carr IM, Robinson JJ, Mackie SL, Morgan AW. 2020. Standardized protocols for differentiation of thp-1 cells to macrophages with distinct m(ifngamma+lps), m(il-4) and m(il-10) phenotypes. *J Immunol Methods*. 478:112721. [PubMed: 32033786]
- Bonneh DY, Lowenthal MN. 1988. [thyroid disease and bronchial asthma: Is their simultaneous occurrence casual?]. *Harefuah*. 114(10):490–492. [PubMed: 3396980]
- Boonpiyathad T, Sozener ZC, Satitsuksanoa P, Akdis CA. 2019. Immunologic mechanisms in asthma. *Semin Immunol*. 46:101333. [PubMed: 31703832]
- Brindicci C, Ito K, Barnes PJ, Kharitonov SA. 2007. Effect of an inducible nitric oxide synthase inhibitor on differential flow-exhaled nitric oxide in asthmatic patients and healthy volunteers. *Chest*. 132(2):581–588. [PubMed: 17550932]
- Chung S, Kim JY, Song MA, Park GY, Lee YG, Karpurapu M, Englert JA, Ballinger MN, Pabla N, Chung HY et al. 2019. Foxo1 is a critical regulator of m2-like macrophage activation in allergic asthma. *Allergy*. 74(3):535–548. [PubMed: 30288751]
- Chung S, Lee TJ, Reader BF, Kim JY, Lee YG, Park GY, Karpurapu M, Ballinger MN, Qian F, Rusu L et al. 2016. Foxo1 regulates allergic asthmatic inflammation through regulating polarization of the macrophage inflammatory phenotype. *Oncotarget*. 7(14):17532–17546. [PubMed: 27007158]
- Dudekula DB, Panda AC, Grammatikakis I, De S, Abdelmohsen K, Gorospe M. 2016. Circinteractome: A web tool for exploring circular rnas and their interacting proteins and micrnas. *RNA biology*. 13(1):34–42. [PubMed: 26669964]
- 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]
- Ferrazzoni S, Scarpa MC, Guarnieri G, Corradi M, Mutti A, Maestrelli P. 2009. Exhaled nitric oxide and breath condensate ph in asthmatic reactions induced by isocyanates. *Chest*. 136(1):155–162. [PubMed: 19225065]
- 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). 1700196 [PubMed: 28899935]
- Gau SY, Huang JY, Yong SB, Cheng-Chung Wei J. 2022. Higher risk of hyperthyroidism in people with asthma: Evidence from a nationwide, population-based cohort study. *J Allergy Clin Immunol Pract*. 10(3):751–758 e751. [PubMed: 34563740]
- 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]

- Glažar P, Papavasileiou P, Rajewsky N. 2014. Circbase: A database for circular rnas. *Rna*. 20(11):1666–1670. [PubMed: 25234927]
- Gomez Huelgas R, Garcia Calvo MI, Martin Villascargas JJ, Hidalgo San Juan MV. 1989. [bronchial asthma and thyroid dysfunction]. *Med Clin (Barc)*. 92(18):718.
- Gong B, Zheng Y, Li J, Lei H, Liu K, Tang J, Peng Y. 2022. Luteolin activates m2 macrophages and suppresses m1 macrophages by upregulation of hsa_circ_0001326 in thp-1 derived macrophages. *Bioengineered*. 13(3):5079–5090. [PubMed: 35152837]
- Gu F, Zhang J, Yan L, Li D. 2020. Circchipk3/mir-381-3p axis modulates proliferation, migration, and glycolysis of lung cancer cells by regulating the akt/mTOR signaling pathway. *Open Life Sci*. 15(1):683–695. [PubMed: 33817257]
- Holgate ST. 2008. Pathogenesis of asthma. *Clin Exp Allergy*. 38(6):872–897. [PubMed: 18498538]
- Huang Z, Cao Y, Zhou M, Qi X, Fu B, Mou Y, Wu G, Xie J, Zhao J, Xiong W. 2019. Hsa_circ_0005519 increases il-13/il-6 by regulating hsa-let-7a-5p in cd4(+) t cells to affect asthma. *Clin Exp Allergy*. 49(8):1116–1127. [PubMed: 31148290]
- 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]
- Jiang M, Fang H, Dang E, Zhang J, Qiao P, Yu C, Yang A, Wang G. 2021. Small extracellular vesicles containing mir-381-3p from keratinocytes promote t helper type 1 and t helper type 17 polarization in psoriasis. *J Invest Dermatol*. 141(3):563–574. [PubMed: 32712160]
- Lane C, Knight D, Burgess S, Franklin P, Horak F, Legg J, Moeller A, Stick S. 2004. Epithelial inducible nitric oxide synthase activity is the major determinant of nitric oxide concentration in exhaled breath. *Thorax*. 59(9):757–760. [PubMed: 15333851]
- Liang D, Wilusz JE. 2014. Short intronic repeat sequences facilitate circular rna production. *Genes Dev*. 28(20):2233–2247. [PubMed: 25281217]
- Liang Q, Fu J, Wang X, Liu L, Xiao W, Gao Y, Yang L, Yu H, Xie X, Tu Z et al. 2023. Circs100a11 enhances m2a macrophage activation and lung inflammation in children with asthma. *Allergy*. 78(6):1459–1472. [PubMed: 36104951]
- 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. [PubMed: 34775880]
- Lin CC, Law BF, Hettick JM. 2023. 4,4'-methylene diphenyl diisocyanate exposure induces expression of alternatively activated macrophage-associated markers and chemokines partially through kruppel-like factor 4 mediated signaling in macrophages. *Xenobiotica*. 53(12):653–669. [PubMed: 38014489]
- Lin CC, Law BF, Hettick JM. 2024a. Circular rna hsa_circ_0008726 targets the hsa-mir-206-3p/klf4 axis to modulate 4,4'-methylene diphenyl diisocyanate-glutathione conjugate-induced chemokine transcription in macrophages. *Cells*. 13(20):1725. [PubMed: 39451243]
- Lin CC, Law BF, Hettick JM. 2024b. MicroRNA-mediated kruppel-like factor 4 upregulation induces alternatively activated macrophage-associated marker and chemokine transcription in 4,4'-methylene diphenyl diisocyanate exposed macrophages. *Xenobiotica*. 54(9):730–748. [PubMed: 38568505]
- 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]
- Lin CC, Liu LZ, Addison JB, Wonderlin WF, Ivanov AV, Ruppert JM. 2011. A klf4-mirna-206 autoregulatory feedback loop can promote or inhibit protein translation depending upon cell context. *Mol Cell Biol*. 31(12):2513–2527. [PubMed: 21518959]
- Liu M, Wang Q, Shen J, Yang BB, Ding X. 2019. Circbank: A comprehensive database for circrna with standard nomenclature. *RNA biology*. 16(7):899–905. [PubMed: 31023147]

- Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative pcr and the 2(-delta delta c(t)) method. *Methods*. 25(4):402–408. [PubMed: 11846609]
- 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. [PubMed: 24269601]
- McCarthy NE, Jones HA, Marks NA, Shiner RJ, Ind PW, Al-Hassi HO, English NR, Murray CM, Lambert JR, Knight SC et al. 2007. Inhaled allergen-driven cd1c up-regulation and enhanced antigen uptake by activated human respiratory-tract dendritic cells in atopic asthma. *Clin Exp Allergy*. 37(1):72–82. [PubMed: 17210044]
- Nikrodhanond AA, Ortiga-Carvalho TM, Shibusawa N, Hashimoto K, Liao XH, Refetoff S, Yamada M, Mori M, Wondisford FE. 2006. Dominant role of thyrotropin-releasing hormone in the hypothalamic-pituitary-thyroid axis. *J Biol Chem*. 281(8):5000–5007. [PubMed: 16339138]
- NIOSH. 1994. 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.
- Parshad O, Kumar M, Melville GN. 1985. Thyroid-gonad relationship in bronchial asthma. *West Indian Med J*. 34(2):109–113. [PubMed: 4036096]
- Patop IL, Wust S, Kadener S. 2019. Past, present, and future of circrnas. *EMBO J*. 38(16):e100836. [PubMed: 31343080]
- Rong SJ, Yang CL, Wang FX, Sun F, Luo JH, Yue TT, Yang P, Yu Q, Zhang S, Wang CY. 2022. The essential role of foxo1 in the regulation of macrophage function. *Biomed Res Int*. 2022:1068962. [PubMed: 35993049]
- Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. 2011. A cerna hypothesis: The rosetta stone of a hidden rna language? *Cell*. 146(3):353–358. [PubMed: 21802130]
- Schomburg L, Turwitt S, Prescher G, Lohmann D, Horsthemke B, Bauer K. 1999. Human trh-degrading ectoenzyme cDNA cloning, functional expression, genomic structure and chromosomal assignment. *Eur J Biochem*. 265(1):415–422. [PubMed: 10491199]
- Settipane GA, Schoenfeld E, Hamolsky MW. 1972. Asthma and hyperthyroidism. *J Allergy Clin Immunol*. 49(6):348–355. [PubMed: 4554357]
- Shang Y, Sun Y, Xu J, Ge X, Hu Z, Xiao J, Ning Y, Dong Y, Bai C. 2020. Exosomes from mmu_circ_0001359-modified adscs attenuate airway remodeling by enhancing foxo1 signaling-mediated m2-like macrophage activation. *Mol Ther Nucleic Acids*. 19:951–960. [PubMed: 32018116]
- Statista. 2024. Demand for methylene diphenyl diisocyanate worldwide from 2011 to 2023. 2024. [accessed 2024 October 10].
- Sun L, Liu J, Sun X, Zhang Y, Cui X. 2022. Circrthde knockdown protects wi-38 cells against lps-induced inflammatory injury. *Autoimmunity*. 55(4):233–242. [PubMed: 35481453]
- 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]
- Uhlen M, Fagerberg L, Hallstrom BM, Lindskog C, Oksvold P, Mardinoglu A, Sivertsson A, Kampf C, Sjostedt E, Asplund A et al. 2015. Proteomics. Tissue-based map of the human proteome. *Science*. 347(6220):1260419. [PubMed: 25613900]
- Vandenplas O, Suojalehto H, Aasen TB, Baur X, Burge PS, de Blay F, Fishwick D, Hoyle J, Maestrelli P, Munoz X et al. 2014. Specific inhalation challenge in the diagnosis of occupational asthma: Consensus statement. *Eur Respir J*. 43(6):1573–1587. [PubMed: 24603815]
- Wen Q, Zhou C, Xiong W, Su J, He J, Zhang S, Du X, Liu S, Wang J, Ma L. 2016. Mir-381-3p regulates the antigen-presenting capability of dendritic cells and represses antituberculosis cellular immune responses by targeting cd1c. *J Immunol*. 197(2):580–589. [PubMed: 27296666]
- Wisnewski AV. 2007. Developments in laboratory diagnostics for isocyanate asthma. *Curr Opin Allergy Clin Immunol*. 7(2):138–145. [PubMed: 17351466]
- 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*. 65(3):166–172. [PubMed: 35028957]

