Mettl14 Mediates the Inflammatory Response of Macrophages in Atherosclerosis Through the NF- κ B/IL-6 Signaling Pathway

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Abstract

The inflammatory response of macrophages has been reported to play a critical role in atherosclerosis. The inflammatory state of macrophages is modified by epigenetic reprogramming. m⁶A RNA methylation is an epigenetic modification of RNAs. However, little is known about the potential roles and underlying mechanisms of m⁶A modification in macrophage inflammation. Herein, we showed that the expression of the m⁶A modification “writer” Mettl14 was increased in coronary heart disease and LPS-stimulated THP-1 cells. Knockdown of Mettl14 promoted M2 polarization of macrophages, inhibited foam cell formation and decreased migration. Mechanistically, the expression of Myd88 and IL-6 was decreased in Mettl14 knockdown cells. Through m⁶A modification, Mettl14 regulated the stability of Myd88 mRNA. Furthermore, Myd88 affected the transcription of IL-6 via the distribution of p65 in nuclei rather than directly regulating the expression of IL-6 through m⁶A modification. In vivo, Mettl14 gene knockout significantly reduced the inflammatory response of macrophages and the development of atherosclerotic plaques. Taken together, our data demonstrate that Mettl14 plays a vital role in macrophage inflammation in atherosclerosis via the NF-κB/IL-6 signaling pathway, suggesting that Mettl14 may be a promising therapeutic target for the clinical treatment of atherosclerosis.

Introduction

Monocytes and macrophages are regarded as the main actors in atherosclerosis because of their ubiquitous involvement during all stages of atherosclerosis[4, 8]. Considerable amounts of evidence support that atherosclerosis is a chronic inflammatory disease starting with the entry of monocytes from peripheral blood into the endothelium: rolling, adhesion, activation and migration. Then, monocytes differentiate into macrophages[5, 27]. Depending on the markers, production of specific factors and biological functions of the macrophages, macrophages can be divided into two major subtypes: classically activated macrophages (M1) and alternatively activated macrophages (M2). High levels of the glycoprotein Ly6C (Ly6C^{high}) monocytes in mice, known as CD14 (CD14⁺⁺CD16⁻) in humans, differentiate into M1 macrophages. However, Ly6C^{low} monocytes (CD14^{+/low}CD16⁺ in humans) differentiate into M2 macrophages[12, 38]. M1 macrophages initiate and sustain inflammation, producing inflammatory cytokines (TNF-α, IL-1β and IL-6) and leading to foam cell formation. M2 macrophages counteract inflammation and secrete anti-inflammatory cytokines (IL-10 and TGF-β)[5, 25]. The biological characteristics of macrophages in atherosclerotic plaques determine the size, composition and stability of the lesion[22]. Recent studies suggest that a substantial proportion of patients retain the progression of the plaque despite achieving guideline-directed therapeutic targets caused by inflammation[31, 35]. Regulating the inflammatory state and function of macrophages raises hope for atherosclerosis regression.

Macrophages are associated with epigenetic reprogramming and are modified by epigenetic enzymes[20]. N⁶-methyladenosine (m⁶A) RNA methylation is a posttranscriptional epigenetic modification of eukaryotic RNAs, including coding and noncoding RNAs[9]. The m⁶A modification

process is jointly regulated by three types of enzymes: “writers” (methyltransferases), “erasers” (demethylases) and “readers” (m⁶A-related binding proteins)[36]. Previous research has established that m⁶A modification is associated with RNA metabolism, including changes in RNA structure, splicing, export and translation[16]. Numerous studies have demonstrated that regulators of m⁶A methylation are involved in multiple physiological and pathological processes, such as immune system development, human cancers and cardiovascular disease[16].

However, our understanding of the regulation of m⁶A modification in atherosclerosis is still in its infancy. Guo et al. showed that IFN regulatory factor-1 induced macrophage pyroptosis by Mettl3-mediated m⁶A modification of circ_0029589 in atherosclerosis[13]. Zhang et al. demonstrated that Mettl14 increased the m⁶A modification of pri-miR-19a to promote endothelial cell proliferation and invasion in vitro[44]. Similar studies have reported that Mettl14 regulates endothelial inflammation and that Mettl14 knockdown reduces the development of atherosclerotic plaques[15]. However, the amount of published data on the m⁶A modification of macrophages in atherosclerosis is limited.

Here, we found that the Mettl14 “writer” can regulate the inflammatory state of macrophages in atherosclerosis. Knockdown of Mettl14 promoted M2 polarization and inhibited foam cell formation. Mechanistically, RNA sequencing (RNA-seq) analysis revealed that Mettl14 regulated the expression of Myd88 through m⁶A modification. Myd88 affected the transcription of IL-6 through p65 by regulating the distribution of p65 in nuclei. In vivo, Mettl14 gene knockout in mice significantly reduced the development of atherosclerosis by decreasing the inflammatory response of macrophages. Collectively, our study offers important insights into the m⁶A modification in atherosclerosis and highlights a potential target for treatment.

Materials And Methods

Study population and ethics statement

A total of 54 participants were selected for our study from the Second Affiliated Hospital of Harbin Medical University between December 2020 and June 2021: 9 controls, 18 patients with ST-segment elevation myocardial infarction (STEMI), 11 patients with non-ST-segment elevation myocardial infarction (NSTEMI) and 17 patients with unstable angina (UA). Inclusion and exclusion criteria were previously described in detail[23]. Briefly, patients with UA, STEMI and NSTEMI were enrolled according to the guidelines[39]. All participants or their families provided informed consent for inclusion before participation in the study, conforming to the Declaration of Helsinki. The current study was approved by the Ethics Committee of the Second Affiliated Hospital of Harbin Medical University, China (KY2020-156).

