Supplemental Material

Methods and Materials

CRISPR/Cas9 to ablate the miR-206 binding site within the 3'UTR of *Klf4* in the genome of KCs of mice

The locations of sgRNA pairs were selected at the boundary of the miR-206 binding site within the 3'UTR of *Klf4* (kruppel like factor 4). Two sgRNAs were designed by CRISPOR and synthesized in IDT (Coralville, IA).[1] The sgRNAs were further cloned into a pX601-AAV8-CMV-SaCas9 (Addgene, Watertown, MA) backbone, termed AAV8-SaCas9-sgRNA. The AAV8 viruses were packaged and tittered in the Viral Vector Core of the University of Minnesota. AAV virus was delivered to mice intravenously via lateral tail vein injection. To ablate the miR-206 binding site within the 3'UTR of *Klf4* in the genome of KCs of AKT/Ras mice, Group I mice (*n*=6) received 4 µg pT3-EF1α-myr-AKT, 4 µg NRasV12/pT2-CAGGS, 10 µg pT3-CD68p-scramble and 0.72 µg pCMV/SB; Group II mice (*n*=6) received 4 µg pT3-EF1α-myr-AKT, 4 µg NRasV12/pT2-CAGGS, 10 µg pT3-CD68p-miR-206, 0.72 µg pCMV/SB, and 5×10^{11} GC AAV8-control viruses; and Group III mice (*n*=6) received 4 µg pT3-EF1α-myr-AKT, 4 µg NRasV12/pT2-CAGGS, 10 µg pT3-CD68p-miR-206, 0.72 µg pCMV/SB, and 5×10^{11} GC AAV8-SaCas9-sgRNA viruses. Eight weeks post-injection, mice were sacrificed.

Identification of miR-206 Targets.

miRNAs inhibit gene expression by binding to their 3'UTRs. However, miR-206 significantly induced expression of *Ccl2* in KCs. We, therefore, speculated that miR-206 promoted expression of *Ccl2* by an unknown dual inhibitory mechanism by which miR-206 promoted transcription of

Ccl2 by directly targeting a transcription repressor that has a binding site within the promoter of *Ccl2*. Since we intended to explore how miR-206 induces *Ccl2* in KCs, we first identified genes that were specifically expressed in macrophages versus hepatocytes. Such an analysis identified 341 genes that were abundantly and specifically expressed in macrophages (**Supplemental Table 3**). To identify the genes whose 3'UTRs contain miR-206 binding motifs, we downloaded the target gene databases of miR-206 based on PITA, PICTAR, and Starbase (HITS-CLIP database).[2-4] Only hits from PITA and PICTAR software that were confirmed in the database of Ago HITS-CLIP (high-throughput sequencing of RNAs isolated by crosslinking immunoprecipitation from Argonaute protein complex) were selected. Microsoft Access 2000 was used to compare predicted targets for miR-206 with 341 genes that are highly and specifically expressed in macrophages. Only those genes that contained miR-206 binding sites and were highly and specifically expressed in macrophages were selected for further analysis, yielding seven potential targets of miR-206 (**Supplemental Table 4**). Geno Ontology analysis revealed that only KLF4 functions as a transcription repressor, leading us to focus on *Klf4*.

H&E Staining. Livers were fixed in 10% Formalin (Thermo Fisher Scientific, Agawam, CA) and examined using 4 μm thick, serial sections stained with hematoxylin and eosin which was carried out in the Comprehensive Cancer Center at the University of Minnesota. Images were captured on Panoptiq Digital Slide Imaging System.

Cell Lines and Culture. RAW264.7 cells were purchased from ATCC (TIB-71) and cultured in DMEM (Gibco, Agawam, CA) supplemented with penicillin/streptomycin (Gibco) and 10% fetal bovine serum (FBS) (Invitrogen) at 37 °C in a 5% CO_2 atmosphere. THP-1 cells were purchased from ATCC (TIB-202) and cultured in RPMI-1640 (Gibco) supplemented with 50 μM 2-mercaptoethanol and 10% FBS. $CD8^+$ T cells were isolated from mouse spleen using EasySep

Mouse $CD8^+$ T Cell Isolation Kit per the manufacture's instructions (Stemcell, Cambridge, MA) and cultured in DMEM supplemented with penicillin/streptomycin, 10% FBS, anti-CD3 antibody (1 µg/ml, Biolegend), anti-CD28 antibody (1 µg/mL, Biolegend) and IL-2 (20 ng/mL, Stemcell).

