Materials and methods

Generation of MED1$^{\Delta Mac}$ mice

Mice with macrophage-specific MED1 knockout were generated by using the Cre-loxP recombination system. Mice homozygous for the floxed MED1 allele (MED1$^{fl/fl}$)[1] were bred with a mouse containing the Lyz2-Cre transgene on the C57BL/6 background; genotypes were assessed by RT-PCR (Supplemental Figure IA). Mice with macrophage-specific MED1 knockout (Lyz2$^{Cre/+}$/MED1$^{fl/fl}$, designated MED1$^{\Delta Mac}$) were viable and fertile, with no notable differences from MED1$^{fl/fl}$ littermates in body weight or plasma lipid levels. The mRNA level of MED1 was greatly reduced in MED1$^{\Delta Mac}$ peritoneal macrophages (PMs) as compared with MED1$^{fl/fl}$ macrophages, as shown by semi-quantitative PCR (Supplemental Figure IB) or quantitative real-time PCR (RT-qPCR) (Supplemental Figure IC). MED1 mRNA was detected in kidney, adipose, or liver tissue but not peritoneal macrophages from MED1$^{\Delta Mac}$ mice (data not shown). Furthermore, immunostaining confirmed the lack of MED1 in the nuclei of macrophages from MED1$^{\Delta Mac}$ mice (Supplemental Figure ID).

Animals

Mice with "floxed" MED1 (MED1$^{fl/fl}$) were described previously.[1] Lyz2-Cre transgenic, ApoE$^{-/-}$ and LDLR$^{-/-}$ mice in the C57/BL6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in a pathogen-free facility (12-hr light/dark cycle) and fed a standard rodent chow or a Western diet consisting of 21% fat and 0.15% cholesterol and water *ad libitum*. Animal care and experimental procedures were approved by the Animal Care and Use Committee of Xi'an Jiaotong University.

Bone marrow transplantation

Male LDLR$^{-/-}$ mice 8 weeks old (n=8 per group) were lethally irradiated with a single 9-Gy radiation dose from a cesium gamma source. Bone marrow from male MED1$^{\Delta Mac}$ mice or MED1$^{fl/fl}$ littermates was harvested by flushing femurs and tibias. Irradiated recipients received 5×10^6 bone-marrow cells by intravenous injection in the tail vein. At 6 weeks after transplantation, bone-marrow–transplanted mice were fed a Western diet for 12 weeks.

Plasma lipid and intracellular cholesterol measurement

Mice were fasted for 4 hr and blood was collected from tail veins in heparinized capillary tubes. Plasma levels of total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were determined by use of reagents from Biosino Bio-technology and Science, Beijing. For intracellular cholesterol measurements, peritoneal macrophages from MED1$^{fl/fl}$ and MED1$^{\Delta Mac}$ mice were incubated with or without oxLDL (50 μg/ml) for 24 hr. Cells were lysed and then centrifuged at 2,000g for 5 min. Supernatants were collected and

measured using the cholesterol assay kit (Applygen Technologies Inc., Beijing).

Analysis of atherosclerotic lesions

Mice were killed by pentobarbital overdose and flushed with 10 ml 0.01 M phosphate-buffered saline (PBS), then perfused with 4% paraformaldehyde through the left ventricle. The entire aortic tree including the heart was dissected free of fat and other tissues. Aortas were open longitudinally, stained with Oil Red O and digitally scanned. *En face* lesion area was assessed by use of WinROOF 6.5 (Mitani Co., Fukui, Japan).

Histology and immunohistochemistry

Cross sections of aortic roots were analyzed by the method of Cheng et al.[2] with minor modification. Each heart was cut in a plane between the lower tips of the right and left atria. The upper portion was embedded in OCT compound. Then, the aortic root was sectioned (8 μm) serially from the start of the aortic valve to the start of the ascending aorta until the valve cusps were no longer visible. Six sections from each of the aortae were quantified and averaged. All morphometric analyses involved hematoxylin and eosin (H&E) and Oil Red O staining. Adjacent sections on separate slides were immunostained with rat monoclonal antibodies for macrophages (MOMA2; 1:100; Cat. No. ab-33451, Abcam). Sections were pre-incubated with 2% horse serum for 2 hr, then incubated with primary antibodies overnight at 4°C, followed by a secondary antibody against rat IgG (1:200, Thermo Scientific) for 1 hr at room temperature. Sections were visualized with use of a DAB substrate kit (Vector Laboratories). Histological analysis and image processing were carried out with the use of a Leica DMRE microscope equipped with Spot digital image analysis software and camera. Images of the sections stained for Oil Red O and MOMA2 were analyzed by using WinROOF 6.5. The TUNEL assay (Beyotime Biotechnology) was performed with fluorescein-dUTP following the manufacturer's instructions.