- Wisnewski AV, Liu J, Colangelo CM. 2015. Glutathione reaction products with a chemical allergen, methylene-diphenyl diisocyanate, stimulate alternative macrophage activation and eosinophilic airway inflammation. *Chem Res Toxicol*. 28(4):729–737. [PubMed: 25635619]
- Wisnewski AV, Liu J, Redlich CA. 2013. Connecting glutathione with immune responses to occupational methylene diphenyl diisocyanate exposure. *Chem Biol Interact*. 205(1):38–45. [PubMed: 23791970]
- 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]
- Yang LJ, Sui SX, Zheng QH, Wang M. 2024. Circuqrc2 promotes asthma progression in children by activating the vegfa/nf-kappab pathway by targeting mir-381-3p. *Kaohsiung J Med Sci*. 40(8):699–709. [PubMed: 39031804]
- Zeng H, Gao H, Zhang M, Wang J, Gu Y, Wang Y, Zhang H, Liu P, Zhang X, Zhao L. 2021. Atractylon treatment attenuates pulmonary fibrosis via regulation of the mmu_circ_0000981/mir-211-5p/tgfr2 axis in an ovalbumin-induced asthma mouse model. *Inflammation*. 44(5):1856–1864. [PubMed: 33855682]
- Zhang PF, Pei X, Li KS, Jin LN, Wang F, Wu J, Zhang XM. 2019. Circular rna circfgfr1 promotes progression and anti-pd-1 resistance by sponging mir-381-3p in non-small cell lung cancer cells. *Mol Cancer*. 18(1):179. [PubMed: 31815619]
- Zhou M, Xiao MS, Li Z, Huang C. 2021. New progresses of circular rna biology: From nuclear export to degradation. *RNA biology*. 18(10):1365–1373. [PubMed: 33241761]

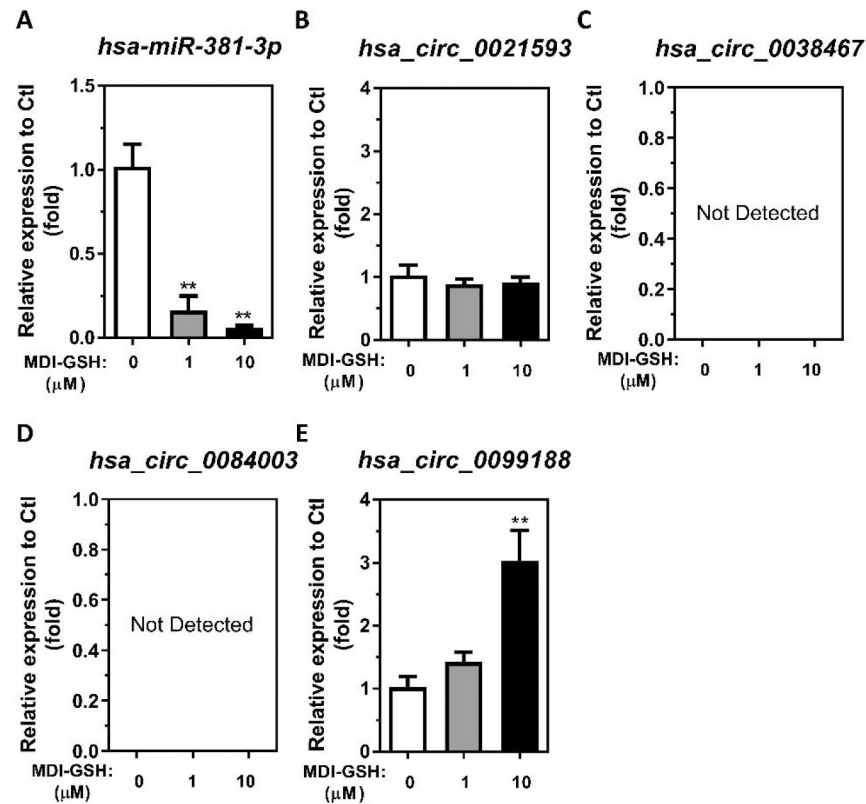


Figure 1. Treatment of MDI-GSH conjugate in differentiated/enhanced THP-1 macrophages induces endogenous circRNA *hsa_circ_0099188* while downregulating endogenous *hsa-miR-381-3p*.

Total RNA was extracted from MDI-GSH conjugate-treated THP-1 macrophages using the *mirVana*TM miR isolation kit, then reverse transcribed, and analyzed by TaqMan or SyBR green based RT-qPCR. Endogenous miR/circRNA expressions of (A) *hsa-miR-381-3p*, (B) *hsa_circ_0021593*, (C) *hsa_circ_0038467*, (D) *hsa_circ_0084003* and (E) *hsa_circ_0099188* were measured 24 h after MDI-GSH conjugate treatments ($N=3$; bars, SEM). MDI: 4,4'-methylene diphenyl diisocyanate. GSH: Glutathione. (** $P<0.01$)