Animal and atherosclerosis induction

Mettl14 heterozygous mice (Mettl14^{+/-}) were established from C57/BL6 mice by Cyagen Biosciences, Inc. (Suzhou, Jiangsu, China), using CRISPR/Cas9-based targeting and homology-directed repair. C57/BL6

and APOE^{-/-} mice were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China). Mettl14^{+/-}APOE^{-/-} mice were generated by breeding Mettl14^{+/-} mice with APOE^{-/-} mice. Eight- to 10-week-old male APOE^{-/-} (WT) mice and Mettl14^{+/-}APOE^{-/-} (KO) mice were fed a high-cholesterol diet (D12108C, Opensource diets) for 12 weeks. Then, the mice were euthanized for further analysis. All mice were housed under specific pathogen-free (SPF) conditions with controlled temperature and a 12-hour light/dark cycle at the Second Affiliated Hospital of Harbin Medical University. All experimental protocols were approved by the Institutional Animal Care and Use Committee at the Second Affiliated Hospital of Harbin Medical University (sydwggr2020-095). This study was conducted in accordance with the Guide for the Care of Use of Laboratory Animals (Institute of Laboratory Animal Resources/National Institutes of Health, Bethesda, MD, USA).

Peripheral blood mononuclear cells isolated

Overnight fasting blood samples were collected by venipuncture from all participants when they were hospitalized on the first day. The peripheral blood of mice was collected when the mice were fed a high-cholesterol diet for 12 weeks. Peripheral blood mononuclear cells (PBMCs) of humans and mice were isolated using the density gradient technique (TBD; Tianjin, China). Human PBMCs were lysed with TRIzol reagent (Invitrogen, Carlsbad, USA) for RNA extraction. The PBMCs of mice were further analyzed via flow cytometry.

RNA extraction and qRT-PCR

Total RNA from PBMCs of patients and THP-1 cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA) and converted to complementary DNA by a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). qRT-PCR was performed with a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). The settings were as follows: 40 cycles of 10 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C. All relative mRNA expression levels were analyzed using the $2^{-\Delta\Delta C_t}$ method. The primers used are listed in Table S1.

Dot-blot assays

Total RNA from PBMCs of patients and THP-1 cells was extracted using TRIzol reagent (Invitrogen, Carlsbad, USA). The RNAs (200, 100 and 50 ng) were double diluted, denatured by heating at 95°C for 5 min and chilled on ice immediately. The RNAs were then spotted onto nitrocellulose membranes (Solarbio, Beijing, China). Then, the membranes were ultraviolet (UV) crosslinked, blocked and incubated with an m6A-specific antibody (Synaptic Systems, Gottingen, Germany). The other membrane was stained with methylene blue as a loading control.

Culture and transfection of THP-1 cells

The human macrophage cell line THP-1 was purchased from the American Type Culture Collection. The cells were cultured in RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) consisting

of 10% FBS (Biological Industries, Israel) at 37°C with 5% CO₂.

The siRNAs for siMettl14 and siMyd88 and the plasmid DNA were purchased from GenePharma (Shanghai, China). All the sequences of the siRNAs are provided in Table S1. The siRNAs were transfected into cells by Lipofectamine RNAiMax (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) for 48 h. The plasmid DNAs were then transfected into cells by Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) for 48 h. Then, the media were removed and replaced with fresh media. The cells treated with LPS (500 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) for 24 h.

Western blot and antibodies

Proteins were isolated from THP-1 cells in RIPA buffer (Beyotime, Shanghai, China) together with a 1% cocktail of protease (Roche, Basel, Switzerland) and 1 mM PMSF (Beyotime, Shanghai, China). Protein quantification was performed using a BCA protein detection kit (Beyotime, Shanghai, China). Protein lysates were separated by 8%-12.5% SDS-PAGE and transferred onto 0.22 µm Immobilon-NC gels (Merck Millipore, Billerica, MA, USA). The primary antibodies used were anti-Mettl14, anti-p65, anti-p-p65, anti-IκBα, anti-ABCA1, anti-ABCG1, anti-PPAR-γ, anti-LXR-α, anti-Myd88, anti-IL-6, and anti-β-actin, anti-GAPDH. The membranes were blocked in blocking buffer and then incubated with the primary antibodies overnight. The protein bands were visualized using chemiluminescence in a Tanon 5100 system (Tanon, Shanghai, China).

Flow cytometry analysis

THP-1 cells were treated with LPS (500 ng/ml) after transfection. The positive control groups for M1 and M2 were treated with LPS (500 ng/ml) or IL-4 (20 ng/ml), respectively. The cells were harvested and stained with PE-CD86 and APC-CD163 antibodies (BioLegend, San Diego, CA, USA) for 30 min at 4°C in the dark. Then, the cells were washed, fixed and permeabilized using Fix/Perm buffer (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). FITC-CD68 (BioLegend, San Diego, CA, USA) was added to the cells for 30 min at room temperature. The PMBCs of mice were stained with FITC-Ly-6c and PE-CX3CR1 (BioLegend, San Diego, CA, USA) for 30 min at 4°C in the dark. The data were analyzed using FACSDiva version 6.1.3 (BD Biosciences, San Jose, CA, USA) and FolwJo_V10 (TreeStar, Ashland, OR, USA).

