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KLF7 promotes macrophage activation by activating the NF-κB signaling pathway in epicardial adipose tissue in patients with coronary artery disease

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Abstract. – OBJECTIVE: Inflammatory accumulation in epicardial adipose tissue (EAT) may influence the formation and development of coronary artery disease (CAD). EAT macrophages exhibit M1 polarization and the secretion of a large number of inflammatory factors in CAD patients. Emerging data demonstrate that Krüppel-like factor-7 (KLF7), contributes to the regulation of adipocyte differentiation and the secretion of adipose tissue inflammation. However, the function of KLF7 in EAT inflammation still remains to be uncovered. This study aims to investigate the role of KLF7 in macrophage activation in EAT.

PATIENTS AND METHODS: The levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in cell supernatant were measured by enzyme-linked immunosorbent assay (ELISA). The mRNA expression levels were measured by Real Time-PCR. The protein expression level was detected by Western blot.

RESULTS: The expression of inflammatory factors and KLF7 were markedly increased in CAD EAT than non-CAD EAT. KLF7 is highly expressed in human THP-1-derived macrophages induced by inflammatory stimuli, such as LPS. The knockdown of KLF7 inhibited the release of inflammatory factors and significantly decreased the expression of KLF7 in human THP-1-derived macrophages stimulated by LPS. Moreover, transfection with KLF7-siRNA caused the marked inhibition of LPS-induced phosphorylation of JNK-MAPKs and also suppressed the levels of p-p65 and inhibited the activation of p-IκBα.

CONCLUSIONS: Taken together, these results indicate that KLF7 enhances macrophage activation, mediated by JNK-NF-κB signaling pathways in EAT. This suggests that KLF7 may be a potential therapeutic target for cardiovascular diseases such as CAD.

Key Words:
KLF7, Macrophage, NF-κB signaling pathway, Coronary artery disease.

Introduction

Coronary artery disease (CAD) is the leading cause of death worldwide, resulting in 9.48 million deaths in 2016[1]. Endothelial dysfunction is the first sign in the development of atherogenesis (AS)[2,3], followed by the invasion of pro-inflammatory cytokines, such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). This occurs in the epicardial adipose tissue (EAT) and the artery wall around the coronary arteries.

EAT evolves from brown adipose tissues, it is in close proximity to the coronary arteries[4] and shares the same origin with omental adipose tissue. As a result, EAT can secrete pro-inflammatory cytokines and adipokines[5]. The tissue is also an active endocrine organ that secretes substantial TNF-a[6] and IL-6[7] *via* paracrine or vasocrine effects. It is able to infiltrate the myocardium and coronary artery walls because of the lack of fascia between the coronary arteries and EAT[8-10].

Macrophage activation is an integral process in the development of atherosclerosis. The best described immunocompetent cells within EAT are macrophages. Some scholars[11,12] have shown

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that EAT macrophages exhibit M1 polarization in CAD patients, and the percentage of these cells is higher in CAD than non-CAD patients. EAT is characterized by the increased infiltration of macrophages, suggesting that they might represent an important source of inflammation in adipose tissue. As pattern recognition receptors, Toll-like receptors (TLRs) associate their signaling pathways with the infiltration of macrophages in the EAT of patients with CAD[11]. TLRs can initiate the activation of nuclear factor-kB (NF-kB) and induce the expression of inflammatory genes. In CADs, TLR-4 is the main TLR isoform involved in EAT inflammation[13].

Kruppel-like factor 7 (KLF7) is a member of the 18 KLF family that plays different roles in gene expression and regulation[14,15]. This ubiquitous transcription factor is expressed in cardiovascular, digestive, respiratory, and immune systems and is involved in cardiovascular disease, cancer, and inflammatory conditions[16]. KLF7, which localizes in the nucleus, is a member of the pluripotency transcriptional network and plays a vital role in transcriptional activation[17,18]. There has been little research into the role of KLF7 in causing inflammation. In a recent study, the expression of KLF7 was remarkably and positively correlated with TLR4 and IL-6 in adipose tissue samples[19]. However, the functional role of KLF7 in macrophage activation in EAT remains unknown.

This study found that KLF7 had a significant positive correlation with inflammatory factors in CAD EAT. Moreover, KLF7 protein levels were markedly increased in CAD EAT compared to non-CAD EAT. Our results indicate that knockout of KLF7 decreases LPS-induced expression of cytokines, including IL-6 and TNF-α by activating JNK-NF-κB signaling pathways in human THP-1-derived macrophages. These results suggest that KLF7-NF-κB may form a positive feedback loop to drive macrophage activation and the production of inflammatory factors in EAT.

Patients and Methods

Ethical Note

All procedures involving human subjects were approved by the medical Ethics Committee at the First Affiliated Hospital of Shihezi University School of Medicine. Written informed consent was obtained from all subjects and all associated methods were conducted in accordance with the approved guidelines for human experimental research.

Study Population

Thirty patients with CAD who underwent off-pump coronary artery bypass grafting (CABG) were enrolled in this study. Other 30 patients who underwent atrial septal defect repair or valvular replacement surgery without coronary artery stenosis were included as the control group. EAT biopsy samples (average weight 0.5-1.0 g) were collected from the anterior wall of the left ventricle, which was adjacent to the diseased segment, one hour after the administration of anesthesia. All samples were immediately stored in an RNA stabilizer and then transferred to -80°C before total RNA extraction. Patients with acute myocardial infarction, severe heart failure, active infections, hematologic diseases, rheumatologic diseases, steroid or immunomodulatory drug use, and histories of chemotherapy or radiotherapy were excluded. The baseline characteristics and laboratory parameters of the patients are shown in Table I.