Hepatocyte and Kupffer Cell Isolations. Mouse livers were enzymatically digested and hepatocytes and Kupffer cells (KCs) were isolated by Percoll (GE Healthcare, Maple Grove, MN) density gradient centrifugation. M1 and M2 state KCs were separated using a MACS-Based Method.[5] Briefly, dissected livers were washed with cold HBSS and teared into pieces with forceps, and then incubated with Digestion Buffer (10 mL Collagenase IV (1 mg/mL) and 5 µL DNase I (100 U/µl)) at 37 °C for 30 minutes. The cells were poured through 70 µm sterile nylon filter and centrifuged at 50 g for 5 minutes at 4 °C to collect hepatocytes. Cell supernatants were then centrifuged at 450 g for 8 minutes at 4 °C, washed twice with DMEM and treated with red blood lysis buffer for 5 minutes. After centrifugation at 400 g for 5 minutes at 4 °C, the cells were subjected to Percoll density gradient centrifugation as previously described.[6] Isolated KCs were further separated into M1 and M2 populations. KCs were incubated with APC-conjugated iNOS antibody (M1) (eBioscience) or APC-conjugated CD206 antibody (M2) (Biolegend) at 4 °C for 15 minutes. After the cells were washed twice, they were further incubated with anti-APC MicroBeads at 4 °C for 15 minutes. Cells were washed twice and subjected to the magnetic column separation.

Cell Transfection. RAW264.7 cells were cultured in DMEM with 10% FBS, and THP-1 cells were cultured in RPMI-1640 (Gibco) supplemented with 50 µM 2-mercaptoethanol, 20 ng/ml PMA (Phorbol 12-myristate 13-acetate, Sigma, St. Louis, MO) and 10% FBS. IFNγ (20 ng/ml, R&D systems, Minneapolis, MN) and LPS (10 ng/ml, Sigma) were added to the medium for 24

hours to induce M1 state, and IL-4 (20 ng/ml, R&D systems) and IL-13 (20 ng/ml, R&D systems) were used for M2 state activation. 5×10^4 of RAW264.7 or THP-1 cells were seeded in a 24-well plate and allowed to adhere overnight. 24 hours later, the cells were transfected with pT3-CD68-scramble (Control), pT3-CD68p-miR-206 (miR-206), or pT3-CD68p-miR-206 and AAV8-SaCas9-sgRNAs using jetPEI-Macrophage DNA transfection reagent (Polyplus transfection, New York, NY) or Lipofectamine LTX with Plus Reagent (Invitrogen). Forty-eight hours post-transfection, cells were washed with cold PBS and collected for further analysis.

Immunofluorescence Staining. Liver samples were embedded in Tissue-Tek OCT embedding compound, and quickly frozen on dry ice. 6 µm-thick sections were cut with a Leica CM3050 S cryostat, air-dried, and fixed in 10% formalin (Thermo Fisher Scientific, Agawam, MA). Raw264.7 and THP-1 cells were plated on slides and fixed with cold acetone. Liver tissue and cell slides were blocked with PBS supplemented with 10% goat normal serum (Thermo Fisher Scientific, Agawam, MA) and 0.3% Triton X-100 at room temperature for 1 hour. After washing with PBST buffer (PBS with 0.1% Tween 20), slides were incubated with primary antibodies (CD8: Biolegend, San Diego, CA; iNOS: Abcam, Cambridge, MA; CD206: Bio-RAD, Hercules, CA; CLEC4F: R&D systems, Minneapolis, MN; CCL2: Novus Biologicals, Centennial CO; DC-SIGN: R&D systems; AKT PHOSPHO S473: Rockland, Limerick, PA) at 4 °C overnight. The slides were then washed for 4 times and incubated with fluorescence-conjugated secondary antibodies (Alexa Fluor 594 Goat anti-Mouse IgG (H+L), Alexa Fluor 594 Goat anti-Rat IgG (H+L) and Alexa Fluor 647 Donkey anti-Goat IgG (H+L), Invitrogen) at room temperature for 1 hour, and then covered with ProLong Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific, Agawam, MA). Images were captured by Motorized Zeiss Axio Observer Z1 inverted scope with 40 x magnification in the immersion medium of air at room temperature. The images

were organized by Zen 3.2 software and processed by gramma adjustment. The ratios of iNOS$^+$ or CD206$^+$ to CLEC4F$^+$ staining were calculated according to the Image J quantification.