Intracellular cholesterol assessment

THP-1 cells were treated with 50 µg/ml ox-LDL (YB-002, Yiyuan Biotechnologies, Guangzhou, China) after transfection for 24 h. Then, the cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min. Afterward, the cells were washed with 60% isopropanol and stained with an oil red O staining kit (cultured cells; Solarbio, Beijing, China).

Wound healing

After stimulation with LPS for 24 h, the cell monolayer was scratched with a sterile pipette tip and washed with PBS. The scratch wound areas were observed at 0 and 12 h.

RNA-seq and bioinformatics analysis

RNA from the siM14 and NC groups was isolated using TRIzol reagent. RNA-seq and data analysis were carried out by Sangon Biotech (Shanghai, China). The cDNA library was constructed using a TruSeq PE Cluster Kit v4-cBot-HS (Illumina, USA). Sequencing was performed on a MGISEQ-2000 platform. The RNA-seq reads were mapped by the human reference genome (hg19) from the NCBI using HISAT2. The differentially expressed genes (DEGs) were filtered based on P values <0.05 and fold-changes >1.5. GO enrichment and KEGG pathway enrichment analyses of DEGs were performed by using DAVID Bioinformatics Resources 6.8 (<https://david.ncifcrf.gov/>).

Immunofluorescence

The cells were fixed with 4% paraformaldehyde for 20 min. The tissue was cut into 7 µm thin slices and fixed in acetone. Then, the cells or cryosections were immersed in Triton X-100 (Biosharp, Hefei, China) for 30 min followed by supplementation with 10% normal goat serum (Solarbio, Beijing, China) for 30 min. The cells or cryosections were incubated overnight with primary antibodies. Secondary antibodies were added to the cells or cryosections and then labeled with DAPI. The images were captured using a confocal laser scanning microscope (Zeiss LSM 700).

mRNA decay analysis

THP-1 cells were treated with actinomycin D at a final concentration of 5 µg/ml for 5 or 10 h. Total RNA was extracted at the indicated time points for reverse transcription and qRT-PCR. The mRNA decay rate was normalized to that at 0 h.

RNA-binding protein immunoprecipitation assays

RIP assays were performed using an RNA Immunoprecipitation Kit (Geneseed Biotech, Guangzhou, China) according to the manufacturer's instructions. In brief, the cells were harvested in RIP buffer on ice for 10 min. Then, 100 µL RIP lysis was used as input. Then, the cell lysates were incubated with 5 µg anti-m⁶A antibody or control IgG containing protein A/G-agarose beads. After rotation at 4°C for 2 h, the beads were washed. The immunoprecipitated and input RNAs were isolated and subjected to RIP-qPCR analysis. The PCR primers used are listed in Table S1.

Chromatin immunoprecipitation (CHIP) assays

ChIP assays were performed using a SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology, Danvers, MA, USA). The cells were crosslinked with 1% formaldehyde for 10 min and then sonicated in lysis buffer. Ten microliters of the lysate was used as an input. The remaining lysate was subjected to a ChIP assay using p65 or IgG antibodies. The PCR primers used are provided in Table S1.

Pathological staining

The hearts from the mice were perfused with PBS and immediately embedded in Tissue OCT-Freezing Medium (Tissue-Tek, Sakura, Torrance, CA). Serial cryosections (7 μm) were made using a cryostat. The serial cryosections from the caudal aortic sinus to the proximal aorta were stained with hematoxylin and eosin (HE) to visualize the plaques, oil red O to evaluate the lipid content, and Masson trichrome to analyze lesion size, collagen content, fibrous cap thickness and necrotic core formation. The HE staining kit, oil red O staining kit and Masson trichrome staining kit were purchased from Solarbio (Beijing, China).

ELISA analysis

Plasma was collected from the mice. Then, the levels of IL-6 were assessed with ELISA kits (Lianke Biotech, Hangzhou, China) according to the manufacturer's instructions. The absorbance was measured using a microplate reader (Tecan Infinite M200).

Serum lipid determination

Sera were collected from the mice. Serum lipids, including triglycerides, total cholesterol, LDLs and HDLs, were determined based on commercial kits (Jiancheng, Nanjing, China) using a microplate reader (Tecan Infinite M200).

Statistical analysis

The results are presented as the means $\pm$ SDs. The statistical analyses were performed using GraphPad Prism version 9.1.0 or SPSS 23.0 software (IBM). Two-group comparisons were performed by unpaired two-tailed Student's t-test, and three or more group comparisons were performed by ordinary one-way analysis of variance (ANOVA). *P* values <0.05 were considered statistically significant. All experiments were performed at least three times.

