eIF6 Promotes Inflammatory Macrophage Phenotype, Foam Cell Formation and Atherosclerosis

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Research Article

Keywords: atherosclerosis, eIF6, inflammation, macrophages, mice

Posted Date: August 27th, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-820763/v1>

Abstract

Background Atherosclerosis is a chronic inflammatory disease, caused by accumulation of lipid-laden and inflammatory macrophages in the artery wall. Understanding its molecular mechanisms and developing novel therapeutic targets to promote atherosclerotic regression is an important clinical goal.

Methods : ApoE^{-/-} and eIF6^{+/-}/ApoE^{-/-} mice were fed Western diet (WD) for 16 weeks. Molecular biology technology were performed to analyze the differences between them.

Results: The mechanism by which Eukaryotic initiation factor 6 (eIF6) affects macrophages and atherosclerosis remains to be elucidated. Western blotting and real-time polymerase chain reaction (PCR) analysis indicated significantly higher expression levels of eIF6 than those in the control in RAW264.7 cells induced by Lipopolysaccharide (LPS) and Interleukin-4 (IL4). We constructed eIF6^{+/-}/ApoE^{-/-} mice, the hematoxylin (HE) and Oil Red O staining analysis indicated that these mice showed a significant decrease in atherosclerotic lesion formation increased anti-inflammatory cell content in aortas, and reduced necrotic core content compared with ApoE^{-/-} mice on a western diet for 16 weeks. eIF6 deficiency suppressed foam cell formation and promoted the anti-inflammatory macrophage phenotype in primary macrophages. More anti-inflammatory populations were observed in blood and atherosclerotic aortas of eIF6^{+/-} ApoE^{-/-} mice by flow cytometry. Immunofluorescent staining analysis obtained the same results.

Conclusions: eIF6 deficiency protects against atherosclerosis by promoting the anti-inflammatory macrophage phenotype and reducing macrophage uptake of low-density lipoprotein (LDL), indicating that new insight into eIF6 may reveal a potential novel therapeutic target for the resolution of inflammation in atherosclerosis.

1. Introduction

Atherosclerosis is a chronic inflammatory disease that is harmful to human health, and is also a leading cause of morbidity and mortality worldwide [1]. Atherosclerotic inflammation does not readily resolve, and plays an important role in all stages of the atherosclerotic process, the inflammatory process resulting in arterial plaque initiation and development. A particular hallmark of atherosclerotic lesions is the accumulation cholesterol-loaded macrophages or foam cells [2].

Macrophages play an important role during the pathogenesis of atherosclerosis [3]. Macrophages take up oxLDL (oxidized low-density lipoprotein), leading to the development of foam cells and atherosclerotic lesions [3]. Macrophages initially display efficient efferocytosis, which plays a major mechanistic role in the resolution of inflammation, and increasing anti-inflammatory cytokines to reduce the inflammatory environment, therefore restricting plaque progression [4, 5]. However, with plaque development, macrophages reduce the efferocytic capacity for continuing to take up oxLDL, leading to post apoptotic necrosis of apoptotic cells and inflammation. Thus, enhancing macrophage efferocytosis ability and

regulating the macrophage anti-inflammatory phenotype presents a significant therapeutic method for atherosclerosis.

Eukaryotic initiation factor 6 (eIF6) is necessary for 60S ribosomal subunit biogenesis and efficient initiation of translation[6]. eIF6 plays an important role in tumor progression, and eIF6 heterozygosity can restrain tumorigenesis and increase tumor-free survival in mouse model[7]. eIF6 levels have been shown to correlate with insulin resistance and obesity. Diet-induced obesity and lipid steatosis are restrained in eIF6 het mice [8]. High eIF6 levels were detected in lymphoid cells and activating T cells in vivo [9, 10]. The mechanism by which eIF6 affects macrophages and atherosclerosis remains to be elucidated. Through a combination of in vivo mouse studies and ex vivo macrophage studies, the study tried to find the effects of eIF6 on macrophage, further influencing the progression and development of atherosclerosis.