Flow Cytometry Analysis of iNOS Staining. RAW264.7 and THP-1 cells were collected and stained with APC-conjugated anti-iNOS antibody (eBioscience, Agawam, MA). Briefly, RAW264.7 and THP-1 cell suspension was washed with 1× Permeabilization Buffer (eBioscience, Agawam, MA) and centrifuged at 450 g for 5 minutes at room temperature. Cell pellets were further suspended in 100 µL of 1× Permeabilization Buffer with anti-mouse CD16/CD32 antibody (BD pharmingen, San Diedo, CA). After incubation at 4°C for 10 minutes, cells were washed twice and stained with conjugated antibodies at 4°C for 30 minutes. Stained cells were then suspended in an appropriate volume of PBS and subjected to flow cytometry analyzer.

Flow cytometry analysis of liver lymphocytes. Liver cells were collected and stained with antibodies to CD3 (Biolegend, San Diedo, CA), CD4 (BD pharmingen, San Diedo, CA), CD8 (Biolegend), CD25 (Biolegend), FoxP3 (Biolegend) and Granzyme B (Biolegend). Briefly, cell suspensions were washed with 1× Permeabilization Buffer (eBioscience, Agawam, MA) and centrifuged at 450 x g for 5 minutes at room temperature. Cell pellets were further suspended in 100 µL of 1× Permeabilization Buffer with anti-mouse CD16/CD32 antibody (BD pharmingen, San Diedo, CA). After incubation at 4°C for 10 minutes, cells were washed twice and stained with conjugated antibodies at 4°C for 30 minutes. Stained cells were then suspended in an appropriate volume of PBS and subjected to flow cytometry. For liver lymphocytes suspension preparation, dissected livers were digested in the Digestion Buffer (10 mL Collagenase IV (1 mg/mL) and 5 µL DNase I (100 U/µL)) at 37 °C for 30 minutes. The cells were poured through 70 µm sterile nylon filter and centrifuged at 50 g for 5 minutes at 4 °C to remove hepatocytes.

The supernatant was centrifuged at 400 g for 5 minutes at 4 °C, washed for three times with 2% FBS PBS buffer, and then subjected to flow cytometry analysis. Levels of oxidative stress in livers was detected by CellROX™ Deep Red Flow Cytometry Assay Kit (Thermo Fisher scientific).

LPS Treatment with RAW264.7 cells. pT3-KLF4 and pT3-p65 expression vectors were generated by insert KLF4 coding region into pT3-EF1α. To determine whether KLF4 is a repressor of M1 polarization of RAW264.7 cells, RAW264.7 cells were seeded in 1 mL Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin in each well of a 12-well cell culture plate. 24 hours later, RAW264.7 cells were transfected with pT3-EF1a, pT3-KLF4 or a combination of pT3-KLF4 and pT3-p65. After 24 hours of transfection, the cells were then washed with PBS before adding 1 mL serum-free DMEM to each well. The cells were treated with 10 ng/mL lipopolysaccharide (LPS) (Sigma) for 24 hours at 37°C.

Reporter Vector Construction and Luciferase Assay. To generate the luciferase reporter 3'UTR vectors, the *Klf4* 3'UTR was amplified from mouse cDNA using PCR, and inserted into the pMiR-Reporter vector (Ambion, Agawam, CA), referred as pMiR-Klf4. Two bases of the binding sites for miR-206 within the 3'UTR of *Klf4* was mutated using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies, Santa Clara, CA) per the manufacture's instructions, and referred to as pMiR-Mu-Klf4. Luciferase Assay were performed as previously described[7].

CFSE Staining. $CD8^+$ T cells were stained with carboxyfluorescein succinimidyl ester (CFSE) (Sigma, St. Louis, MO) and subjected to flow cytometry analysis. Briefly, splenic $CD8^+$ T cells

were washed twice with DMEM, and then incubated with CFSE (200 nM) at 37°C for 10 minutes. After quenching CFSE via two volumes of ice cold FBS, T cells were washed twice with cold DMEM, and then cultured at 37°C with 5% CO_2 for 2 days. After that, cells were suspended in an appropriate volume of PBS and subjected to flow cytometry analysis.