Results

Mettl14 is upregulated in coronary heart disease patients and macrophages

The role of m^6A in PBMCs of patients with coronary heart disease (CHD) was evaluated using an m^6A mRNA dot blot assay. We divided patients with CHD into the UA group, STEMI group and NSTEMI group. The levels of m^6A modification, including UA, STEMI and NSTEMI, were significantly increased in CHD (Fig. 1a). Given that the m^6A modification is primarily catalyzed by methyltransferase (writers) and demethylase (erasers), we hypothesized that the abnormal m^6A modification in CHD was caused by the dysregulation of m^6A writers and erasers. Then, we measured the expression of writers (Mettl3, Mettl14, Mettl16 and WTAP) and erasers (FTO and ALKBH5) in the PBMCs of CHD patients. Interestingly, the expression of Mettl3 and Mettl14 was significantly increased in CHD patients, including those in the UA group, STEMI group and NSTEMI group (Fig. 1b,c). The level of WTAP was increased in the UA group and NSTEMI group but did not change in STEMI group (Fig. 1e). FTO was upregulated in the UA group but did not change in the STEMI or NSTEMI group (Fig. 1f). The level of ALKBH5 was decreased in the STEMI

and NSTEMI groups but did not change in the UA group (Fig. 1g). In addition, Mettl16 did not change significantly in CHD patients (Fig. 1d). Consistent with the results in CHD patients, m⁶A modification in LPS-stimulated THP-1 cells (a human monocyte cell line) increased (Fig. 2a). Moreover, the expression of Mettl3, Mettl14, Mettl16 and WTAP was increased in LPS-stimulated THP-1 cells, and ALKBH5 and FTO expression decreased in LPS-stimulated THP-1 cells (Fig. 2b-c, Fig. S1a-e). In particular, the expression level of Mettl14 was highest in LPS-stimulated THP-1 cells. Taken together, these results prompted us to explore the consequences of increased Mettl14 in macrophages caused by atherosclerosis.

Mettl14 knockdown conditioned macrophages toward M2 polarization and suppressed NF-κB signaling

The m⁶A writer Mettl14 plays a critical role in the regulation of m⁶A modification in macrophages in atherosclerosis. Hence, we knocked down Mettl14 in THP-1 cells through two different siRNAs. qRT-PCR and Western blot analysis showed that both siM14-1 and siM14-2 caused a decrease in the expression of Mettl14 (Fig. S1f-h). LPS- and IL-4-stimulated THP-1 cells were used as positive controls for M1 and M2 polarization, respectively (Fig. 2d, Fig. S1i-j). As shown in Fig. 2d and Fig. S1i-j, knockdown of Mettl14 significantly increased the proportion of M2 macrophages (CD68⁺CD163⁺ cells) and decreased the proportion of M1 macrophages (CD68⁺CD86⁺ cells). Like flow cytometry, qRT-PCR confirmed that the mRNA expression of IL-1β and TNF-α was downregulated and that IL-10 and CD163 were upregulated in siM14-1- and siM14-2-treated THP-1 cells (Fig. 2e-h). Previous studies have demonstrated that the NF-κB pathway plays an important role in macrophage polarization by inducing the expression of inflammatory genes. Then, we investigated the NF-κB pathway using western blotting to evaluate whether the NF-κB pathway was involved in macrophage polarization. Knockdown of Mettl14 markedly reduced the phosphorylation of NF-κB-p65, but the level of p65 was not significantly changed (Fig. 2i, Fig. S1k-l). The expression of IκBα increased in M2 phenotype macrophage cells (Fig. 2i, Fig. S1m). Taken together, these data suggested that knockdown of Mettl14 prevents the macrophage inflammatory response by promoting M2 polarization via the NF-κB pathway.

Mettl14 deficiency inhibited foam cell formation and macrophage migration

In arteries, foam cell formation and macrophage migration play important roles in the development of atherosclerosis. We asked whether Mettl14 has a role in foam cell formation and macrophage migration. As shown in Fig. 3a and 3b, the siM14-1 and siM14-2 groups had much less lipid accumulation than the NC group stained with oil red O did. Then, we measured the expression level of lipid metabolism-related proteins. Several studies have shown that ABCA1 and ABCG1 have an essential role in cholesterol efflux during foam cell formation and that PPAR-γ and LXR-α regulate ABCG1/ABCA1[6]. The expression of ABCA1, ABCG1, PPAR-γ and LXR-α was increased in the Mettl14 knockdown group compared to the NC group (Fig. 3c-g), indicating that the protective function of Mettl14 in macrophages may be related to the PPARγ-LXRα-ABCA1/ABCG1 pathway. Next, using a scratch test, we confirmed that Mettl14 affected migration. The results showed that knockdown of Mettl14 significantly decreased macrophage migration (Fig. 3h-i). Together, the above findings demonstrated that Mettl14 deficiency inhibited foam cell formation and macrophage migration.

Mettl14 modifies Myd88 mRNA stability by m⁶A modification

To determine Mettl14-regulated genes in macrophages, we used RNA-seq. For this, we used three samples of Mettl14 knockdown THP-1 cells and three samples of THP-1 cells treated with NC. The RNA-seq data showed that 150 mRNAs were upregulated and that 128 mRNAs were downregulated in the Mettl14 knockdown group compared with the NC group (Fig. 4a, Table S2). Gene Ontology (GO) analysis of these DEGs showed that they were involved mainly in the immune response, inflammatory response, chronic inflammatory response, positive regulation of interleukin-6 production and positive regulation of I- κ B kinase/NF- κ B signaling (Fig. 4b). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway was mainly enriched in cytokine-cytokine receptor interaction, rheumatoid arthritis, pertussis, Toll-like receptor signaling pathway and inflammatory bowel disease (Fig. 4c). These data suggested that Mettl14 regulated the inflammatory response of macrophages.