4. Material And Methods

Animal model. *ApoE*^{-/-}/*eIF6*^{+/-} double knockout mice were obtained by crossing *ApoE*^{-/-} with *eIF6*^{+/-} mice. To induce atherosclerosis, mice were fed a Western diet (WD) (protein 20% kcal, fat 40% kcal, carbohydrate 40% kcal; D12108C, Research diets, New Brunswick, USA) beginning at 6 to 8 weeks of age for 16 weeks at which point mice were euthanized and peripheral blood, heart and aorta were harvested. Peritoneal macrophages (Mφs) were harvested from *eIF6*^{+/-} and *WT* mice. The *ApoE*^{-/-}/*eIF6*^{+/-} control mouse cohort consisted of both *ApoE*^{-/-} and *eIF6*^{+/-}. Both male and female mice were used throughout this study in separate groups. Ten to twelve animals were analyzed per group. Randomization and blinding were adopted for animal studies. *ApoE*^{-/-} mice were obtained from the Model Animal Center of Nanjing University and *eIF6*^{+/-} mice were obtained from San Raffaele Scientific Institute. All mice were retained in a pathogen-free facility of Xuzhou Medical University. Animal experimental procedures were performed in accordance with the ethical guidelines of Xuzhou Medical University (Xuzhou, China).

Antibodies and reagents. Unless specified otherwise, common laboratory chemicals and reagents were from either Sigma-Aldrich or Fisher Scientific. Media were from KeyGEN BioTECH and additives for cell culture were obtained from Gibco. Alexa Fluor conjugated goat anti-rabbit 594 (VA027) and goat anti-mouse 488 (VA1012) were from VICMED. The reagents and materials for RNA isolation included RNAiso Plus (Takara 9108), DEPC treated water (VICMED VR908), CHCl₃ and absolute Ethanol. Reagents for Western blotting include the NP-40(VP1005), and a protease inhibitor cocktail (VP10112) were from VICMED. SYPR Green PCR Master Mix reagent for qRT-PCR (40346000) was from Roche and HiScript Q RT SuperMix for qPCR (+ gDNA wiper) was purchased from Vazyme. Oil Red O (0625) was from Sigma-Aldrich. Hematoxylin and eosin were from KeyGEN. DAPI (MA0222) was obtained from Meilun Biotechnology. Oxidized LDL was obtained from Peking Union Biology Co. Anti-β-actin (66009; 1:5000 Western blotting dilution), anti-CD86 (13395; 1:100 immunofluorescent dilution), and anti-CD206 (18704; 1:100 immunofluorescent dilution) were from Proteintech Group. Anti-eIF6 (SC-390441; 1:500 Western blotting dilution; 1:200 immunofluorescent dilution) was obtained from Santa Cruz. Fluorochrome

conjugated anti-CD45 (103112), anti-F4/80 (123110), anti-CD11b (101226), anti-Ly6C (128017), anti-CX3CR1 (149019), anti-CCR2 (150609), anti-CD86(105014), and anti-CD206 (141704) antibodies were purchased from BioLegend. Collagenase I (SCR103) was from Millipore and DNase I (D5025) and hyaluronidase I-S (H3506) were from Sigma-Aldrich.

Atherosclerosis analysis. Mice were sacrificed with carbon dioxide. Total plasma cholesterol, plasma glucose and triglyceride concentrations were collected and measured from fasted mice. Vascular perfusion was performed with cold PBS to remove the remaining blood. Hearts isolated from mice were fixed in 4% PFA and cryopreserved in OCT, and the aortic sinus in the heart was sectioned from scryoblock blocks at 6 microns. Cryosections were stained with Oil Red O or immunofluorescent staining was performed as described below with anti-CD68 and eIF6 or anti-CD206 and eIF6 antibodies. The en face aortas were isolated after PBS perfusion, split, pinned, and fixed in 4% PFA followed by Oil Red O staining. Imaging of aortas was performed using a Leica stereomicroscope. Quantification of lesions was performed by manually tracing the aorta and lesion areas with Image-Pro Plus 6.0 software. Studies analyzing mouse phenotypes including Oil Red O, HE, and IF staining were performed.

Oil Red O staining. The cryosections were washed 3 times with PBS for 5 min each, and incubated in 60% isopropanol for 2 min followed by incubation in 0.5% Oil Red O solution for 10 min. Slides were then differentiated in 60% isopropanol for several seconds followed by 3 washes with PBS, incubation with hematoxylin for 30 sec and differentiation in 1% hydrochloric alcohol 3 sec followed by 3 washes with water tap water, and mounting coverslips. Coverslips were washed 3 times with PBS for 5 min each and fixed in 4% PFA for 30 min followed by 3 washes with PBS. Then the coverslips were incubated in 60% isopropanol for 2 min followed by incubation in 0.5% Oil Red O solution for 30 min. Slides were washed 3 times for 5 min each in PBS, incubated with hematoxylin for 30 secs, washed with PBS 3 times, and mounted coverslips onto slides. Quantification of lesions or foam cells was performed as described above.