Cell Culture

Human monocytic THP-1 cells were purchased from the cell bank of the Chinese Academy of Sciences in Shanghai, China. THP-1 cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), penicillin (100 U/mL), and streptomycin (100 μg/mL) at 37°C in a humidified atmosphere containing 5% CO_2. The THP-1 cells were seeded in 12-well plates at a density of $2x10^5$ cells per well and differentiated into macrophages by using 100 μg/mL phorbol-12-myristate 13-acetate (Invitrogen, Carlsbad, CA, USA) for 24 hours. The cells were then incubated with 20 ng/mL IFN-γ (Gibco, Rockville, MD, USA) and 10 ng/mL LPS (Sigma-Aldrich, St. Louis, MO, USA) for 24 hours to induce M1 macrophages before their use in experiments.

Real-Time Polymerase Chain Reaction (PCR)

The total RNA from CAD EATs, non-CAD EATs, and the experimental groups of cells were extracted using TRIzol (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in accordance with the manufacturer's instructions. The purity and concentration of RNAs were measured using a NanoDrop 1000 spectrophotometer (Thermo

Fisher Scientific, Waltham, MA, USA). Reverse transcription was performed using the PrimeScript RT reagent Kit with genomic deoxyribonucleic acid (gDNA) eraser (TaKaRa Bio, Inc., Otsu, Shiga, Japan) with 500 ng of total RNA isolated for each sample. Quantitative Real Time-PCR (qRT-PCR) was performed with the QuantiNova SYBR Green PCR kit (Qiagen, Hilden, Germany) using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The gene expression levels of beta-actin, KLF7, TLR4, IL-6, CD68, TNF-α, JNK, and NF-kB were quantified, and the primer sequences are listed in Table II. All assays were performed in triplicate. The relative amounts of the target gene transcripts were calculated using the comparative cycle time method. The relative expression levels for each tissue and control samples were calculated by the comparative critical threshold method.

Western Blot

Cells or human tissues were harvested and lysed using phenylmethylsulfonyl fluoride (PMSF), phosphatase inhibitors, and radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Shanghai, China). Approximately 30 μg to 50 μg of total protein was heated for 10 minutes at 100°C, separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% gels, and then, transferred onto polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). After incubation in 5% skimmed milk for two hours at room temperature to block non-specific binding, the membranes were incubated at 4°C overnight with primary antibodies. This was followed by incubations with the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) and by detection using the Pierce Fast Western Blot Kit (Thermo Fisher Scientific, Waltham, MA, USA). Antibodies used in the study were β-actin (ZSGBBIO, Beijing, China), KLF7 (Abcam, Cambridge, MA, USA), p-IkBα (S32/S36), JNK, p-JNK (T183/ Y185), p-p65 (Cell Signaling Technology, Danvers, MA, USA), and mouse IgG and rabbit IgG (ZSGBBIO, Beijing, China). β-actin protein was probed with mice antibodies to β-actin (ZSGBBIO, Beijing, China, TA-09; 1:1000 dilution). KLF7 protein was probed with rabbit antibodies to KLF7 (Abcam, Cambridge, MA, USA, ab129149; 1:1000 dilution). Phospho-IkBα protein was probed with mice antibodies to Phospho-IkBα (CST, Danvers, MA, USA, #9246; 1:1000 dilution). JNK protein was probed with rabbit antibodies to JNK (CST, Danvers, MA, USA, #9252; 1:1000 dilution). Phospho-JNK protein was probed with rabbit antibodies to phospho-JNK (CST, Danvers, MA, USA, #4668; 1:1000 dilution). ERK (1/2) protein was probed with rabbit antibodies to ERK(1/2) (CST, Danvers, MA, USA, #4695; 1:1000 dilution). Phospho-ERK (1/2) protein was probed with rabbit antibodies to phospho-ERK(1/2) (CST, Danvers, MA, USA, #4370; 1:1000 dilution). p38 protein was probed with rabbit antibodies to p38 (CST, Danvers, MA, USA, #8690; 1:1000 dilution). Phospho-p38 protein was probed with rabbit antibodies to phospho-p38 (CST, Danvers, MA, USA, #4511; 1:1000 dilution). Phospho-NF-κB p65 protein was probed with rabbit antibodies to Phospho-NF-κB p65 (CST, Danvers, MA, USA, #3033; 1:1000 dilution). NF-κB p65 protein was probed with rabbit antibodies to NF-κB p65 (CST, Danvers, MA, USA, #6956; 1:1000 dilution).

Table I. The baseline characteristics and laboratory parameters of the patients.

	CAD (n=30)	non-CAD (n=30)	*p*-value
Age (years)	61.4±6.84	59.87±6.11	ns
BMI (kg/m2)	23.22±2.18	22.28±1.76	ns
SBP (mmHg)	123.77±12.16	121.73±8.69	ns
DBP (mmHg)	72.1±7.96	69.17±8.67	ns
LDL (mmol/L)	2.15±0.81	2.13±0.66	ns
HDL (mmol/L)	1.17±0.88	1.13±0.3	ns
TC (mmol/L)	3.82±1.15	3.96±0.94	ns
TG (mmol/L)	1.92±1.32	1.69±1	ns

Data are shown as mean ± SD, CAD: coronary artery disease; BMI: Body mass index; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; LDL: low-density lipoprotein; HDL: high-density lipoprotein; TC: total cholesterol; TG: triglycerides; ns: no significant.

Enzyme-Linked Immunosorbent Assay (ELISA)

The cell culture supernatants were collected and then cleared through centrifugation to assess the secretion of TNF-α and IL-6. The samples were then stored at -80°C until further analysis was performed. For the quantification of human IL-6 and TNF-α, human IL-6 ELISA and TNF-α kits (Invitrogen, Carlsbad, CA, USA) were used respectively. Assays were performed in accordance with the manufacturer's protocols.