Among the DEGs, Myd88 and interleukin-6 (IL-6) were very interesting because they play an important role in the inflammatory response of macrophages[1, 3, 7]. First, we detected whether the expression of Myd88 was regulated by Mettl14. qRT-PCR analysis revealed that the mRNA expression levels of Myd88 were markedly lower in Mettl14 knockdown THP-1 cells than in those in the NC group, whereas the mRNA expression of Myd88 was increased in the Mettl14 overexpression group (Fig. 4d). Moreover, using western blots and immunofluorescence staining, we obtained similar results of Myd88 protein expression (Fig. 4e-f, Fig. S2a). To determine the regulatory mechanism by which Mettl14 regulates Myd88, we examined the mRNA stability of Myd88. Similar to our hypothesis, the stability of Myd88 mRNA was decreased in the Mettl14 knockdown group (Fig. 4i). RNA immunoprecipitation (RIP) analysis revealed that knockdown of Mettl14 reduced the m⁶A modification on site 4 of Myd88 mRNA (Fig. 4j). These results suggested that Mettl14 modifies Myd88 mRNA stability by m⁶A modification.

Mettl14 regulates the expression of IL-6 by affecting the nuclear distribution of p65 through Myd88

IL-6 is a key inflammatory factor involved in the activation and regulation of macrophages[40]. To validate the RNA-seq results, we performed qRT-PCR to detect the expression of IL-6 mRNA. The level of IL-6 was significantly decreased in the Mettl14 knockdown group, and the expression of IL-6 was the opposite in the Mettl14 overexpression group (Fig. 4h). Next, we identified the mechanism by which Mettl14 regulates IL-6. Unfortunately, the stability of IL-6 mRNA and translation was not significantly affected by Mettl14 (Fig. 4g-k, Fig. S2b). Therefore, Mettl14 does not regulate IL-6 through m⁶A modification. Therefore, how does Mettl14 regulate the expression of IL-6? We assumed that Mettl14 regulated IL-6 through the Myd88/NF- κ B pathway according to the prediction of p65 binding to the promoter region of IL-6. Mettl14 did not affect the expression level of p65 (Fig. 2i). Immunofluorescence staining showed that knockdown of Mettl14 impeded p65 translocation from the cytosol to the nucleus (Fig. 4l). However, p65 translocation from the cytosol to the nucleus was facilitated in THP-1 cells treated with siM14 and overexpressing Myd88 (Fig. 4l). As expected, ChIP analysis revealed that knockdown of Mettl14 inhibited p65 binding to the IL-6 promoter (Fig. 4m, Fig. S2c). P65 binding to the IL-6 promoter

was increased in cotransfected THP-1 cells. These results indicated that Mettl14 regulates its expression through the Myd88/NF- κ B pathway.

Knockdown of Mettl14 induces a low inflammatory response in macrophages through Myd88

We showed that Mettl14 knockdown induced a low inflammatory response in macrophages (Fig. 2 and Fig. 3) and that Myd88 was regulated by Mettl14 (Fig. 4). We next examined whether the functions of Mettl14 are mediated by Myd88 in macrophages. For this, we conducted rescue experiments. We generated a Mettl14 expression vector, which was cotransfected with siMyd88. Overexpression of Mettl14 promoted M1 polarization rather than M2 polarization in THP-1 cells (Fig. 5a-c). As expected, siMyd88 reversed Mettl14 overexpression-induced M1 polarization (Fig. 5a-c). The expression level of p65 protein did not significantly change in the OE-M14 group or cotransfection group. The p-p65 protein level increased in Mettl14-overexpressing THP-1 cells, and its expression was decreased by siMyd88 (Fig. 5d, Fig. S3a-c). We found that the inflammatory factors IL-1 β and TNF- α increased and that the anti-inflammatory factors IL-10 and CD163 decreased in Mettl14-overexpressing cells (Fig. 5e-h). Moreover, the levels of inflammatory factors and anti-inflammatory factors were reversed in cells cotransfected with siMyd88. Furthermore, Mettl14 overexpression increased foam cell formation and decreased the abundance of lipid metabolism-related proteins (Fig. 5i-k). However, foam cell formation decreased in response to siMyd88, and lipid metabolism-related proteins increased (Fig. 5i-k, Fig. S2d-g). The migration of macrophages had similar results. Overexpression of Mettl14 promoted the migration of macrophages (Fig. 5l-m). However, siMyd88 reversed OE-M14-induced migration. Collectively, these results suggested that Mettl14 regulates the functions of macrophages through Myd88.

Mettl14 deficiency attenuates atherosclerosis progression in vivo

Given that Mettl14 regulates the functions of macrophages in vitro, we further assessed the effect of Mettl14 on atherosclerosis progression in vivo. Previous studies have demonstrated that Mettl14 gene knockout causes embryo death. Mettl14 heterozygous knockout mice were used for research[15]. To do this, age- and weight-matched male APOE^{-/-}(WT) mice and Mettl14^{+/-}APOE^{-/-}(KO) mice were fed a western diet for three months. After three months, the aortas and peripheral blood of these mice were dissected. Compared to the WT mice, the KO mice did not exhibit changes in the plasma lipid profiles (Fig. S4a-c). As shown in Fig. 6a, images of aortic arches suggested that KO mice were significantly protected from atherosclerosis. HE staining of the aortic roots revealed that the lesion areas were smaller in KO mice than in WT mice (Fig. 6b, Fig. S4d). The extent of proximal aortic atherosclerosis was reduced in KO mice using oil red O (Fig. 6c, Fig. S4e). These results suggested that Mettl14 knockout can significantly reduce atherosclerosis.