Macrophage isolation and culture

Primary peritoneal macrophages were isolated from 6- to 8-week-old $MED1^{fl/fl}$ and $MED1^{\Delta Mac}$ mice injected with 3% thioglycolate for 3 days. Cells were plated for 2 hr in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and penicillin/streptomycin. Cultures were washed and treated with lipopolysaccharide (LPS; 50 ng/mL; Cat. No. L2630, Sigma) for 6 hr or IL-4 (10 ng/mL; Cat. No. 214-14, Peprotech) for 16 hr or were infected with adenovirus MED1 (Ad-MED1) or Ad-null for 24 hr. The mouse macrophage RAW264.7 cell line (American Type Culture Collection) was grown in DMEM supplemented with 10% FBS. For siRNA knockdown, a combination of three MED1 siRNAs was used: 5'-GAGGAAGGCTGAAACCATA-3', 5'-GAAGGAACCTCTGCTATGT-3',

5’-GAATCTCGCTTCAGTGTAT-3’. Cells were grown to 70% confluency and transfected with control (Ctrl) siRNA or MED1 siRNA at 50 nmol/L with lipofectamine 2000 (Life Technologies).

Immunofluorescence microscopy

$MED1^{fl/fl}$ and $MED1^{\Delta Mac}$ PMs or cryosections of proximal aortas were fixed in 4% paraformaldehyde for 20 min, then incubated with permeabilization solution (0.1% Triton ×100 and 0.1% sodium citrate) on ice for 2 min and blocked with horse serum for 2 hr. Cells or sections were incubated with anti-MED1 antibody (Cat. No. A300-793A, Bethyl Labs), or anti-iNOS (Cat. No.sc-8310, Santa Cruz) and anti-MOMA2, and then incubated with the secondary antibody goat anti-rabbit Alexa Fluor 488 (Cat. No. A11008, Invitrogen) or donkey anti-rat Alexa Fluor 594 (Cat. No. A21209, Invitrogen). Images were captured by using a Leica DMRE microscope equipped with Spot digital image analysis software and camera or laser scanning confocal microscope.

RNA preparation and real-time PCR

Total RNA was extracted from mouse aortas or peritoneal macrophages by using RNAiso Plus (TaKaRa). Reverse transcription involved 1 μg total RNA with the PrimeScript RT reagent kit and gDNA eraser (TaKaRa). Quantitative PCR (qPCR) was performed in triplicate for amplifying specific genes and normalized to β-actin level. Each PCR reaction involved 0.8 μl (10 μM) forward and reverse primers and 10 μl 2×SYBR Green PCR Master Mix for a final volume of 20 μl. PCR reaction was performed with Thermal Cycler Dice Real Time System (TP-800, TaKaRa). The generation of specific PCR products was confirmed by melting curve analysis. Relative gene expression was measured by the comparative Ct method, $X=2^{-\Delta\Delta Ct}$. Primer sequences are shown in below.

Genes	Primers Forward	Primers reverse
MED1	GAGACTCCGCCCACTTACCTG	GGACACACTTCAAACTGGAGG
IL-1β	CGTGGACCTTCCAGGATGAG	CATCTCGGAGCCTGTAGTGC
IL-6	CGGCCTTCCCTACTTCACAA	TTCTGCAAGTGCATCATCGT
COX2	CTGACCCCCAAGGCTCAAAT	TCCATCCTTGAAAAGGCGCA
iNOS	GCTTGCCCCTGGAAGTTTCT	CCTCACATACTGTGGACGGG
Gro1	GACCATGGCTGGGATTCACC	CGCGACCATTCTTGAGTGTG
MCP-1	AGATGCAGTTAACGCCCCAC	CCCATTCCTTCTTGGGGTCA
TNFα	TGAGCACAGAAAGCATGATCC	GCCATTTGGGAACTTCTCATC
NLRP3	TGTGAGAAGCAGGTTCTACTCT	GACTGTTGAGGTCCACACTCT

VCAM-1	AGTTGGGGATTCGGTTGTTCT	CCCCTCATTCCTTACCACCC
ICAM-1	GCTACCATCACCGTGTATTCG	TAGCCAGCACCGTGAATGTG
CCL5	GTGCCCACGTCAAGGAGTAT	CTCTGGGTTGGCACACACTT
NOX2	GGCTGGGATGAATCTCAGGCCAA	ACTGGTTTCCTGGTGAAAGAGCGG
Arg1	CTTGCGAGACGTAGACCCTG	CTTCCTTCCCAGCAGGTAGC
Mrc1	GTGGAGTGATGGAACCCCAG	CTGTCCGCCCAGTATCCATC
Retnla	CTGGGATGACTGCTACTGGG	CAGTGGTCCAGTCAACGAGTA
Chi3l3	CCAGCAGAAGCTCTCCAGAAG	TCAGCTGGTAGGAAGATCCCA
PPARγ	CCACAGTTGATTTCTCCAGCATTTC	CAGGTTCTACTTTGATCGCACTTTG
IL-10	TAACTGCACCCACTTCCCAG	AAGGCTTGGCAACCCAAGTA
CD36	GGAGGCATTCTCATGCCAGT	CTGCTGTTCTTTGCCACGTC
ABCA1	TCTGGGTGAACGAGTTTCGG	AGCATGCCAGCCCTTGTTAT
ABCG1	AACGTGGATGAGGTTGAGACA	CACCAGCTCTCCACTGTTGAA
C/EBPβ	GACGAGTACAAGATGCGGCG	GAACAAGTTCCGCAGGGTG
β-actin	CATCCGTAAAGACCTCTATGCCAAC	ATGGAGCCACCGATCCACA