Transfection of KLF7 Small Interfering RNA (siRNA)

siRNA was designed and synthesized by GenePharma (Shanghai, China). THP-1-derived macrophages were transfected with KLF7 siRNA or the negative control and Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's protocol. Transfection was validated after 24 hours by assessing the levels of the transfected KLF7 siRNA by qRT-PCR. The siRNA sequences used in this study are listed in Table II.

Statistical Analysis

The GraphPad Prism 8 statistical software package (GraphPad Software, La Jolla, CA, USA) was used for data analysis. Data are expressed as mean ± SD (standard deviation) unless stated otherwise. Student *t*-tests and Wilcoxon rank sum tests were used to compare groups. Correlations were assessed using Spearman's rank correlation for non-normally distributed data, and $p<0.05$ was defined as statistically significant.

Results

mRNA Expression Levels of TLR4, TNF-α, IL-6, JNK, NF-kB and Protein Expression of JNK, p-JNK, p-P65 in EAT Taken from CAD and Non-CAD Subjects

To correlate KLF-7 expression with the inflammatory factors of patients with CAD, this study began by detecting the mRNA expression levels of TLR4, TNF-α, IL-6, JNK, and NF-kB in EAT (Figure 1A). The mRNA levels of these factors were also markedly higher in CAD EAT. The results of JNK, p-JNK, and p-p65 were consistent with Western blotting results showing differences in CAD and non-CAD EAT (Figure 1B-1C).

Expression of KLF-7 in CAD and Non-CAD Samples

This study examined the KLF-7 expression in EAT from CAD and non-CAD samples. It validated KLF-7 mRNA by qRT-PCR and CAD EAT protein expression levels by Western blot. As indicated in Figure 1D, KLF-7 mRNA levels were markedly high in CAD EAT, and this result is consistent with Western blot analysis (Figure 1E).

Table II. Sequences of the primers for qPCR and sequences of siRNAs are used in this study.

Name	Primer sequence (5′-3′)
h-KLF7 F	5'-GGTGAGCCAGACAGACTGACAA-3'
h-KLF7 R	5'-GAAGTAGCCGGTGTCGTGGA-3'
h-TLR4 F	5'-CTGCAATGGATCAAGGACCA-3'
h-TLR4 R	5'-TCCCACTCCAGGTAAGTGTT-3'
h-IL-6 F	5'-AGAGGCACTGGCAGAAAACAAC-3'
h-IL-6 R	5'-AGGCAAGTCTCCTCATTGAATCC-3'
h-TNF-a F	5'-CCCCAGGGACCTCTCTCTAAT-3'
h-TNF-a R	5'-GGTTTGCTACAACATGGGCTAC-3'
h-JNK F	5'-ACACCACAGAAATCCCTAGAAG-3'
h-JNK R	5'-CACAGCATCTGATAGAGAAGGT-3'
h-NF-κB F	5'-AATCATCCACCTTCATTCTCAACTTG-3'
h-NF-κB R	5'-CTCCACCACATCTTCCTGCTTAG-3'
h-CD68 F	5'-CCCAGATTCAGATTCGAGTTCGAGTCAT-3'
h-CD68 R	5'-GTTTTGTTGGGGTTCAGTACAG-3'
h- si-KLF7-1	5'-GCCUUGAAUUGGAACGCUATTUACCGUUCCAAUUCAAGGCTT-3'
h- si-KLF7-2	5'-GCUCUUCUCUAGACAGCUATTUAGCUGUCUAGAGAAGAGCTT-3'
h- β-actin F	5'-CTCCATCCTGGC CTCGCTGT-3'
h- β-actin R	5'-GCTGTCACCTTCACCGTTCC-3'

F: forward; R: reverse.