Next, using Masson trichrome staining, we examined the characteristics of plaque stabilization between WT and KO mice. Compared to the WT mice, the KO mice had an increased collagen content in the proximal aorta (Fig. 6d, Fig. S4f). In addition, the thickness of the fibrous cap was greater in the lesions of KO mice than in those of WT mice (Fig. 6d, Fig. S4g). Importantly, the necrotic area was decreased in KO

mice vs. WT mice (Fig. 6d, Fig. S4h). Taken together, these data showed that Mettl14 deficiency suppressed the characteristics of vulnerable plaque formation.

Mettl14 regulates the function of macrophages via Myd88/IL-6 in vivo

To verify the function and mechanism of macrophages regulated by Mettl14 in vivo, we analyzed the subtypes of monocytes in the peripheral blood of the mice. The subtypes of monocytes include proinflammatory Ly6C^{high}CX3CR1^{low} monocytes and anti-inflammatory Ly6C^{low}CX3CR1^{high} monocytes. We found that the subpopulations of Ly6C^{high}CX3CR1^{low} monocytes were lower in KO mice than in WT mice and that Ly6C^{low}CX3CR1^{high} monocytes were higher (Fig. 6e-g). These data indicate that Mettl14 deficiency reprograms monocytes/macrophages to anti-inflammatory effects. Then, we detected the expression of Myd88 in plaque macrophages. Fig. 6i shows that the expression of Myd88 significantly decreased in plaque macrophages in KO mice. Surprisingly, we also found that the plasma level of IL-6 was reduced in KO mice (Fig. 6h). In conclusion, the results suggest that Mettl14 can regulate the function of macrophages in atherosclerosis via Myd88/IL-6 in vivo.

Discussion

The presence of macrophages is a common condition that has considerable impact on all stages of atherosclerosis[4, 8]. The functions of macrophages are mainly regulated by epigenetic reprogramming[20]. Recent evidence suggests that epigenetic modifications can be grouped into three categories: epigenetic triad, DNA methylation, and histone modification and nucleosome positioning[30]. In recent years, the mechanism of epigenetic modifications has been updated with the development of specific methylated RNA immunoprecipitation and next-generation sequencing[28]. m⁶A is the most common epitranscriptomic modification of mRNA from yeast, plants, flies, humans and other mammals[45]. m⁶A methylation marks are dynamic and reversible. Briefly, m⁶A methylation occurs at the consensus sequence RRACH (R=G or A; H=A, C or U) through methyltransferases (writers) and demethylases (erasers). Then, the m⁶A-related binding proteins (readers) selectively bind the site of m⁶A modification. After this regulatory event, RNAs are cleaved, stable, degraded and translated[18, 21, 34]. Several studies have demonstrated a link between m⁶A modification and human diseases[34].

Zhang et al. demonstrated that m⁶A modification and Mettl14 were significantly increased in the atherosclerotic vascular endothelial cells of patients with carotid stenosis[44]. Guo et al. showed that the expression of Mettl3 was significantly elevated in macrophages in patients with acute coronary syndrome[13]. Another study showed opposite results, which may be caused by different detection methods for m⁶A modification. The m⁶A levels were significantly decreased in peripheral blood mononuclear cells, as detected by colorimetry. These three studies suggest that m⁶A modification plays an important role in atherosclerosis. Here, we observed that the levels of m⁶A modification and Mettl14 were increased in the peripheral blood mononuclear cells of patients with coronary heart disease and LPS-stimulated THP-1 cells. It is possible, therefore, that Mettl14 has a pivotal role in macrophages in

atherosclerosis. Existing studies recognize the critical role played by m⁶A modification in the immune response of macrophages. An earlier study showed that the reader YTHDF2 was upregulated after LPS stimulation and that YTHDF2 knockdown promoted the inflammatory response in LPS-stimulated macrophages[43]. Subsequent studies confirmed that m⁶A modification regulated M1/M2 polarization and cholesterol efflux[24, 29, 41, 42]. However, the function of Mettl14 in macrophages in atherosclerosis has not been reported. In this study, we first reported that Mettl14 regulated the inflammatory state of macrophages in atherosclerosis. Knockdown of Mettl14 in macrophages promotes M2 polarization. Moreover, foam cell formation and migration were inhibited in Mettl14 knockdown macrophages. Atherosclerotic plaques and the inflammatory response were significantly reduced in Mettl14 knockout mice. These data provide evidence that Mettl14 plays an essential role in the regulation of macrophages in atherosclerosis.

The mechanisms of m⁶A modification are diverse, including the fold, stability, degradation and cellular interactions of the modified RNA[28]. Jian et al. demonstrated that Mettl14 enhances the transcription factor FOXO1 by increasing its translation, not RNA stabilization, in endothelial inflammation[15]. In bacterial infection, Mettl14 depletion blocked m⁶A methylation of SOCS1, diminishing its RNA stability[10]. In addition, Mettl14 forms a complex with Mettl3, called the Mettl3/Mettl14 complex, modifying nascent transcripts whose translation is enhanced[14]. We performed RNA-seq of Mettl14-knockdown macrophages to explore the regulatory mechanism of Mettl14. The DEGs regulated by Mettl14 were enriched in the inflammatory response, indicating that Mettl14 plays an important role in the inflammatory response in macrophages. We found two interesting DEGs, those encoding Myd88 and IL-6. qRT-PCR further confirmed that the expression of Myd88 and IL-6 was consistent with the RNA-seq results. Most Toll-like receptors (TLRs) and several cytokine receptors signal through Myd88 to initiate a rapid immune response when alarmins stimulate macrophages[7]. In addition, Myd88 knockout mice showed smaller atherosclerotic plaques and less macrophage activation, lipid accumulation and foam cell formation[3, 26, 37]. Myd88 plays a central role in the inflammatory response of macrophages. Interestingly, Myd88 was a target gene of Mettl14 in our study. Through what mechanism does Mettl14 regulate Myd88? We measured Myd88 mRNA stability in Mettl14 knockdown THP-1 cells. As expected, the stability and m⁶A modification of Myd88 mRNA were decreased in Mettl14 knockdown cells. Thus, Mettl14 regulates Myd88 through m⁶A modification.

