MATERIALS AND METHODS

Caution

4,4'-methylene diphenyl diisocyanate (MDI) is a strongly reactive, hazardous, irritating, and well-known immune sensitizing chemical. Appropriate personal protective equipment (PPE) such as nitrile gloves, protective clothing, and goggles must be utilized while handling MDI.

Chemicals and Reagents

HPLC grade acetone (CAS No. 67-64-1), 4,4'-methylene diphenyl diisocyanate (MDI, 98%) (CAS No. 101-68-8), 3Å molecular sieve (4–8 mesh), 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), 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 performed as previously described [1]. 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 acquired from American Type Culture Collection (ATCC; Manassas, VA) and maintained at 0.5 - 1 × 10^6/ml in complete RPMI media (RPMI-1640 media supplement, 10% FBS, 1 × PSG) at 37°C in a humidified atmosphere with 5% CO_2 [1]. 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. Differentiation by PMA at a concentration of 10 ng/ml has been shown to enhance response to polarizing stimuli [2,3]. 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 [4-6].

MDI-GSH conjugation reactions

MDI-GSH conjugate was prepared as previously described [1,7]. 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, resulting in an approximate MDI concentration of 800 µM. MDI-GSH conjugates 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. Freshly prepared MDI-GSH conjugate was immediately added into differentiated/enhanced THP-1 macrophages at 0, 1, and 10 µM as indicated or at 10 µM MDI concentrations.

Plasmid construction

Plasmid constructed in current study was similarly performed as our previous reports [7,8]. The circular RNA overexpression vector pcDNA3.1$^{(+)}$_CircRNA_Mini Vector was obtained from Addgene (plasmid id#60648; Watertown, MA) which was deposited by Dr. Jeremy Wilusz at Baylor College of Medicine [9]. To generate a wildtype (WT) *hsa_circ_0008726* overexpression plasmid, a 0.28-kb cDNA fragment representing the full length of human circular RNA *hsa_circ_0008726* was generated by PCR using a *PacI* restriction site containing forward primer (cccttaattaaATATCGGAAACTGGCACTG) and a *SacII* restriction site containing reverse primer (cccccgcggCCCATTTTCATTTGACTTC) on THP-1 cell cDNA. The PCR amplified *hsa_circ_0008726* cDNA fragment was treated with *PacI* and *SacII*. 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_0008726* overexpression plasmid.

Plasmid, microRNA mimics/inhibitors and siRNA transfection

Nucleic acid transfections were performed as previously described [1,7,10]. 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_0008726*, or pcDNA3.1$^{(+)}$_CircRNA_Mini Vector using Mirus *Trans*IT®-2020 transfection reagent for 48 h, after which total RNA was 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_0008726*, 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 (4464076), and *mirVana*™ miRNA Mimic, negative control #1 (4464058). For circRNA *hsa_circ_0008726* knockdown studies, two Dharmacon custom siRNAs to *hsa_circ_0008726* and ON-TARGETplus Non-targeting Control Pool (Cat#

D-001810-10-05) were purchased from Horizon Discovery (Lafayette, CO). The custom designed siRNA sequences are as follows: si-*hsa_circ_0008726*#1 (Sense): 5'-ACUUCUUUGAUAUCGGAAACUUU-3'; si-*hsa_circ_0008726*#1 (Antisense) 5'-AGUUUCCGAUAUCAAAGAAGUUU-3'; si-*hsa_circ_0008726*#2 (Sense): 5'-UUUGACUUCUUUGAUAUCGGAUU-3'; si-*hsa_circ_0008726*#2 (Antisense): 5'-UCCGAUAUCAAAGAAGUCAAAUU-3'. As previously described, differentiated/enhanced THP-1 macrophages were reverse transfected and forward transfected after 24 hours [11]. Total RNA was prepared for RT-qPCR expression analyses 24 hours following forward transfection.

Expression analyses

To determine whether endogenous circRNA *hsa_circ_0008726* is expressed within differentiated/enhanced THP-1 macrophages, total RNA was extracted from THP-1 macrophages using *mirVana*™ miR Isolation Kit (ThermoFisher Scientific). To deplete linear RNA species in isolated THP-1 total RNA, 3 µg of purified THP-1 total RNA was treated with 20 U Ribonuclease R (RNase R; LGC Biosearch Technologies, Teddington, UK). Briefly, a 20 µl reaction mix containing 3 µg of total RNA, 20 U of RNase inhibitor (Cat# N8080119; ThermoFisher Scientific), 1 × RNase R buffer and 20 U of RNase R and incubated for 30 min at 37°C. Control reactions were performed similarly, but without RNase R. After RNAase R treatment, the processed RNA was further purified by using *mirVana*™ miR Isolation Kit (ThermoFisher Scientific) according to manufacturer's instructions. RNase R protein was removed during the RNA isolation step using the *mirVana*™ miR Isolation Kit, which eliminates all protein from the sample. Total RNA with or without RNAse R treatment was reverse transcribed to cDNA using a High-Capacity cDNA Synthesis Kit (Thermo Fisher Scientific) according to manufacturer's instructions. PCR reactions were conducted using either *DNAJB6* convergent primers (*DNAJB6*-F: TAAAGTCCTTAACAATAAATG; *DNAJB6*-R: GAGGCCGGCAGGCTGGGCTGGC) or circRNA *hsa_circ_0008726* divergent primers (see Supplemental Table 1).