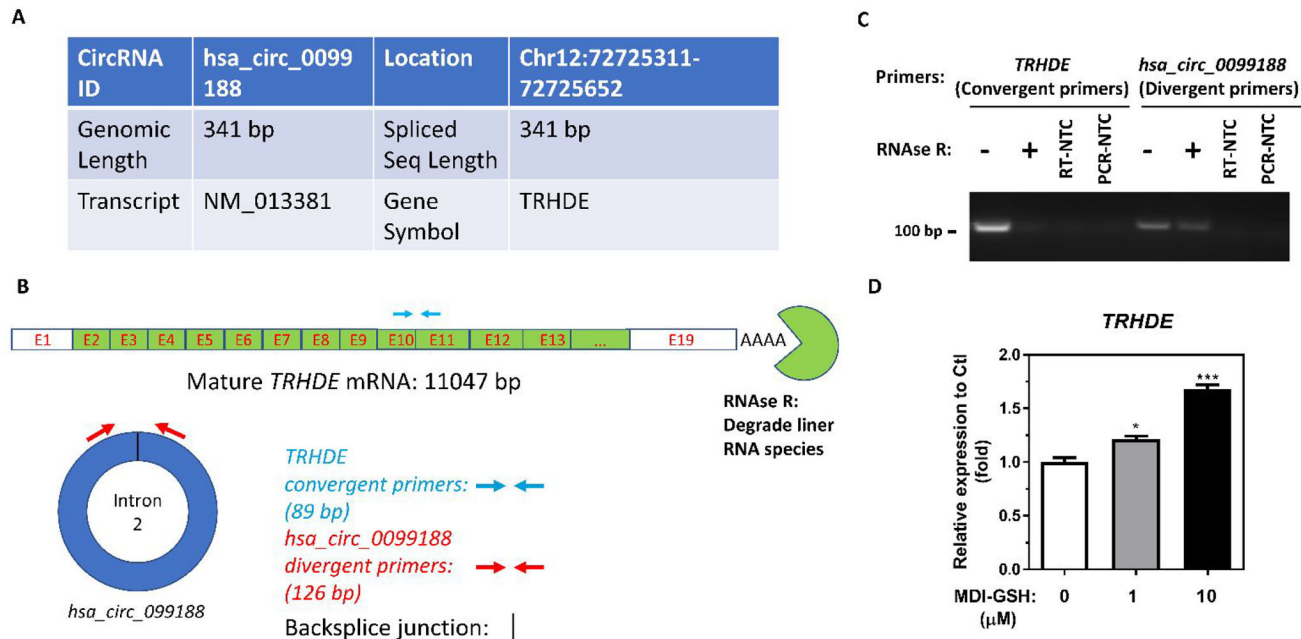


Figure 2. Circular RNA *hsa_circ_0099188* is presented in THP-1 macrophages and MDI-GSH conjugates upregulate endogenous *hsa_circ_0099188* parental host gene transcript *TRHDE*. (A) The information table of *hsa_circ_0099188* obtained from the circBase and Circular RNA Interactome database. (B) A diagram showing the exon numbers and the locations of the convergent and divergent primer sites on the mature *TRHDE* transcripts. The cartoon illustrates the degradation of linear RNA species, including the *TRHDE* transcript, by RNase R. It also shows that *hsa_circ_0099188* is backspliced from intron 2 of the *TRHDE* transcript. (C) Total RNA was isolated from THP-1 macrophages using *mirVana*TM miR isolation kit, treated with or without RNase R, further purified by *mirVana*TM miR isolation kit, reverse transcribed, and subjected to RT-PCR using convergent or divergent primers. RT-NTC lane using templates from cDNA synthesis reaction without adding reverse-transcriptase. PCR-NTC lane using only water to replace cDNA templates during PCR reaction. (D) Total RNA was isolated from differentiated/enhanced THP-1 macrophages treated with MDI-GSH at indicated concentration for 24 h, using *mirVana*TM miR isolation kit. The isolated RNA was reverse transcribed and analyzed by TaqMan RT-qPCR assays. Endogenous levels of *TRHDE* were measured at 24 h after MDI-GSH conjugate treatments ($N=3$; bars, SEM). MDI: 4,4'-methylene diphenyl diisocyanate. GSH: Glutathione (* $P<0.05$, *** $P<0.001$).

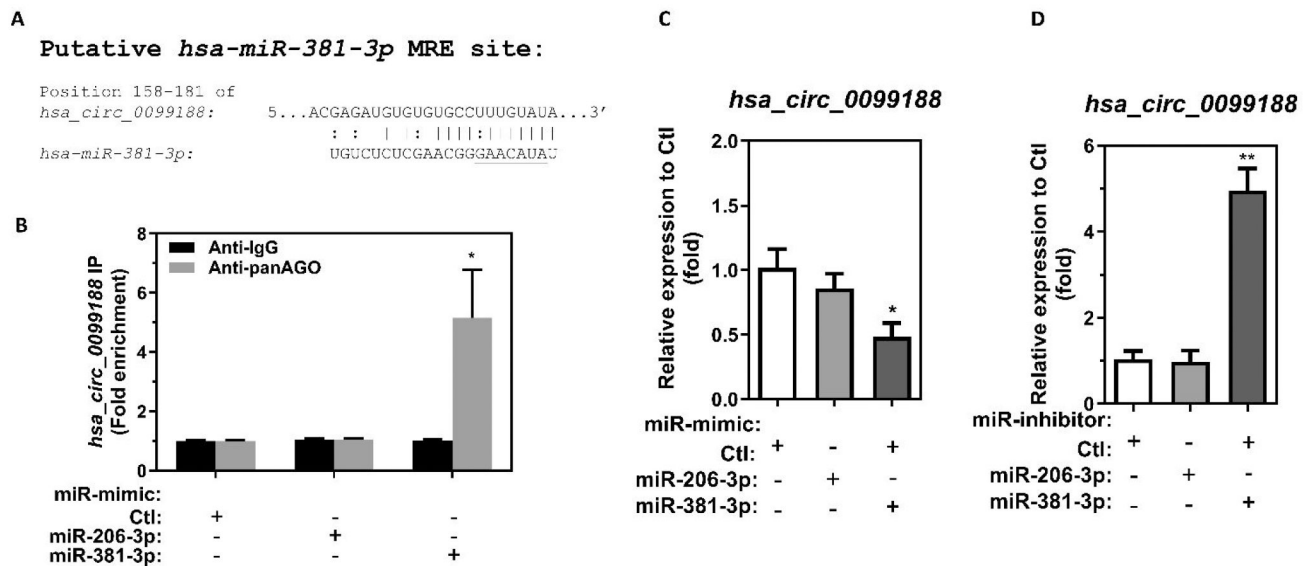


Figure 3. Human circular RNA *hsa_circ_0099188* is a target of *hsa-miR-381-3p*.