H & E staining. Aortic root cryosections were washed with PBS for 3 times and then stained with hematoxylin followed by washes in tap water. Slides were differentiated in 1% hydrochloric ethanol for 3 sec followed by washes in tap water and then stained with eosin followed by washes in tap water, dehydration in 80%, 90%, 95% and 100% ethanol, incubation in 100% xylene, and mounting coverslips with neutral resin.

Immunofluorescent staining. For cryosections, slides were washed 3 times with PBS for 5 minutes each, fixed for 10 min with 4% paraformaldehyde and then permeabilized for 5 min with 0.25% Triton X-100. After blocking with 5% bovine serum albumin in PBS containing 0.5% Tween-20, cells were stained with specific primary antibodies, followed by blotting with fluorescent-conjugated secondary antibodies. Nuclei were labeled with DAPI. The stained cells were visualized and photographed with an Olympus Cell Observer. For coverslips with cells, coverslips were fixed for 15 min with 4% cold paraformaldehyde followed by 3 washes for 5 minutes each and then permeabilized for 10 min with 0.25% Triton X-100. After blocking with 0.5% bovine serum albumin in PBS containing 0.5% Tween-20, cells were stained with

specific primary antibodies, followed by blotting with fluorescent-conjugated secondary antibodies. Nuclei were labeled with DAPI. The stained cells were visualized and photographed with an Olympus fluorescence inverted microscope.

Primary mouse peritoneal macrophage isolation and culture. Mice were injected with 1 mL of 2.5% thioglycolate to elicit peritoneal macrophages (M ϕ s). At day 3 postinjection, mice were sacrificed and 5 mL of sterile 1X PBS containing 1% FBS was injected into the peritoneal cavity. The abdomen was massaged for approximately 5 minutes and then PBS was extracted from the peritoneal cavity, repeat which was repeated 3–5 times. Cells were centrifuged (600xg, 10 minutes), washed with PBS twice, and plated in RPMI (10% FBS, 1% PennStrep). Cells were washed with PBS to remove non-M ϕ cells after 24 hours. Cells were cultured at 37°C with 5% CO₂. Elicited peritoneal M ϕ s were primarily used for Oil Red O and foam cell formation.

Cell culture. The peritoneal macrophages and RAW264.7 cells were cultured in DMEM containing 10% FBS, and 1% PennStrep at 37°C with 5% CO₂. Afterward, the cells were stimulated with interleukin 4 (20 ng/ μ L) or lipopolysaccharide (100 ng/ μ L) for 24h and then used for Western blots and qPCR. The overexpressed cells and the control were used for qPCR and migration assays.

RNA isolation and quantitative real-time PCR. Total RNA was extracted from cells with RNAiso Plus according to the phenol-chloroform method. One microgram of total RNA was treated with 4 μ L of 4xgDNA wiper Mix and 14–16 μ L of double distilled water to eliminate genomic DNA. RNA was reverse transcribed using HiScript Q RT SuperMix according to the manufacturer's instructions. RT-PCR analysis was conducted using SYBR Green PCR Master Mix reagent on Roche a 480 real-time detection system. PCR amplification was performed in triplicate on 96-well optical reaction plates and replicated in at least three independent experiments. The fold change in mRNA expression was calculated using the comparative cycle method ($\Delta\Delta C_t$). The Ct of β -actin cDNA was used to normalize all samples. Primers are listed in Table 1.