***Chemotaxis Assay of CD8$^+$ T Cells*.** Three groups of RAW264.7 or THP-1 cells were transfected with pT3-CD68p-scramble (Control), pT3-CD68p-miR-206 or a combination of pT3-CD68p-miR-206 and the sgRNAs used to ablate the miR-206 binding site within 3'UTR of *klf4*. In the bottom of 24-well plate, RAW264.7 or THP-1 cells were seeded at 1×10^5 per well in 500 µL DMEM medium in the absence or presence of anti-CCL2 (10 µg/mL, R&D System, Cat. AF-479-NA) or 10 nM CCR2 antagonist (Sigma, St. Louis, MO, CAS 445479-97-0). IgG1 isotype served as a control. 2×10^5 splenic CD8$^+$ T cells were seeded in 100 µL medium in the upper 8.0-µm pore size insert (Corning, New York, NY). After four hours of incubation at 37 °C in a 5% CO_2 atmosphere, the upper insert was removed, the number of CD8$^+$ T cells that had migrated into the bottom plate was counted under the microscope, and the migration ratio calculated.

RNA Isolation and Quantitative Reverse Transcription-PCR (qRT-PCR). Total RNA was isolated with Monarch Total RNA Miniprep Kit (Biolabs, Boston, MA). Briefly, 1 µg RNA was used for cDNA synthesis with Superscript III reverse transcription reagent (Invitrogen, Carlsbad, CA) for assessing gene expression; and 50 ng RNA was used for miRNA-specific cDNA synthesis with the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) for detecting miRNA expression. PCR amplification was performed as previously described.[7] Primers for qRT-PCR were designed with Primer Express software (Applied Biosystems). Relative changes in gene and miRNA expression were determined using the $2^{-\Delta\Delta Ct}$ method.

Western Blots and Antibodies. Proteins were extracted from cells and tissues using RIPA buffer (Cell Signaling Technology, Beverly, MA) with proteases inhibitors (Roche, Indianapolis, IN, USA). Protein concentration was measured using Pierce BCA Protein Assay Kit and 25~50 μg of total lysate was loaded and immunoblotted. KLF4 antibody (Cat# sc-166238) was purchased from Santa Cruz; CCL2 antibody (AF-479-NA) was purchased from R&D System and Anti-β-actin (NB600-501) was purchased from Novus Biologicals.

ELISA Assay. Quantitative measurement of TNFα, IL-6, IL-10, CCL2 and TGFβ in cell culture medium or mice serum was performed following standard protocols. Mouse TNFα Immunoassay was purchased by R&D systems (Minneapolis, MN), CCL2 ELISA Kit was purchased from PromoCell (Heidelberg, Germany), IL-10 Mouse Instant ELISA Kit and Mouse IL-6 ELISA Kit were purchased from Invitrogen, and Mouse TGFβELISA Kits were purchased from Abcam.

References

1. Concordet J-P, Haeussler M. CRISPOR: intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. *Nucleic Acids Res.* 2018;46:W242-W245.
2. Yang J-H, Li J-H, Shao P, et al. starBase: a database for exploring microRNA–mRNA interaction maps from Argonaute CLIP-Seq and Degradome-Seq data. *Nucleic Acids Res.* 2011;39:D202-D209.
3. Kertesz M, Iovino N, Unnerstall U, et al. The role of site accessibility in microRNA target recognition. *Nat. Genet.* 2007;39:1278-1284.
4. Krek A, Grün D, Poy MN, et al. Combinatorial microRNA target predictions. *Nat. Genet.* 2005;37:495-500.
5. Zhang C, Lu Y, Zhou H, et al. Acquiring Kupffer cells in mice using a MACS-based method. *Transplant. Proc.* 2015;47:553-7.
6. Zhang T, Hu J, Wang X, et al. MicroRNA-378 promotes hepatic inflammation and fibrosis via modulation of the NF-κB-TNFα pathway. *J. Hepatol.* 2019;70:87-96.
7. Wu H, Tao J, Li X, et al. MicroRNA-206 prevents the pathogenesis of hepatocellular carcinoma by modulating expression of met proto-oncogene and cyclin-dependent kinase 6 in mice. *Hepatology* 2017;66:1952-1967.