(A) Alignment of potential *hsa-miR-381-3p* binding sites with the *hsa_circ_0099188* sequence regions. (B) Differentiated/enhanced THP-1 macrophages were transfected with 25 nM of indicated miR-mimics or a non-targeting miR-mimic control (miR-mimic-Ctl) for 24 h. After 24 h, transfected cells were collected, lysed, and immunoprecipitated using either pan-AGO or isotype IgG antibodies. Total RNA was isolated and the fold enrichment of *hsa_circ_0099188* was measured by SYBR Green based RT-qPCR ($N=3$; bars, SEM). (C) Differentiated/enhanced THP-1 macrophages were transfected with 25 nM of either miR-mimic-206-3p, miR-mimic-381-3p, or non-targeting miR-mimic control (miR-mimic-Ctl) for 24 h. Total RNA was isolated using the *mirVana*TM miR isolation kit, reverse transcribed, and subjected to SYBR Green RT-qPCR to measure endogenous *hsa_circ_0099188* levels ($N=3$; bars, SEM). (D) Differentiated/enhanced THP-1 macrophages were transfected with 25 nM of miR inhibitors targeting *hsa-miR-206-3p* or *hsa-miR-381-3p* or non-targeting miR inhibitor control for 24 h. Total RNA was isolated and *hsa_circ_0099188* levels were determined using SYBR Green RT-qPCR assays ($N=3$; bars, SEM). (* $P<0.05$, ** $P<0.01$).

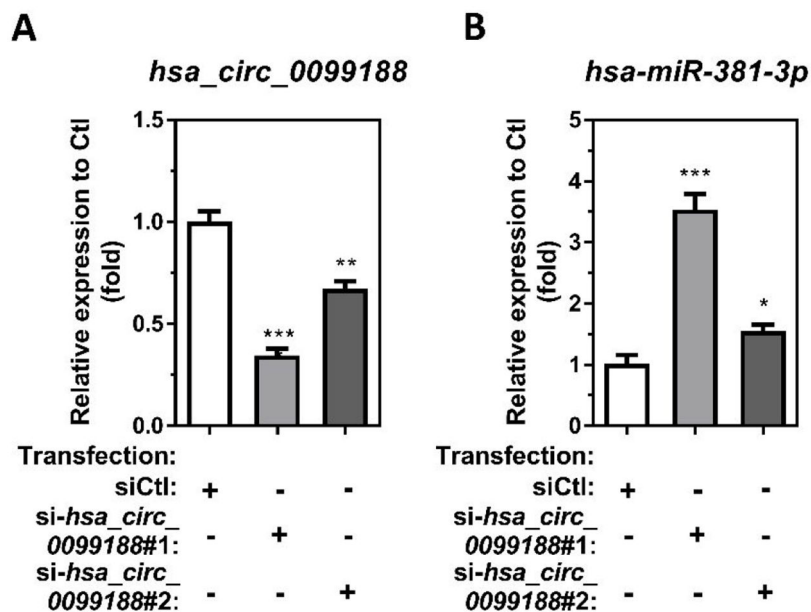


Figure 4. Transfection of *hsa_circ_0099188* siRNA knocks down endogenous *hsa_circ_0099188* levels while upregulating *hsa-miR-381-3p* in differentiated/enhanced THP-1 macrophages. Differentiated/enhanced THP-1 macrophages were transfected with 25 nM of either si-*hsa_circ_0099188*#1, si-*hsa_circ_0099188*#2 siRNA or nontargeting siRNA control (siCtl). After 24 h, the endogenous levels of (A) *hsa_circ_0099188*, and (B) *hsa-miR-381-3p* were measured by RT-qPCR ($N = 3$; bars, SEM). (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