Table 1
Primers for qPCR

Gene	Primer sequence(5'to3')
eIF6	Forward: AGAGCGTCGTTCGAGAACAAC
	Reverse: CGGGAATGGCATCGGAGAG
IL-1 β	Forward: GAAGAAGAGCCCATCCTCTG
	Reverse: TCATCTCGGAGCCTGTAGTG
IL-6	Forward: AGTCCGGAGAGGAGACTTCA
	Reverse: TTCCACGATTTCCCAGAG
IL-10	Forward: CCCAGAAATCAAGGAGCATT
	Reverse: TCACTCTTCACCTGCTCCAC
iNOS	Forward: CCCTTCCGAAGTTTCTGGCAGC
	Reverse: GGCTGTCAGAGCCTCGTGGCTTTGG
MCP-1	Forward: GAAGGAATGGGTCCAGACAT
	Reverse: ACGGGTCAACTTCACATTCA
TNF- α	Forward: CCTGTAGCCCACGTCGTAG
	Reverse: GGGAGTAGACAAGGTACAACCC
CD11c	Forward: GCAGGAGTGTCCAAAGCAAGA
	Reverse: CGTGTGCTAGGTCTCTGAAGC
CD163	Forward: GGGTCATTGAGAGGCACACTG
	Reverse: CTGGCTGTCCTGTCAAGGCT
TGF- β 1	Forward: CCGCAACAACGCCATCTATG
	Reverse: CTCTGCACG GGACAGCAAT
GAPDH	Forward: CAACTTTGGCATTGTGGAAGG
	Reverse: ACACATTGGGGGTAGGAACAC

Western blotting. Proteins (30–50 μ g) were separated by SDS-PAGE (10%) followed by electroblotting to polyvinylidene difluoride membrane and blocking with 5% BSA 0.1% Tween 20 at 37°C for 30 min. Primary eIF6 antibodies (1:500) were incubated at 4°C overnight followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibodies (1:1000) at room temperature for 1 hour. The immunoreactive proteins were detected by enhanced chemiluminescence with autoradiography.

Total protein levels were normalized to β -actin levels, and the signal intensities were quantified by densitometry using ImageJ software.

Foam cell formation. Peritoneal M ϕ s were isolated and plated on 12-well plates, cultured in RPMI supplemented with 10% serum and 1% PennStrep for 24 hours, and then treated with 50 μ g/mL oxLDL for 24 hours. Cells were fixed in 4% PFA for 30 minutes at room temperature and washed with PBS, stained with Oil Red O, counterstained with hematoxylin, and then mounted on slides.

Analysis of lipid levels of cellular and serum

The cellular levels of cholesterol, free cholesterol and cholesterol ester were measured by HPLC according to conduct as described previously[11]. Biochemical Analysis: serum lipid profiles, including total cholesterol and triglycerides and high-density lipoprotein (HDL) cholesterol (Nanjing Fuji Bioengineering Institute, Nanjing, China), were detected by commercial kits.

Flow cytometry analysis

Flow cytometry of peripheral blood mononuclear cells: 100 microliters of blood from the eye socket was collected into 5 mL tubes containing 100 μ L 20 mM EDTA. Two milliliters of cold Red Blood Cell Lysing Buffer were added, followed by vortexing and incubation for 10 minutes at room temperature, and centrifugation (400xg, 5 minutes), the supernatant was discarded, and the above step was repeated 2 times. Cells were resuspended in 50 μ L of 1 $\times$ PBS with fluorochrome conjugated anti-CD45, an-Ly6C, an-CX3CR1, and-CCR2. The expression of immune and inflammatory cell markers was analyzed as described above. Flow cytometry of aortas: En face aortas were isolated from mice on Western diet for 16 weeks. Cut into small pieces and placed in an enzyme solution containing collagenase type I, hyaluronidase type I-S (60 U/mL), and DNase I (1 U/mL) in PBS for 60 minutes at 37°C with shaking. Enzyme solutions with en face aortas were grinded, and cells were put through a 200-mesh filter and centrifuged (400xg, 5 minutes), and resuspended in 1 $\times$ Red Blood Cell Lysing Buffer (Biolegend) for 10 minutes. Cells were centrifuged (400xg, 5 minutes) and resuspended in PBS containing the following: fluorochrome conjugated anti-CD45, anti-CD11b, anti-F4/80, anti-CD86, anti-CD206. Cells were washed with PBS, centrifuged and resuspended in PBS for analysis.

Statistical analysis The data are shown as the mean $\pm$ standard deviation (SD). Data for experiments with two groups were analyzed by the unpaired Student's test. Data from experiments with multiple groups were analyzed with ANOVA. A P value < 0.01 was considered significant. Statistical analysis was performed by using GraphPad Prism version 8.0.2 for Windows.