Gene expression profiling

Total RNA was prepared from peritoneal macrophages by using the RNeasy Mini Kit (Qiagen). The concentration and purity of RNA were determined by measuring absorbance with a spectrophotometer. cDNA was synthesized with 1 μg total RNA with the RT^2 First Strand Kit (Qiagen). Determination of 84 atherosclerosis-related genes was conducted using SABioscience Mouse Atherosclerosis PCR array (PAMM-038Z) according to the manufacturer's protocol[3] with data analysis software for the ΔΔCt-based fold-change calculations.

Western blot and immunoprecipitation analyses

Nuclear extracts were prepared from PMs or RAW264.7 cells. Protein samples (40 μg) underwent 10% SDS-PAGE, transferred to polyvinylidene fluoride membrane (Millipore), and immunoblotted with PPARγ, MED1, COX2, MCP-1, iNOS, TNFα or p65 antibodies. β-actin or histone blots were loading controls. For immunoprecipitation analyses, 400 μg of total cellular lysate was incubated with the desired antibodies at 4°C overnight in the presence of protein A/G-agarose beads (Santa Cruz). Beads were washed, and bound proteins were resolved on 8% SDS-PAGE, transferred to polyvinylidene fluoride

membrane, immunoblotted with the indicated antibodies, and visualized using enhanced chemiluminescence (Millipore).

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed as described.[4] In brief, 2×10^7 PMs from $MED1^{fl/fl}$ and $MED1^{\Delta Mac}$ mice or cultured RAW264.7 cells were crosslinked for 10 min with 0.75% formaldehyde. Antibodies against MED1, PPARγ, H3K4me1, and H3K27ac were used for immunoprecipitation. Normal rabbit IgG was a negative control. PGC1α lacking the PPRE in the promoter was used as positive control. Primers used for qPCR analysis are shown in below.

Location	Primers Forward	Primers reverse
PPARγ (PPRE1)	GGGTAGAAAAGTCTATCCAG	TGTGTCATTATCACCATAACCT
PPARγ (PPRE2)	TGGATCTGACTGGCTAGGTG	TCCTGTCGGAGTGTGGCTTT
Arg1 (PPRE1)	ACTTGGCATCTGATGGAGAT	AGTCTTGGATGCATGAAGAA
Arg1 (PPRE2)	TGGCTTTACACAGGGACC	GCGATTCTAATCTACACACC
Mrc1 (PPRE1)	GCAGCTTGCAGTCATTCAGT	CTCTCATGTCAGGTCTCTGG
Mrc1 (PPRE2)	GGTCTTAATCCTGTAGAGAC	AACATTGCTCCCTCCCTGTC
Chi3l3 (PPRE1)	CCACAAAGCTATAGAGGGCT	AGGTTCCTGAAAGGCAGCCT
PPARγ promoter	AGGAGCCTGTGAGACCAACA	CACCTAGCCAGTCAGATCCA
Arg1 promoter	AACTGCTTTGGGTTGTCAGG	ACATTTCATGTGTCACCAGG
PPARγ enhancer	GTGCTAACAAGTATGTGGAG	TGAATAGTGATGGGCAATAC
Arg1 enhancer	CACACACACAATCACACAGA	CTTCTGCTCTCTGACTTCCT
PGC1α promoter	TCCGAGTTTCCCTGCTGTGGC	AGGGACTTGCAGCTGTGGTGG

Transfection and luciferase reporter assays

Luciferase reporter assays using PPRE-TK-Luc was used as previously described.[5] Briefly, the reporter plasmids were co-transfected with pRSV-β-gal, along with control or MED1 siRNA into RAW264.7 cells using lipofectamine 2000 (Life Technologies). The transfected cells were then stimulated with PPARγ agonist rosiglitazone (Rosi) (10 mM) for 24 hr. Cell lysates were harvested and luciferase activities were measured and normalized to β-galactosidase activity.

Statistical analysis

Data are expressed as means ± SEM. Student *t* test (for comparison between

2 groups) or one-way ANOVA (for comparison of 3 or more groups) followed by Tukey's post-hoc test was used for statistical analysis with use of GraphPad Prism software (GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered statistically significant.

Supporting references

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