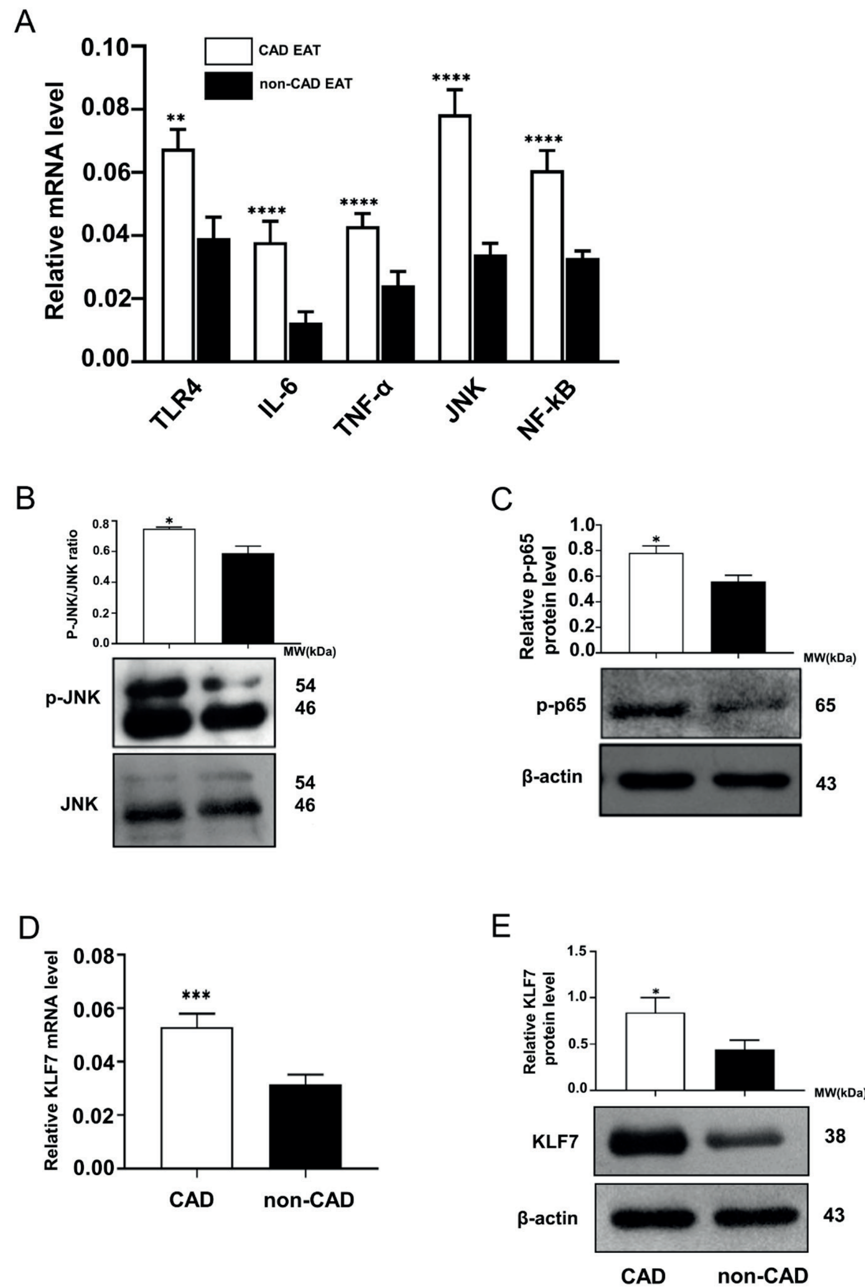

Figure 1. The mRNA and protein expression level of critical gene in inflammation signaling pathways of epicardial adipose tissue in CAD patients. **A,** The mRNA expression level of TLR4, IL-6, TNF-α, JNK, and NF-kB. Values are normalized to β-actin mRNA expression. **B,** and **C**, The protein expression level of JNK, p-JNK and p-p65. **D,** mRNA expression level of KLF7. Values are normalized to β-actin mRNA expression. **E,** Protein expression level of KLF7. Values are normalized to β-actin protein expression. Data are presented as mean ± S.D. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 by Student's *t*-test. CAD coronary artery disease, EAT epicardial adipose tissue.

Correlation of KLF7, TLR4, TNF-α, IL-6, JNK, and NF-kB

To investigate the association between KLF7, TLR4, IL-6, TNF-α, JNK, and NF-kB in CAD EAT, we analyzed the relationship among the gene expression levels of KLF7, TLR4, IL-6, TNF-α, JNK, and NF-kB (Figure 2A-2E). The results indicate that the mRNA expression level of KLF7 positively correlated with TLR4, TNF-α, IL-6, JNK, and NF-kB.