2. Results

2.1 eIF6 deficiency promotes an anti-inflammatory macrophage phenotype

Macrophages play a key role in tissue microenvironments through phenotypic differentiation, and circulating monocytes can be differentiated into M1 and M2 types [12]. M1 are pro-inflammatory phenotypes, and M2 are anti-inflammatory phenotypes, which have significant roles in atherosclerotic inflammation and affect the evolution and complications of atherosclerosis [13]. We first investigated the effects of different stimulations with LPS (100 ng/ μ L) and IL4 (20 ng/ μ L) treatment on RAW264.7 cells for 24 h. The mRNA expression levels of M1 phenotype macrophage markers (MCP-1, IL-1 β and TNF- α) and M2 phenotype macrophage markers (CD163, IL-10 and TGF- β 1) were significantly increased in LPS and IL4 treatments compared with the control (Fig. 1A-F). We successfully induced the different phenotypes of macrophages. The mRNA and protein expression levels of eIF6 were measured in the LPS and IL4 treated RAW264.7 cells, and the results showed that the expression levels of eIF6 were both increased, but the expression of eIF6 in LPS-treated RAW264.7 cells was significantly higher than that in IL4-treated RAW264.7 cells (Fig. 1G H)

To further verify that eIF6 affects the phenotype of macrophages, the peritoneal macrophages of eIF6^{+/-} and eIF6^{+/+} mice were collected. The relative mRNA levels of inflammatory cytokines were measured via real-time PCR, and the inflammatory cytokine expression of M2 macrophages was significantly increased in eIF6^{+/-} macrophages compared with eIF6^{+/+} macrophages. Meanwhile, the inflammatory cytokine expression of M1 macrophages was significantly decreased in eIF6^{+/-} macrophages compared with eIF6^{+/+} macrophages (Fig. 1I).

Proinflammatory cytokines (TNF α , iNOS, IL1 β , IL6 and MCP-1) and anti-inflammatory cytokines (TGF- β 1, CD163 and IL10) were detected by real-time PCR in the aortic sinus of eIF6^{+/-}/ApoE^{-/-} and ApoE^{-/-} mice on Western diet. The results showed that eIF6 deficiency increased the expression of anti-inflammatory factors and reduced pro-inflammatory factors in the aortic sinus of eIF6^{+/-}/ApoE^{-/-} compared with ApoE^{-/-} mice (Fig. 1J). Thus, eIF6 deficiency promotes an anti-inflammatory phenotype in the aortic sinus.

2.1 Macrophage eIF6 deficiency reduces LDL uptake and foam cell formation

To analyze the effect of eIF6 deficiency specifically on foam cell formation, primary peritoneal macrophages were isolated from ApoE^{-/-} and eIF6^{+/-}/ApoE^{-/-} mice, cultured with ox-LDL (50 μ g/mL). eIF6^{+/-}/ApoE^{-/-} peritoneal macrophages had a significant reduction with Oil Red O staining compared with ApoE^{-/-} peritoneal macrophages (Fig. 2A). eIF6^{+/-}/ApoE^{-/-} macrophages had a reduction in total cellular cholesterol, free cholesterol and cholesteryl ester ($P < 0.01$) (Fig. 2B-2D). eIF6^{+/-}/ApoE^{-/-} mice were obtained from eIF6^{+/-} mice crossed with ApoE^{-/-}. ApoE^{-/-} and eIF6^{+/-}/ApoE^{-/-} mice fed a western diet for 16 weeks, then peritoneal macrophages were isolated from eIF6^{+/-}/ApoE^{-/-} mice had a significant reduction in cellular cholesterol content compared with macrophages isolated from ApoE^{-/-} mice (Fig. 2E, 2F; $P < 0.01$).

2.3 eIF6 deficiency protects against atherosclerosis

elF6 can control adipogenic transcription, and increase the levels of lipogenic and glycolytic enzymes[8]. We hypothesized that elF6 may play a critical role in atherosclerosis by regulating the lipid. The serum plasma levels were measured, the results showed the levels of triglyceride and cholesterol had a significantly decrease (Fig. 3A). Concomitantly, plasma levels of HDL- cholesterol showed a significantly decrease in elF6^{+/-}/ApoE^{-/-} mice compared with ApoE^{-/-} mice fed a Western diet for 16 weeks (Fig. 3A).

Oil Red O staining of en face aortas and aortic roots showed that the mean atherosclerotic lesion area was significantly decreased elF6^{+/-}/ApoE^{-/-} mice in compared with ApoE^{-/-} mice (Fig. 3B, 3C). The necrotic core area was decreased significantly via H&E staining in aortic roots in elF6^{+/-}/ApoE^{-/-} mice compared with ApoE^{-/-} mice (Fig. 3D). These results suggest that elF6 deficiency reduces the development of atherosclerosis.