IL-6 is a major contributor to the development of atherosclerosis. The aortas of atherosclerotic mice and rats had higher levels of macrophage-attracting IL-6 than did those of the control, and the expression level of IL-6 was higher with aging[2, 11]. The mice were given an anti-mouse IL-6 receptor antibody, and the atherosclerotic lesion size and inflammation were reduced[1]. In clinical trials, the first cytokine inhibition, IL-1 β inhibition, was used for atherosclerosis treatment and prevention and achieved results. The results showed that IL-6 was the central signaling cytokine of IL-1 β inhibition, in turn reducing the inflammatory response[33]. In a recent RESCUE trial, the IL-6 ligand monoclonal antibody ziltivekimab was highly effective at reducing the inflammatory response and atherosclerotic biomarkers, suggesting that IL-6 has become a new therapeutic target for atherosclerosis[32]. In our study, IL-6 was the target of

Mettl14. The expression of IL-6 was decreased in the Mettl14 knockdown, but the stability and translation of IL-6 mRNA did not significantly change in the Mettl14 knockdown. Mettl14 may therefore not depend on m⁶A modification to regulate the expression of IL-6. On the basis of our results, we found that Mettl14 regulates the expression of Myd88 via m⁶A modification, which is the upstream regulation of the NF-κB pathway. Several studies have demonstrated that NF-κB/p65 can regulate the transcription of IL-6[17, 19]. Therefore, we hypothesized that Mettl14 regulates IL-6 through the Myd88/NF-κB pathway. As expected, Mettl14 regulated the distribution of p65 in nuclei, which regulates the transcription of IL-6, reflecting the mechanism of upstream regulation of IL-6 in macrophages.

In summary, our study explores the regulatory mechanisms of macrophage inflammation from a new epigenetic perspective (Fig. 7). Our findings confirmed that Mettl14 can decrease the inflammatory response of macrophages. In vitro, Mettl14 can reduce the stability of Myd88 mRNA via m⁶A modification, thereby increasing M2 macrophage polarization and decreasing foam cell formation and migration of macrophages. Furthermore, we also demonstrated that Mettl14 regulates the transcription of IL-6 through the NF-κB/p65 pathway. In vivo, Mettl14 knockout in mice decreased atherosclerotic plaques and the inflammatory response. Our data indicate that Mettl14 may be a promising therapeutic target of macrophages in the treatment of atherosclerosis.

Declarations

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Author contributions

JW and YS conceived and designed the research. YZ, YL, RX and DW performed the experiments. YZ and XZ performed the model of atherosclerosis. YZ, MZ and BY analyzed the data. YZ wrote the manuscript.

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Figures

Figure 1

Expression of m6A modification methyltransferases and demethylases in coronary heart disease. **a** The m6A levels of PBMC RNA in CHD diseases, including UA, STEMI, and NSTEMI. mRNA levels of Mettl3 (**b**), Mettl14 (**c**), Mettl16 (**d**), WTAP (**e**), FTO (**f**), and ALKBH5 (**g**) in UA, STEMI and NSTEMI patients. Control=9 volunteers, UA=17 patients, STEMI=18 patients, NSTEMI=11 patients. The data are expressed as the mean $\pm$ SDs. P values were determined by one-way ANOVA with Fisher's least significant difference (LSD) post-hoc test. *, P<0.05; ** P<0.01; ****, P<0.0001; ns, not significant. **h** Protein level of Mettl14 in PBMCs from patients with coronary heart disease. n=5 per group. The data are expressed as the means $\pm$ SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. *, P<0.05. MB, methylene blue. PBMC, peripheral blood mononuclear cell. UA, unstable angina. STEMI, ST-segment elevation myocardial infarction. NSTEMI, non-ST-segment elevation myocardial infarction.

Figure 2

Knockdown of Mettl14 promotes M2 polarization of macrophages. **a** Dot-blot assay using an anti-m6A antibody in THP-1 cells stimulated with IL-4 (20 ng/ml) or LPS (500 ng/ml). **b** Mettl14 mRNA was measured by qRT-PCR in THP-1 cells stimulated with IL-4 (20 ng/ml) or LPS (500 ng/ml). n=5 per group. The data are expressed as the means ± SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. *, P<0.05; ****, P<0.0001. **c** Mettl14 protein was detected by Western blotting. n=3 per group. The data are expressed as the means ± SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. **, P<0.01. **d-i** THP-1 cells treated with NC, siM14-1, or siM14-2 before treatment with IL-4 (20 ng/ml) or LPS (500 ng/ml). **d** The percentage of M1 polarization (CD68⁺CD86⁺) and M2 polarization (CD68⁺CD163⁺) was detected by flow cytometry. The mRNA levels of IL-1β (**e**), TNF-

α (f), IL-10 (g) and CD163 (h) were analyzed by qRT-PCR. n=3 per group. The data are expressed as the means $\pm$ SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. *, P<0.05; **, P<0.01; ****, P<0.0001. i The protein levels of p65, p-p65, and I κ B α were detected by western blotting. MB, methylene blue. NC, negative control.