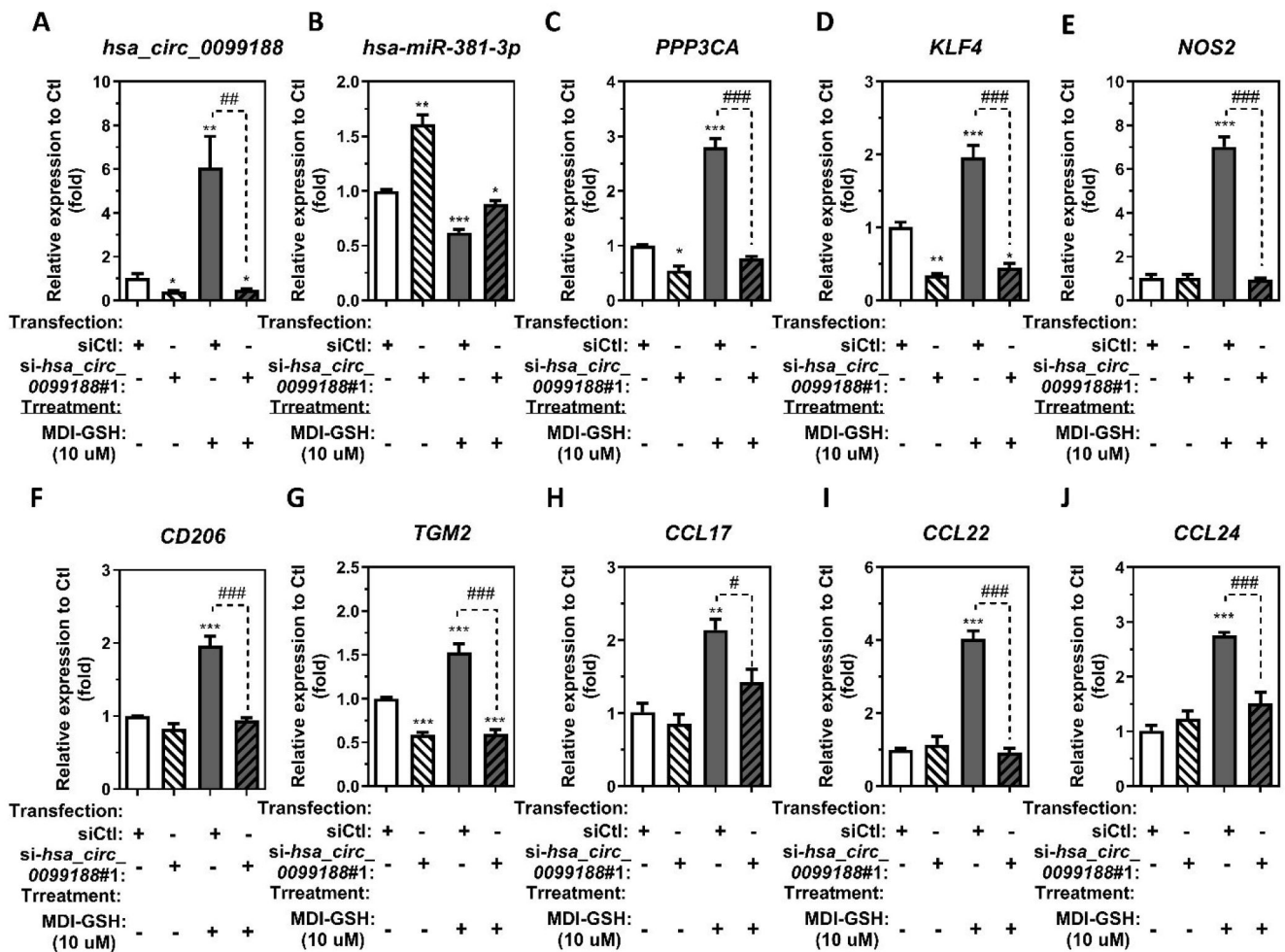


Figure 5. CircRNA *hsa_circ_0099188* as downstream effector to MDI/MDI-GSH exposure for regulating *hsa-miR-381-3p*/*PPP3CA*/*NOS2* and *hsa-miR-381-3p*/*KLF4* and *KLF4*-mediated M2 macrophage-associated markers and chemokines in macrophages.

Differentiated/enhanced THP-1 macrophages were transfected with 25 nM of either *si-hsa_circ_0099188#1* or nontargeting siRNA control (*siCtl*) for 24h followed by treating either with or without 10 μ M MDI-GSH conjugate for 24h. Total RNA were isolated from macrophages with indicated treatments/transfections by *mirVana*TM miR isolation kit, reverse transcribed, and subjected to SYBR green based or TaqMan stem-loop miR RT-qPCR. The endogenous levels of (A) *hsa_circ_0099188* and (B) *hsa-miR-381-3p* as well as the calcineurin catalytic subunit alpha gene (C) *PPP3CA* and calcineurin/NFAT signaling regulated (E) *NOS2*, M2 macrophage-associated transcription factor (D) *KLF4*, markers (F) *CD206*, (G) *TGM2*, (H) *CCL17*, (I) *CCL22*, and (J) *CCL24* mRNA levels were determined in total RNA isolated from macrophages as indicated treatments ($N=3$; bars, SEM). (* $P<0.05$, ** $P<0.01$, *** $P<0.001$ when compared to vehicle treated macrophages with transfection of or nontargeting siRNA control (*siCtl*); # $P<0.05$, ## $P<0.01$, ### $P<0.001$, when compared to macrophages treated with 10 μ M MDI-GSH conjugate as well as with transfection of indicated either *si-hsa_circ_0099188#1* or nontargeting siRNA control (*siCtl*)).