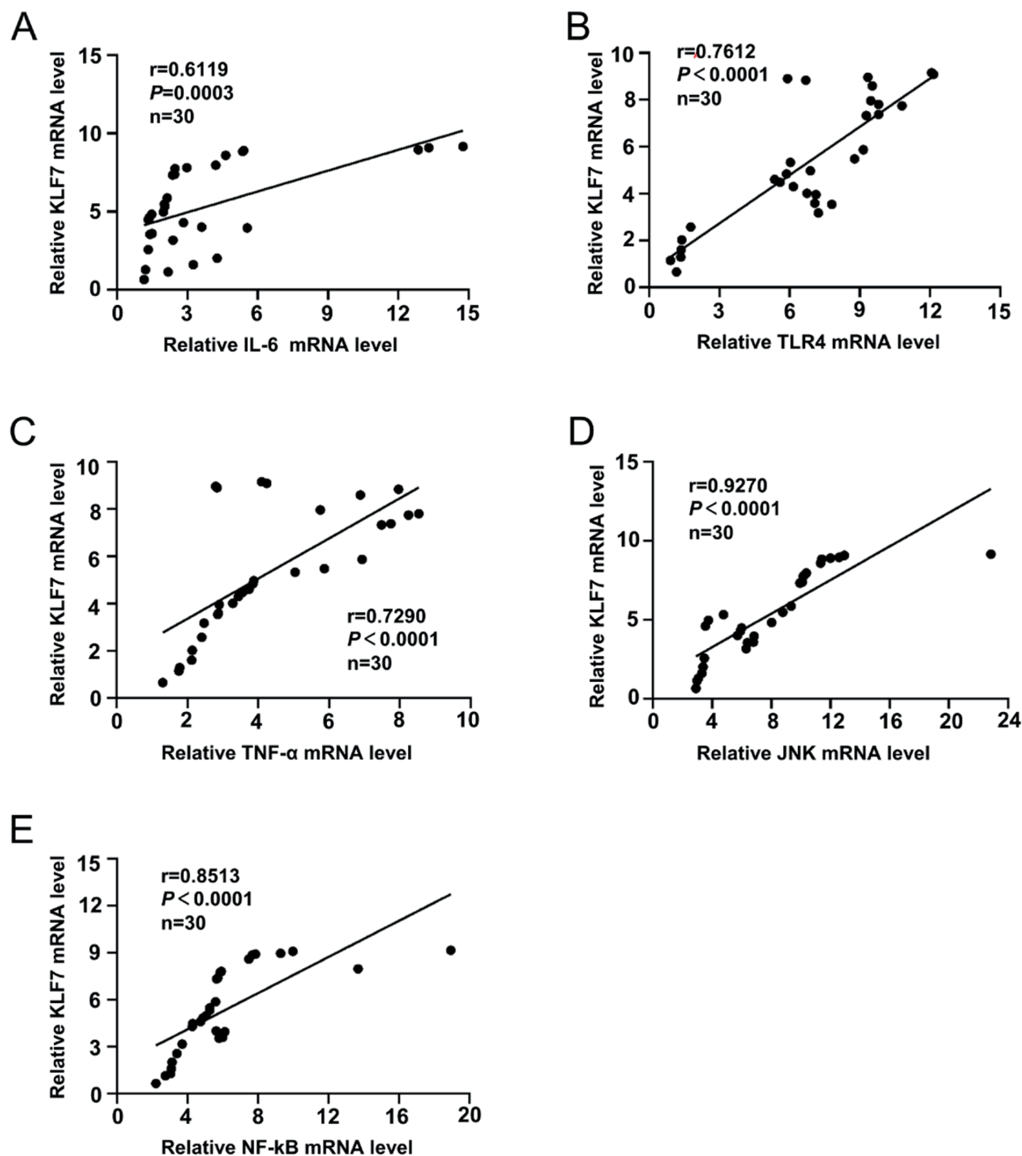

Figure 2. Correlation of KLF7 with TLR4, IL-6, TNF-α, JNK, and NF-kB expression levels was positively correlated with (**A**) IL-6, (**B**) TLR4, (**C**) TNF-α, (**D**) JNK, and (**E**) NF-kB in paired EAT in CAD sample (n = 30). Data are presented as mean ± S.D. Statistics: Spearman analysis. The correlation between the two factors shows statistical significance. CAD coronary artery disease, EAT epicardial adipose tissue.

Macrophages in EAT

To determine the importance of macrophage types in the inflammatory process of EAT, we used the inflammatory cell markers CD68 to detect the presence of macrophages. We found that the mRNA levels of CD68 were markedly higher in CAD EAT than in non-CAD EAT (Figure 3A). We then analyzed the possible correlation between infiltrating macrophages in CAD EAT and the expression of proinflammatory factors. CD68 mRNA expression showed a significantly positive correlation with TLR4, IL-6, and TNF-α in CAD EAT (Figures 3B-3D).

KLF7 Is Highly Expressed in Human THP-1-Derived Macrophages and Induced by Inflammatory Stimuli

To study the role of KLF7 in human THP-1-derived macrophages, we transiently transfected THP-1-derived macrophages with siRNA (siRNA 1–2) specific to KLF7 or with si-NC. As shown in Figure 4A, the si-KLF7-1 and si-

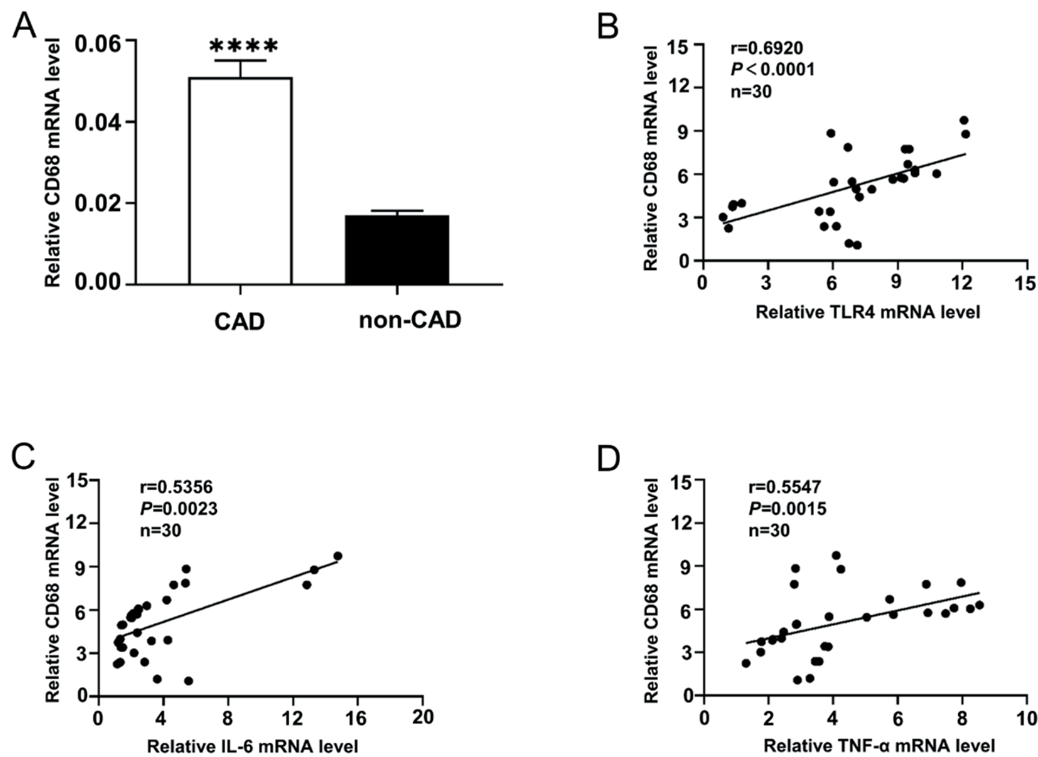

Figure 3. Polarization of macrophage in EAT. **A,** mRNA expression level of CD68. Values are normalized to β-actin mRNA expression. **B-D**, Correlated of CD68 expression level and TLR4, TNF-α, and IL-6 mRNA level in CAD EAT. Data are presented as mean ± S.D. Statistics: Spearman analysis, ****p<0.0001 by Student's t-test. The correlation between the two factors shows statistical significance. CAD coronary artery disease, EAT epicardial adipose tissue.