Figure 3

Knockdown of Mettl14 inhibited foam cell formation and migration. THP-1 cells were treated with NC, siM14-1, or siM14-2 before treatment with LDL (50 µg/ml) or LPS (500 ng/ml). a-b Foam cell formation was assessed by oil red O staining. Scar bar=100 µm. n=3 per group. The data are expressed as the means ± SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. **, P<0.01; ****, P<0.0001. c-g The ABCA1, ABCG1, PPAR-γ, and LXR-α protein levels were measured by western blotting. n=3 per group. The data are expressed as the means ± SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. *, P<0.05; **, P<0.01; ***, P<0.001; ****, P<0.0001. h-i Macrophage migration was detected by wound healing assays. Scar bar=200 µm. n=3 per group. The data are expressed as the means ± SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. *, P<0.05; ***, P<0.001, ****, P<0.0001. NC, negative control.

Figure 4

Mettl14 regulates Myd88 via m6A modification and IL-6 through p65. THP-1 cells were treated with NC, siM14-1, siM14-2, overexpressed (OE) Mettl14 or OE Myd88 before treatment with LPS (500 ng/ml). **a** Heat map showing the DEGs in the siMettl14 group and NC group. $n=3$ per group. **b** Results of a GO analysis performed on the DEGs. **c** Results of a KEGG pathway analysis performed on the DEGs. **d** The expression of Myd88 mRNA was detected by qRT-PCR. $n=3$ per group. The data are expressed as the

means $\pm$ SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. **, $P < 0.01$; ***, $P < 0.001$. e-f Myd88 protein levels were detected by immunofluorescence (e) and western blotting (f), Scar bar=50 μm . g At 24 h after transfection, THP-1 cells were treated with cycloheximide (CHX). IL-6 protein was detected by western blotting. h IL-6 mRNA levels was measured by qRT-PCR. n=3 per group. The data are expressed as the means $\pm$ SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. **, $P < 0.01$; ***, $P < 0.001$. i qRT-PCR analysis of Myd88 mRNA stability following treatment with actinomycin D (ActD, 2 μM). n=3 per group. The data are expressed as the means $\pm$ SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. **, $P < 0.01$. j RIP analysis of the interaction of m6A with Myd88 mRNA. n=3 per group. The data are expressed as the means $\pm$ SDs, Student's t-test. *, $P < 0.05$; ns, not significant. k Results of a qRT-PCR-based analysis of IL-6 mRNA stability following treatment with actinomycin D (ActD, 2 μM). n=3 per group. The data are expressed as the means $\pm$ SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. ns, not significant. l The localization of p65 (green) was observed with immunofluorescence, and the nuclei (blue) were stained with DAPI. Scar bar=50 μm . m ChIP assay results showing the ability of p65 proteins to bind IL-6 promoters. n=3 per group. The data are expressed as the means $\pm$ SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

Figure 5

Mettl14 regulates the inflammatory response of macrophages through Myd88. THP-1 cells were treated with NC, OE Mettl14 or OE Mettl14 and siMyd88 before treatment with LPS (500 ng/ml). a-c The percentage of M1 polarization (CD68⁺CD86⁺) and M2 polarization (CD68⁺CD163⁺) was determined by flow cytometry. n=4 per group. The data are expressed as the means ± SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. **, P<0.01; ***, P<0.001; ****, P<0.0001. d The proteins

p65, p-p65 and I κ B α were measured by western blotting. The mRNA levels of IL-1 β (e), TNF- α (f), IL-10 (g) and CD163 (h) were analyzed by qRT-PCR. n=3 per group. The data are expressed as the means $\pm$ SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. *, P<0.05; **, P<0.01; ***, P<0.001, ****, P<0.0001; ns, not significant. i-j Foam cell formation was detected by oil red O staining. Scar bar=100 μ m. n=3 per group. The data are expressed as the means $\pm$ SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. **, P<0.01, ***, P<0.001. k The ABCA1, ABCG1, PPAR- γ , and LXR- α protein levels were measured by western blotting. l-m Macrophage migration was assessed by wound healing assays. Scar bar=200 μ m. n=3 per group. The data are expressed as the means $\pm$ SDs. P values were determined by one-way ANOVA with Fisher's LSD post-hoc test. ***, P<0.001.

Figure 6

Mettl14 knockout mice undergo an the inflammatory response and atherosclerosis development. Mettl14^{-/-} APOE^{-/-} (KO) mice and APOE^{-/-} (WT) mice were fed a high-cholesterol diet for 12 weeks. a Representative images of aortic arches are shown. Representative images of the aortic root lesion area stained with HE (b), oil red O (c) and Masson trichrome (d). Scar bar=200 μ m. e-g Flow cytometry analysis of monocyte populations in peripheral blood isolated from KO and WT mice. n=3 per group. The

data are expressed as the means $\pm$ SDs. P values were determined by student's t-test. *, $P < 0.05$; **, $P < 0.01$. h The level of IL-6 in plasma isolated from KO and WT mice. The data are expressed as the means $\pm$ SDs. P values were determined by student's t-test. *, $P < 0.05$. i Immunofluorescence staining showing the expression of Myd88-positive cells in atherosclerotic plaques.

Figure 7

Graphic abstract of Mettl14 regulates the development of atherosclerosis via Myd88/IL-6 signaling pathway in macrophages.

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