The mRNA, circRNA and miR levels from THP-1 total RNA were analyzed using RT-qPCR similar as previously described [1,7,8,10-12]. 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. Gene expression and miR specific assays were purchased from ThermoFisher Scientific and include human *KLF4* (Cat#4331182; Assay ID: Hs00358836_m1), *DNAJB6* (Hs00369717_m1), *CD206* (Hs00267207_m1), *TGM2* (Hs01096681_m1), *CCL17* (Hs00171074_m1), *CCL22* (Hs01574247_m1), *CCL24* (Hs00171082_m1), and *B2M* (Hs00187842_m1), *hsa-miR-206-3p* (Cat# 4427975; Assay ID No. 000510; *hsa*/*Homo sapiens*), and *U6* snRNA (No. 001973). For circRNA assays, SYBR Green-based qPCR

reactions were performed using PowerTrack™ SYBR Green Master Mix from ThermoFisher Scientific (Cat#A46110) with divergent primers 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 performed using an ABI 7500 Real-Time PCR System (ThermoFisher Scientific, Waltham, MA, USA). Expression of mRNAs and miRs were determined using the $^{\Delta\Delta}$CT method [7].

Validation of miR-circRNA interaction by Argonaute (AGO) immunoprecipitation

The miR-containing RNA inducing silencing complex (miR/RISC) and miR targeting circRNA(s) were immunoprecipitated using the miRNA target IP kit (Active Motif, Carlsbad, CA) as previously described [1,7,10]. Briefly, differentiated/enhanced THP-1 macrophages were trypsinized and seeded at 8 × 10^6 cells in a 10-cm dish. 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. Two 10-cm dishes using an equal number of 1.6 × 10^7 cells were utilized for each IP reaction. Cells were lysed and the lysates were divided into two equal aliquots. Aliquots 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 purified from the RISC complex using the *mirVana*™ miR Isolation Kit (ThermoFisher Scientific). RNA was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific) and *hsa_circ_0008726* divergent primer was mixed 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_0008726* was calculated from the anti-panAGO and the IgG isotype antibody IP preparations as per manufacturer's instructions.

Chemokine ELISA

Secreted CCL17, CCL22 and CCL24 protein concentrations in conditioned media were determined as previously described [10]. Briefly, conditioned media was collected 48 h following THP-1 macrophage transfection with 2.5 μg of either pcDNA3.1$^{(+)}$_CircRNA_Mini-*hsa_circ_0008726* 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 for each chemokine is as follows: CCL17 (7.8 pg/ml), CCL22 (7.8 pg/ml), and CCL24 (31.2

pg/ml). Human CCL17, CCL22, and CCL24 chemokines released into the conditioned media from plasmid transfected THP-1 macrophages were determined by ELISA per 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_0008726* overexpression plasmid or pcDNA3.1$^{(+)}$_CircRNA_Mini vector only were performed as previously described [1,10]. Chemotaxis/cell migration assays were performed on a 24-well plate with Transwell™ inserts (3 µm pore, Corning™ Transwell™ plates, ThermoFisher Scientific). 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 the upper chamber. 500 µL of cell-free conditioned media from either pcDNA3.1$^{(+)}$_CircRNA_Mini-*hsa_circ_0008726* overexpression plasmid or pcDNA3.1$^{(+)}$_CircRNA_Mini vector plasmid transfected THP-1 macrophages were placed in the lower chamber as chemoattractant. Immune cells were incubated for 6 h at 37 °C in a humidified atmosphere with 5% CO_2. The 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 assembly was washed 2x with PBS. 500 µl cell detaching media (0.25% Trypsin-EDTA, Cat#25200056, ThermoFisher Scientific) were added back to the lower chambers with the upper chambers reinstalled. The whole plate was further incubated at 37 °C for 30 min to detach any remaining migrated cells. After 30 min, the detached cells were combined with the conditioned media/migrated cells previously collected, centrifuged at 300 × g for 5 min, washed with PBS twice, and stored at −80 °C. Total migrated cell numbers in the lower chamber were quantified by using CyQUANT® Cell proliferation assay (ThermoFisher Scientific) according to manufacturer's directions.

References:

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Supplemental Table 1. Candidate *hsa-miR-206-3p* binding circRNA divergent primer sequences.

Candidate circRNA	Forward	Reverse	Host Gene	Host gene Accession #
hsa_circ_0000199	ATCATTGCTTTCAGGGCTCT	CACCCGCTCTCTCGACAAAT	*AKT3*	NM_181690
hsa_circ_0001264	CAGCTCATTAAAAGGCACCA	GGAAGAAGCAGGAGATTTGG	*RAD18*	NM_020165
hsa_circ_0001982	CAATCCAGCTGTTCCCTCAG	TGGTGCATCAGAAGGAATCTC	*RNF111*	NM_017610
hsa_circ_0004662	GTGTGGGAGCACGCTTACTA	CGTTAGGGCTGAGGTTTGTC	*SOD2*	NM_001024465
hsa_circ_0007428	GCAGGAGAGGGTAGTTGTGC	TCCCCAAGTACCAAGTGCAT	*SH3BP4*	NM_014521
hsa_circ_0008726	GGTGGAAGGGACCCATTTTC	TGCCTCCGCTACTTGCTTG	*DNAJB6*	NM_058246
hsa_circ_0056618	GAACCCACCCCACCTCTAC	CTTCCCCGGGATAAACAAACC	*SPOPL*	NM_001001664
hsa_circ_0057558	AGTCACTGCAGGCATGTTC	TGCAACAAGGAATGTAAGA	*SLC39A10*	NM_001127257
hsa_circ_0058141	GCCCTCAATTCATTCCAATG	TGAGGCTCTCTTCTTCATCA	*FN1*	NM_212482
hsa_circ_0072088	ACGCATTCTTCGAGACCTCT	TGCCTGTAACTCCTCTTCAGT	*ZFR*	NM_016107