KLF7-2 were able to attenuate significantly the expression of KLF7, especially si-KLF7-2, compared to the si-NC. To validate KLF7 mRNA results, we also determined the protein expression of KLF7 in the THP-1-derived macrophage lysates transfected with same amount of si-KLF7. The results were same as the ones this study found at the mRNA level (Figure 4B). Consistently, KLF7 mRNA levels were also induced by LPS in time-dependent and dose-dependent ways (Figures 5A and 5C). This suggests that the treatment of human THP-1-derived macrophages with 100 ng/ml LPS for six hours significantly enhanced the expression of KLF7 in human THP-1-derived macrophages. This study then detected that the expression of KLF7 was further induced by LPS in time-dependent and dose-dependent manners (Figures 5B and 5D). Together, KLF7 was highly expressed in human THP-1-derived macrophages and induced by inflammatory stimuli *in vitro*, suggesting that it may involve the regulation of macrophage activation in response to these stimuli.

Knockdown of KLF7 Inhibits LPS-Induced JNK-NF-κB Activation in Human THP-1-Derived Macrophage and Inflammatory Responses

As LPS-induced expression of cytokines mainly attributes to MAPK and NF-κB signaling pathways, this study next investigated the effect of KLF7 on the activation of MAPK and NF-κB signaling pathways. si-KLF7-2 or si-NC (as controls) were transfected into THP-1-derived macrophages for 24 hours, and then, the transfected cells were incubated with or without LPS for six hours (Figure 5E). This result showed that induction by LPS resulted in significant activation of the gene expression of KLF7. Knockdown of KLF7 using siRNAs attenuated LPS-induced phosphorylation of p65, IκBα, and JNK, but did not affect the phosphorylation of ERK1/2 and p38. (Figures 6A-6E). As phosphorylated p65 translocates into the nucleus and activates the transcription of downstream genes, this study next examined whether KLF7 regulates p65 translocation. As shown in Figure 6F, KLF7 knockdown inhibited p65 enter-

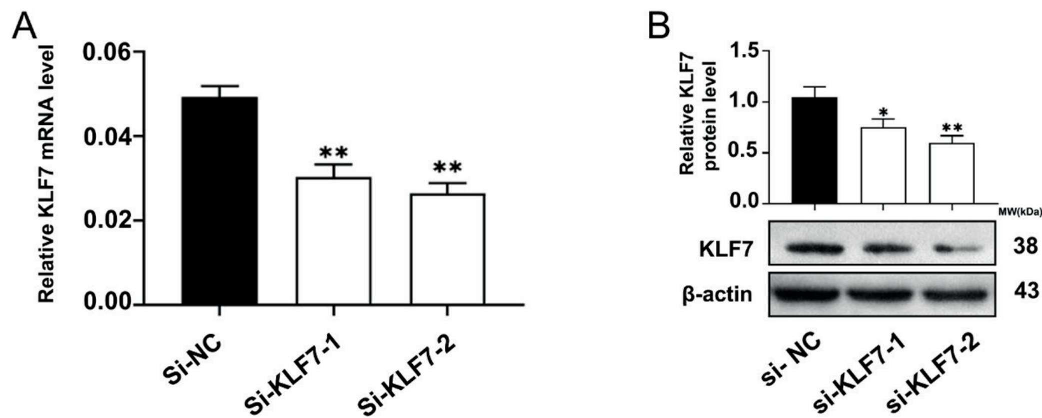

Figure 4. Transfection with KLF7 siRNA suppresses the expression of KLF7 in THP-1-derived macrophage. THP-1-derived macrophage were transiently transfected with si-KLF7- (1-2) or si-NC for 24 h, detect the mRNA levels (**A**) and protein levels (**B**) of KLF7. Data are presented as mean ± S.D. $^*p<0.05$, $^{**}p<0.01$ *vs.* si-NC group by Student's *t*-test.

ing the nucleus. Knockdown of KLF7 also markedly reduced LPS-induced TNF-α and IL-6 production in macrophages, at both the mRNA and protein levels (Figures 7A-7D). Taken together, these results suggest that KLF7 acts as an important regulator in LPS-induced JNK-NF-κB signaling and plays an essential role in regulating the effect of LPS on the production of proinflammatory cytokines.

Discussion

This study first found that the increased inflammatory state of EAT in patients with CAD could be linked to KLF7, which activates immune responses and releases pro-inflammatory cytokines during its upregulation.

The mRNA expression levels of KLF7, TLR4, IL-6, TNF-α, JNK, NF-kB, and CD68 were enhanced in the EAT of patients with CAD compared with those in patients without CAD. Furthermore, KLF7 gene expression in EAT was positively correlated with the gene expression levels of TLR4, IL-6, TNF-α, JNK, and NF-kB in the EAT of the CAD group. CD68 gene expression was also positively correlated with the gene expression levels of TLR4, IL-6, and TNF-α. In the *in vitro* experiment, the transfection of KLF7 siRNA prevented a THP-1-derived macrophage inflammatory response and consequently decreased the levels of IL-6 and TNF-α mRNA and proteins.

This study selected the EAT of patients with CAD who underwent CABG to represent a state of inflammation. The mRNA expression of inflammatory cytokines TNF-α and IL-6 were increased in the EAT of patients with CAD compared with that in patients without CAD. This finding was similar with previous results reporting inflammation in the EAT of patients with CAD, which showed highly increased expression levels of IL-6, TNF-a, and TLR4[13]. The excessive mRNA expression of these factors in EAT may have an important and direct influence on myocardial metabolism. No fascia separates the myocardium from the adipose layer, and both components share the same coronary blood supply[20]. A post-mortem study[21] reported that tissue-specific AT, especially those located in the heart surface and which tightly wrap around the coronary artery, may directly induce a local pro-inflammatory environment in patients with CAD.