To further analyze the effect of elF6 on phenotype macrophages, we analyzed the proportion of M2 macrophages in the aortas of ApoE^{-/-} and elF6^{+/-}/ApoE^{-/-} mice fed WD via flow cytometry. The results showed that elF6^{+/-}/ApoE^{-/-} mice had significantly increased M2 macrophage content compared with ApoE^{-/-} mice (Fig. 4A). We also detected the phenotype of monocytes in peripheral blood and aortas of ApoE^{-/-} and elF6^{+/-}/ApoE^{-/-} mice fed a Western diet, and we obtained an increase in anti-inflammatory monocyte populations in the blood of elF6^{+/-}/ApoE^{-/-} mice compared with ApoE^{-/-} mice (Fig. 4B).

The macrophage content of the aortic root was significantly higher in elF6^{+/-}/ApoE^{-/-} mice than in ApoE^{-/-} mice via immunofluorescent staining. The M2 macrophage (CD206) content of the aortic root was increased via immunofluorescent staining in elF6^{+/-}/ApoE^{-/-} mice compared with ApoE^{-/-} mice (Fig. 4C). These results suggest that elF6 knockdown could promote the phenotype of macrophages to anti-inflammatory, and suppress the atherosclerosis formation.

3. Discussion

Atherosclerosis is generally thought to be caused by an imbalance in lipid metabolism and the accumulation of cholesterol-rich macrophages in the arterial wall. Initially, macrophages engulf oxidized low-density lipoprotein and clear it from the vascular endothelium. When macrophages take up excess lipids, they then result in the formation of lipid-laden macrophages called foam cells [3]. Lipid-laden macrophages die and accumulate to avoid clearing from the vascular endothelium, leading to necrotic core formation[14]. Several studies have demonstrated the importance of elF6 in regulating cell migration and invasion [15, 16]. However, the role of elF6 in the progression of atherosclerosis in macrophages is unknown. In this study, we revealed that elF6 played an important role in macrophages, elF6 deficiency decreases inflammation and inhibits atherosclerosis. The results showed a difference from those of previous studies. Overexpression of elF6 enhances the migration and invasivity of ovarian cancer cells [15]. Therefore, we can speculate that elF6 has different effects in different cells, and much research is needed to elucidate the phenotype.

eIF6 may regulate the lipogenic program and coordinate upregulation of enzymes involved in cholesterol and fatty acid synthesis[8]. eIF6 is expressed in macrophages, in this study, we found that macrophages eIF6 promotes LDL uptake, foam cell formation, and atherosclerosis and that deficiency is protective against atherosclerosis at least in part by reducing LDL uptake.

eIF6 plays a crucial role in translation regulation in addition to ribosome synthesis [17]. eIF6 deficiency affects translation, and causes expression changes in key metabolic genes [8]. eIF6^{+/-}/ApoE^{-/-} mice had reduced cholesterol levels, eIF6 deficiency resulted in a significant reduction in foam cell formation. Macrophages have an important function to phagocytose LDL, so it is possible that increased exposure of macrophages to LDL triggers the transcriptional upregulation of eIF6. Thus, further studies are needed to determine what role eIF6 plays in the regulation of macrophage metabolism.

Macrophages adapt to the tissue microenvironment through phenotypic differentiation, and circulating monocytes can be differentiated into two phenotypes of macrophages: the M1 phenotype is a pro-inflammatory phenotype and the M2 phenotype is an anti-inflammatory phenotype [18, 19]. M1 macrophages promote plaque inflammation and M2 macrophages resolve plaque inflammation; the two macrophage phenotypes were in different locations in the plaques [20]. Thus, regulating macrophage polarization has provided new insights into the effects of M1 and M2 macrophages on atherogenesis.