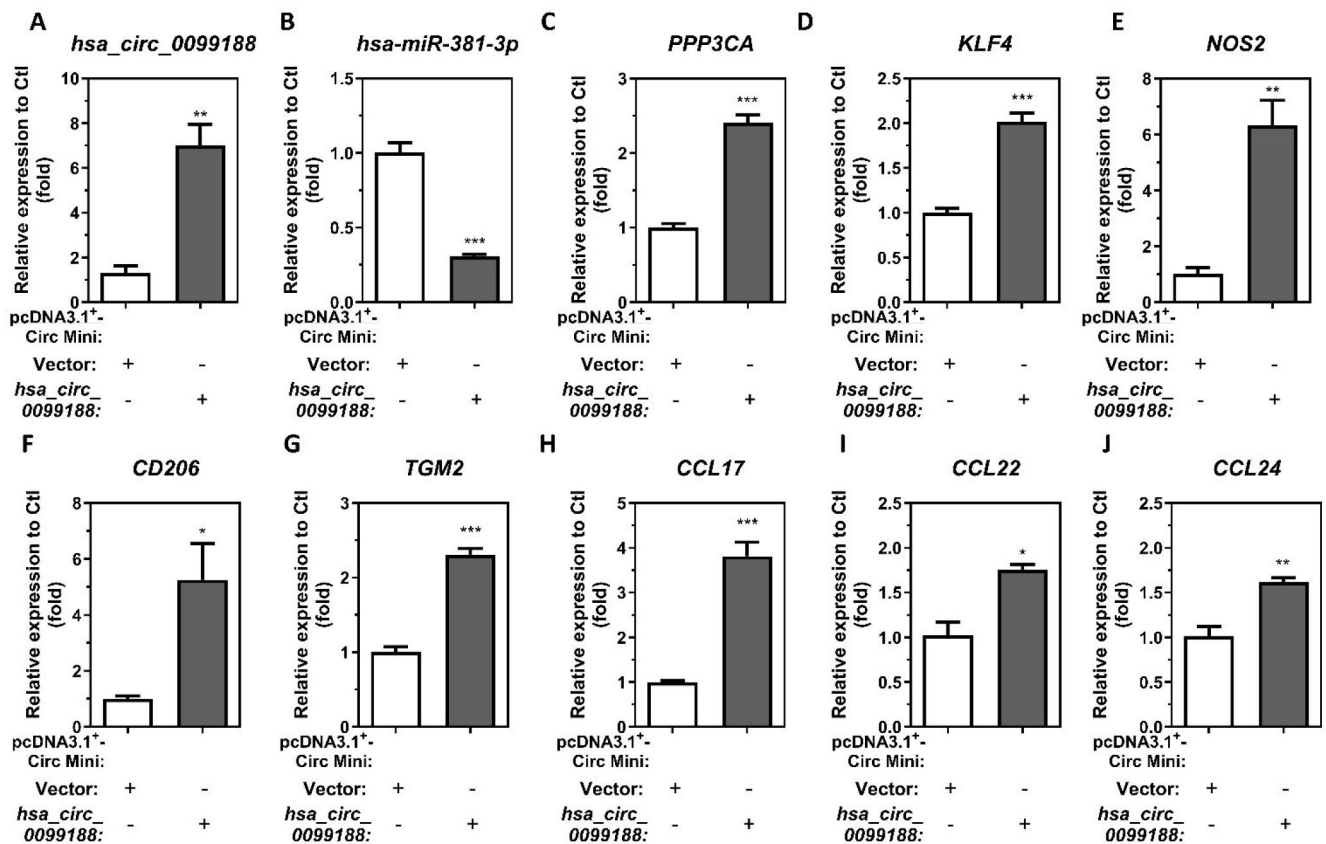


Figure 6. Overexpression of circular RNA *hsa_circ_0099188* led to the induction of *PPP3CA*, *NOS2*, as well as M2 macrophage-associated marker and chemokine transcripts in differentiated macrophages.

Differentiated/enhanced THP-1 macrophages were transfected with 2.5 µg of either pcDNA3.1⁽⁺⁾_Circ_Mini-*hsa_circ_0099188* or pcDNA3.1⁽⁺⁾_Circ_Mini vector plasmids for 48 h. Total RNA was isolated from plasmids transfected THP-1 macrophages using *mirVana*TM miR isolation kit, reverse transcribed, and analyzed by SYBR green or TaqMan RT-qPCR. The transgene of circRNA (A) *hsa_circ_0099188*, and endogenous miRs (B) *hsa-miR-381-3p* as well as the endogenous (C) *PPP3CA*, (E) *NOS2* and M2 macrophage-associated genes (D) *KLF4*, (F) *CD206*, (G) *TGM2*, (H) *CCL17* (I) *CCL22*, and (J) *CCL24* transcript levels were determined by RT-qPCR ($N=3$; bars, SEM). (* $P<0.05$, ** $P<0.01$, *** $P<0.001$).