TLRs play a vital role in the innate immune response, especially in inflammatory signaling pathways[22]. The increased expression of NF-kB and JNK in the epicardial depot indicates the activation of the TLR4 signaling pathway and the release of proinflammatory cytokines. This finding helps us better understand the key inflammatory signaling pathway[23]. NF-kB, a central downstream transcription factor, plays a key role in the TLR4 signaling pathway. This pathway controls the expression of major pro-inflammatory factors and the activation of TLR4-mediated NF-kB. It also enhances the expression of TLR4-dependent genes, such as TNF-α and IL-6, in immune cells[24]. Strains of human adipocyte studies show that the activation of TLR4

triggers the transcription of IL-6 and TNF-α because NF-kB is translocated into the nucleus[25, 26]. Therefore, this study also detected the expression levels of TLR4, IL-6, TNF-α, JNK, and NF-kB. Our study found that the mRNA expression levels of TLR4, IL-6, TNF-α, JNK, and NF-kB were also significantly higher in the EAT of patients with CAD compared with those

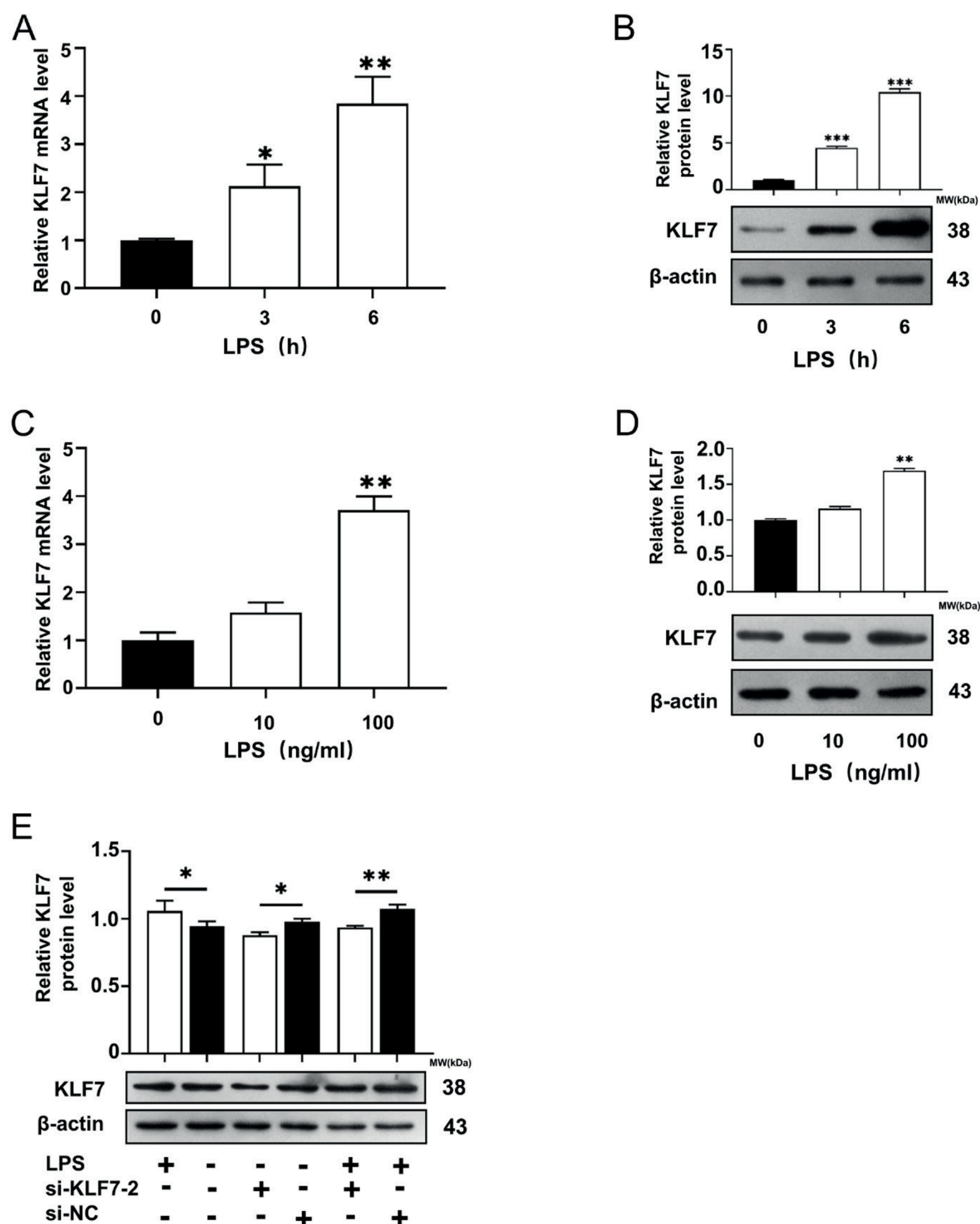

Figure 5. Expression of KLF7 in THP-1-derived macrophage. THP-1-derived macrophage also was treated with LPS in time dependent manners. mRNA expression level of KLF7 (**A**), values are normalized to β-actin mRNA expression, protein expression level of KLF7 (**B**), values are normalized to β-actin protein expression. THP-1-derived macrophage also was treated with LPS in different doses as indicated for 6 h. mRNA expression level of KLF7 (**C**), values are normalized to β-actin mRNA expression, protein expression level of KLF7 (**D**), values are normalized to β-actin protein expression. Data are presented as mean ± S.D. *p<0.05, **p<0.01, ***p<0.001 *vs.* 0 group by Student's t-test. Human THP-1-derived macrophage were transiently transfected with si-KLF7-2 or si-NC for 24 h and then treated with or without 100 ng/mL LPS for 6 h, detect the protein levels of KLF7 (**E**). Data are presented as mean ± S.D. *p< 0.05, **p<0.01 by Student's t-test.