In this study, eIF6 was highly expressed in LPS and IL4 treated RAW264.7 cells. However, the peritoneal macrophages of eIF6^{+/-} and control mice were treated with IL4, and eIF6^{+/-} mRNA levels were highly increased compared with the control. The results showed that eIF6 deficiency promotes the M2 phenotype of macrophages. We further studied the inflammatory factor changes between ApoE^{-/-} and eIF6^{+/-}/ApoE^{-/-} mice fed a Western diet. The anti-inflammatory factors were significantly increased in the aortic sinus. These findings suggest that eIF6 deficiency promotes anti-inflammatory factor expression, and inhibits the pro-inflammatory factors expression. Meanwhile, the mean atherosclerotic lesion area was significantly decreased in eIF6^{+/-}/ApoE^{-/-} mice compared with ApoE^{-/-} mice. FASN is a marker of eIF6 activation, eIF6 could rapidly induce FASN mRNA expression[8]; and FASN could positively regulate the MYD88-dependent inflammation [21]. Thus, based on these findings, we speculate that eIF6 deficiency may inhibit atheroma development by promoting the macrophage anti-inflammatory phenotype.

Monocytes are the precursors of macrophages, and the plaque regression and the resolution of inflammation require the recruitment of new anti-inflammatory monocytes, and increase the levels of M2 macrophages [22]. We performed experiments to confirm the effect of eIF6 for macrophage phenotype. The levels of anti-inflammatory monocytes in blood of ApoE^{-/-} and eIF6^{+/-}/ApoE^{-/-} mice fed a Western diet were detected. Anti-inflammatory monocyte populations in the blood of eIF6^{+/-}/ApoE^{-/-} mice were significantly increased compared with ApoE^{-/-} mice. Furthermore, the macrophage phenotypes of aortas were measured, and M2 phenotype macrophages (CD206 marker) were significantly increased in eIF6^{+/-}/ApoE^{-/-} mice compared with ApoE^{-/-} mice. M2 phenotype macrophages of the aortic root obtained the

same results. Some studies also found that the enrichment of M2 macrophages in plaques promoted atherosclerosis regression [22–24]. Thus, eIF6 deficiency promotes M2 macrophage phenotype, and further drives plaque regression. However, the mechanism by which eIF6 regulates macrophage phenotype is unknown, and more studies will be performed to address eIF6 binding or regulating any other protein or factors that play a critical role in regulating macrophage phenotype and the development and progression of atherosclerosis.

4. Conclusions

The present results provide insights into the inflammatory process and the role of eIF6 in the development and progression of atherosclerosis. eIF6 may be a potential therapeutic target for atherosclerosis. eIF6 deficiency reduces the macrophage lipid uptake and macrophage cholesterol loading, increases the macrophage anti-inflammatory phenotype, and reduces the development and progression of atherosclerosis. Our findings demonstrate that eIF6 may be a potential novel therapeutic target for inhibiting or preventing atherosclerotic cardiovascular disease.

Abbreviations

AS	Atherosclerosis
ApoE	Apolipoprotein E
BSA	Bovine serum albumin
CD163	Scavenger receptor
DMEM	Dulbecco modified eagle's medium
DMSO	Dimethyl sulfoxide
EDTA	Ethylene diamine tetraacetic acid
eIF6	Eukaryotic initiation factor-6
FBS	Fetal bovine serum
IL-10	Interleukin-10
IL-1 β	Interleukin-1 β
IL-4	Interleukin-4
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
MCP-1	Monocyte chemoattractant protein-1
mRNA	Messenger ribonucleic acid
ox-LDL	Oxidized low-density lipoprotein
PCR	Polymerase chain reaction
PVDF	Polyvinylidene fluoride
qPCR	Real-time quantitative
	polymerase chain reaction
RT-PCR	Reverse transcript polymerase chain
	Reaction
SDS-PAGE	Sodium dodecyl sulfate
	polyacrylamide gel electro-phoresis
LDL	Low-density lipoprotein
HE	Hematoxylin
WD	Western diet

AS	Atherosclerosis
TEMED	N, N, N', N'- Tetramethylethylenediamin
	Tetramethylethylenediamin
TGF- β 1	Transforming growth factor- β 1
TNF- α	Tumor necrosis factor- α
WT	Wild type
LDL	Low-density lipoprotein
HE	Hematoxylin
WD	Western diet

Declarations

Ethics approval and consent to participate

Animal experimental procedures were performed in accordance with the ethical guidelines of Xuzhou Medical University (Xuzhou, China).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The author declares that they have no competing interests.

Funding

The Jiangsu Planned Projects for Postdoctoral Research Foundation (1501010B); the China Postdoctoral Science Foundation (2016M590506); Jiangsu Key Laboratory of Animal genetic Breeding and Molecular Design (AGBMD2020001); and the Jiangsu Provincial Government Study Abroad Fund.