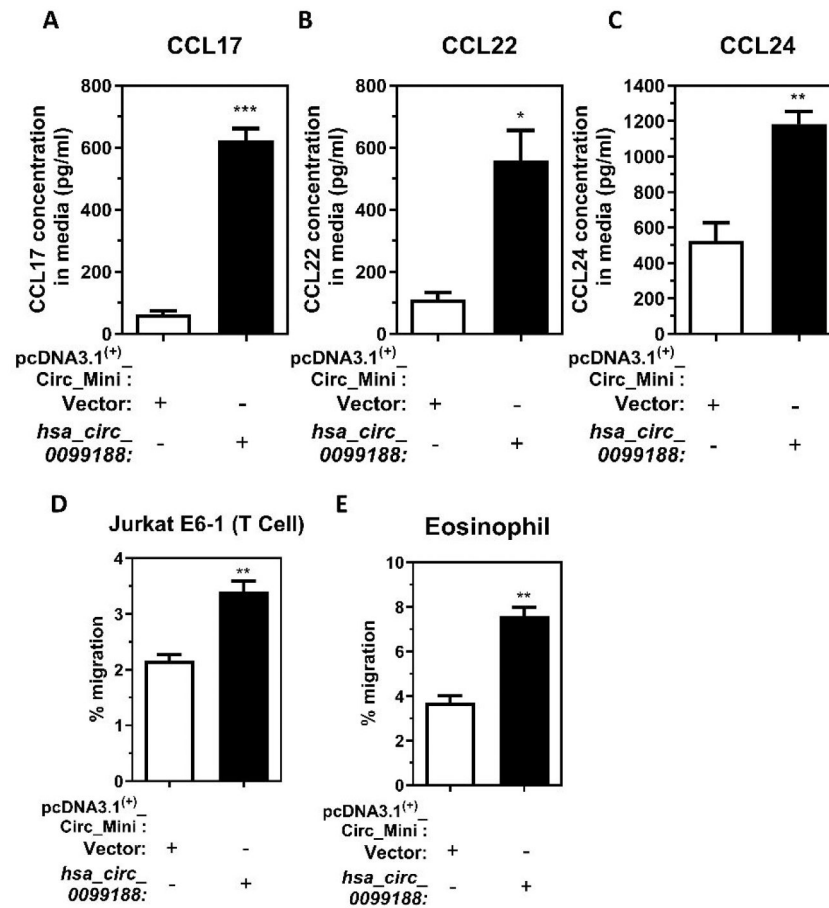


Figure 7. Role of circRNA *hsa_circ_0099188* in chemokine secretion and regulation of T-cell and eosinophil chemotaxis/migration in macrophages.

Cell-free conditioned media were collected from THP-1 macrophages transfected with either *hsa_circ_0099188* overexpression plasmid or the empty vector for 48 h. The levels of secreted proteins (A) CCL17, (B) CCL22, and (C) CCL24 in the conditioned media from *hsa_circ_0099188*-overexpressing or vector-transfected THP-1 macrophages were quantified by ELISA following the manufacturer's instructions. The conditioned media were used as chemoattractant to attract (D) Jurkat T-cells clone E6-1 or differentiated (E) HL-60 C₁₅ eosinophils. T-cell and eosinophil migration responding to the conditioned media was measured after 6 h. The percentage of cells migrating towards the bottom chamber is shown. (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

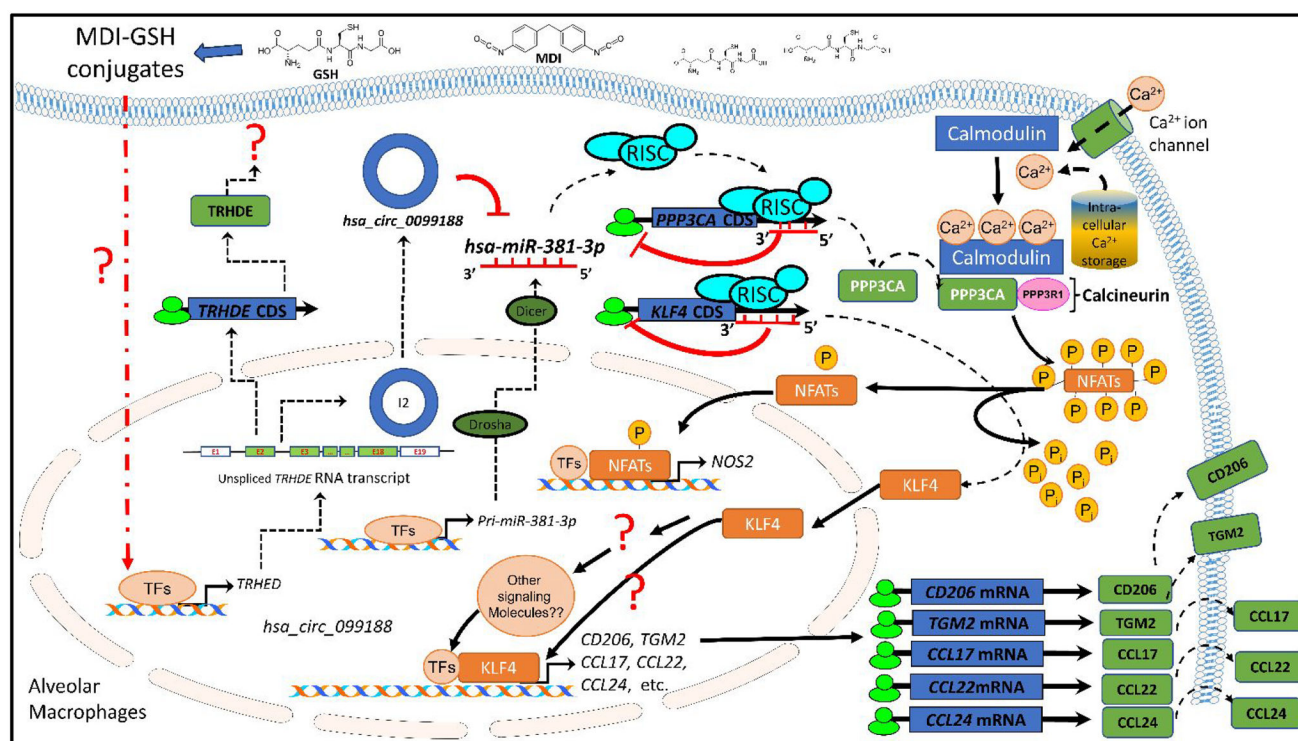


Figure 8. Proposed mechanisms by which MDI-GSH conjugate exposure induces NOS2 and M2 macrophage-associated markers and chemokine CCL17, CCL22, and CCL24, via *hsa_circ_0099188*/*hsa-miR-381-3p* regulated PPP3CA and KLF4 activation in macrophages. MDI: 4,4'-methylene diphenyl diisocyanate; TFs: transcription factors; CDS: coding sequences; PPP3CA: Protein phosphatase 3 catalytic subunit alpha; KLF4: Krüppel-like factor 4. Note. Some illustrated schematics were obtained from motifolio templates (Motifolio Inc., Ellicott City, MD, USA).