Authors' contributions

Junnian Zheng, Renjin Chen conceived the study and designed the experiments. Xuemei Xian, Yingchao Bi, Jiajia Chang, Quangang Chen Xufeng Han performed the experiments. Xuemei Xian, Yuhua Zhu and Zhenzhen Wang analyzed the data with suggestions by Renjin Chen, and Renjin Chen wrote the article. All authors read and approved the final manuscript.

Acknowledgments

Not applicable.

Authors' information

Renjin Chen, Xuemei Xian and Xiaoqiang Zhan are contributed equally to this work and should be considered co-first authors.

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Figures

Figure 1

Expression of M1 and M2 macrophage markers and eIF6 were induced by LPS and IL4. A, B, C, D, E, F. The mRNA expression levels of MCP-1, IL-1 β , TNF- α , CD163, IL-10 and TGF- β 1 were measured by qPCR (n=3). G, H, The mRNA and protein expression levels were measured by qPCR and Western blot (n=3). I, The

peritoneal macrophages of eIF6^{+/-} (n=6) and eIF6^{+/+} (n=6) mice were collected, and the inflammatory cytokine expression levels were measured by qPCR. J, The inflammatory cytokines expressions levels were measured by qPCR in the aortic sinus of ApoE^{-/-} (n=6) and eIF6^{+/-}/ApoE^{-/-} (n=6) mice fed a Western diet. * RAW264.7+LPS, RAW264.7+IL4 vs RAW264.7, P< 0.01.

Figure 2

eIF6 deficiency reduces macrophage cellular cholesterol levels and Oil Red O staining in vivo and in vitro. A, peritoneal macrophages–derived macrophages were cultured and treated with ox-LDL (50 μ g/mL, 24 h) and stained with Oil Red O (n=6). B, C, D, Cellular total cholesterol, Free cholesterol and Cholesterol ester measured by gas chromatography (GC/MS). E, Peritoneal macrophages from mice fed a western diet for 16 weeks were isolated and stained with Oil Red O. F, Cellular total cholesterol of peritoneal macrophages measured by GC/MS. Scale bar=20 μ m. * P< 0.01.

Figure 3

eIF6 deficiency reduces the development of atherosclerosis. A, Serum triglyceride, cholesterol, and glucose levels in ApoE^{-/-} (n=6) and eIF6^{+/-} ApoE^{-/-} (n=6) mice fed Western diet for 16 weeks. B, ApoE^{-/-} (n=6) and ApoE^{-/-}/eIF6^{+/-} (n=6) mice fed a Western diet for 16 weeks. Mouse aortas were dissected and stained with Oil Red O. Scale bar=250 μ m. C, Aortic root sections from ApoE^{-/-} (n=6) and ApoE^{-/-}/eIF6^{+/-} (n=6) mice fed Western diet for 16 weeks were stained with Oil Red O. Scale bar=20 μ m. D, Representative photographs of hematoxylin and eosin stained aortic sinus lesions of ApoE^{-/-} (n=6) and ApoE^{-/-}/eIF6^{+/-} (n=6) mice. Scale bar=20 μ m. *ApoE^{-/-}/eIF6^{+/-} group vs ApoE^{-/-}, P< 0.01.

Figure 4

eIF6 deficiency increases the M2 macrophage phenotype A, Gating strategy for flow cytometric analysis of anti-inflammatory monocyte and M2 macrophage populations of peripheral blood and aortas in *ApoE*^{-/-} (n=6) and *ApoE*^{-/-}*eIF6*^{+/-} (n=6) cells. Dot plots showing the gating strategy used to identify CX3CR1 anti-inflammatory monocytes. * *ApoE*^{-/-}*eIF6*^{+/-} vs *ApoE*^{-/-}, P<0.01. B, Dot plots showing the gating strategy used to identify CD206 M2 phenotype macrophages. * *ApoE*^{-/-}*eIF6*^{+/-} vs *ApoE*^{-/-}, P<0.01.

C, The M2 macrophage (CD206) content of the aortic root was determined via immunofluorescent staining in ApoE^{-/-}/eIF6^{+/-} mice and ApoE^{-/-} mice. Scale bar=100 μ m, ApoE^{-/-}/eIF6^{+/-} vs ApoE^{-/-}, P<0.01.