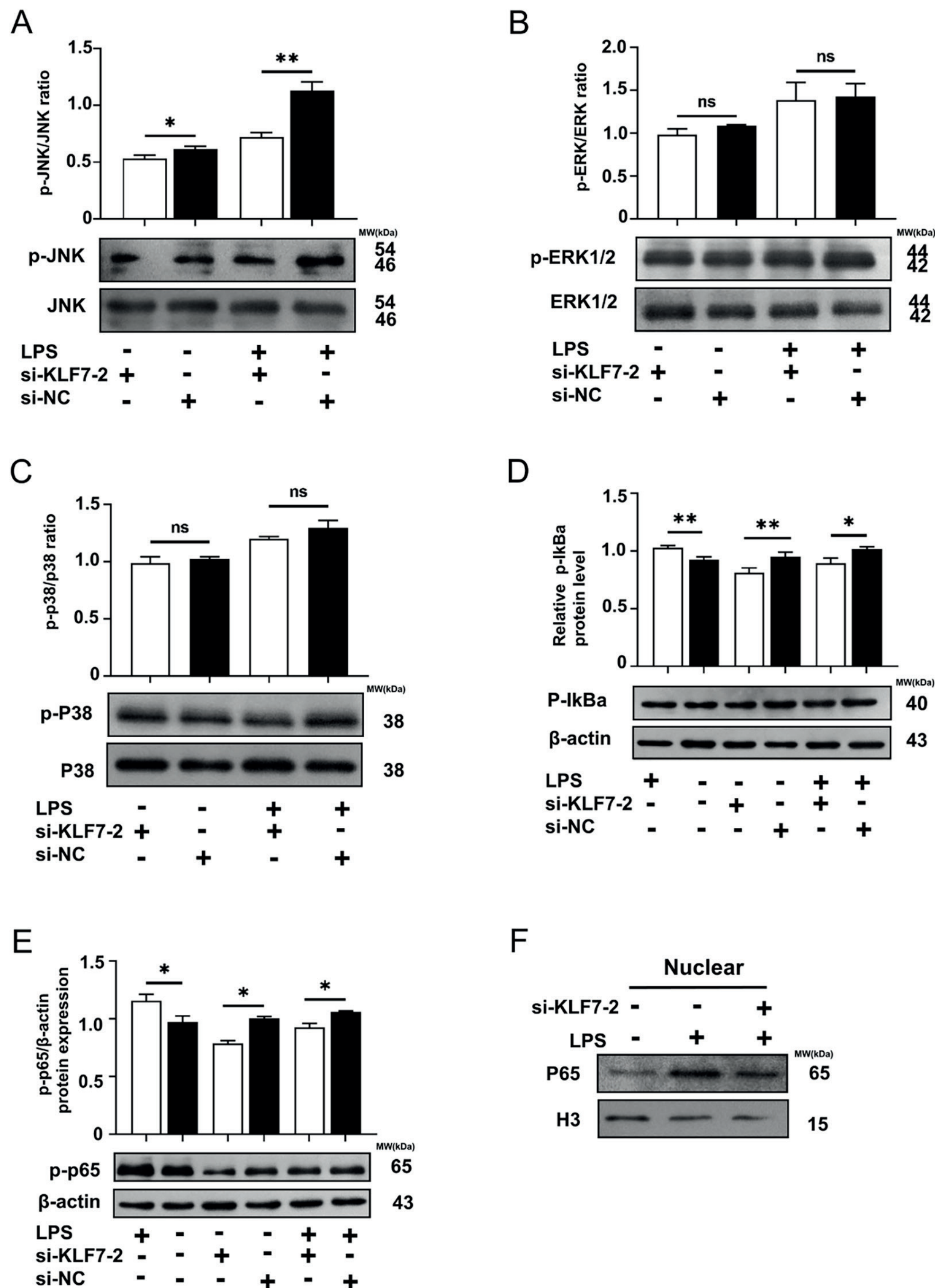

Figure 6. KLF7 facilitated the activation of MAPK and NF-κB signaling pathway. Human THP-1-derived macrophage were transiently transfected with si-KLF7-2 or si-NC for 24 h and then stimulated with or without 100 ng/mL LPS for 6h. Relative fold changes of phosphorylated protein levels were determined by densitometry and normalized to its total protein levels. **A,** The phosphorylation of JNK in si-KLF7-2 transfected human THP-1-derived macrophage. **B,** The phosphorylation of ERK1/2 in si-KLF7-2 transfected human THP-1-derived macrophage. **C,** The phosphorylation of p38 in si-KLF7-2 transfected human THP-1-derived macrophage. Data are presented as mean ± S.D. *$p<0.05$, **$p<0.01$ by Student's *t*-test. **D-E,** Human THP-1-derived macrophage were transiently transfected with si-KLF7-2 or si-NC. 24 h later, cells were stimulated with or without 100 ng/mL LPS for 6 h. Cell lysates were extracted and Western blots were carried out to detect phosphorylation of IκBα and p65. Relative fold changes of phosphorylated proteins was determined by densitometry and normalized to β-actin. Data are presented as mean ± S.D. *$p< 0.05$, **$p<0.01$ by Student's *t*-test. **F,** Human THP-1-derived macrophage were transiently transfected with si-KLF7-2 or si-NC for 24 h and then treated with or without 100 ng/mL LPS for 6 h, cell lysates were harvested and used to extract proteins from nucleus. Western blots were performed to detect protein expression level of p65 in the nucleus.

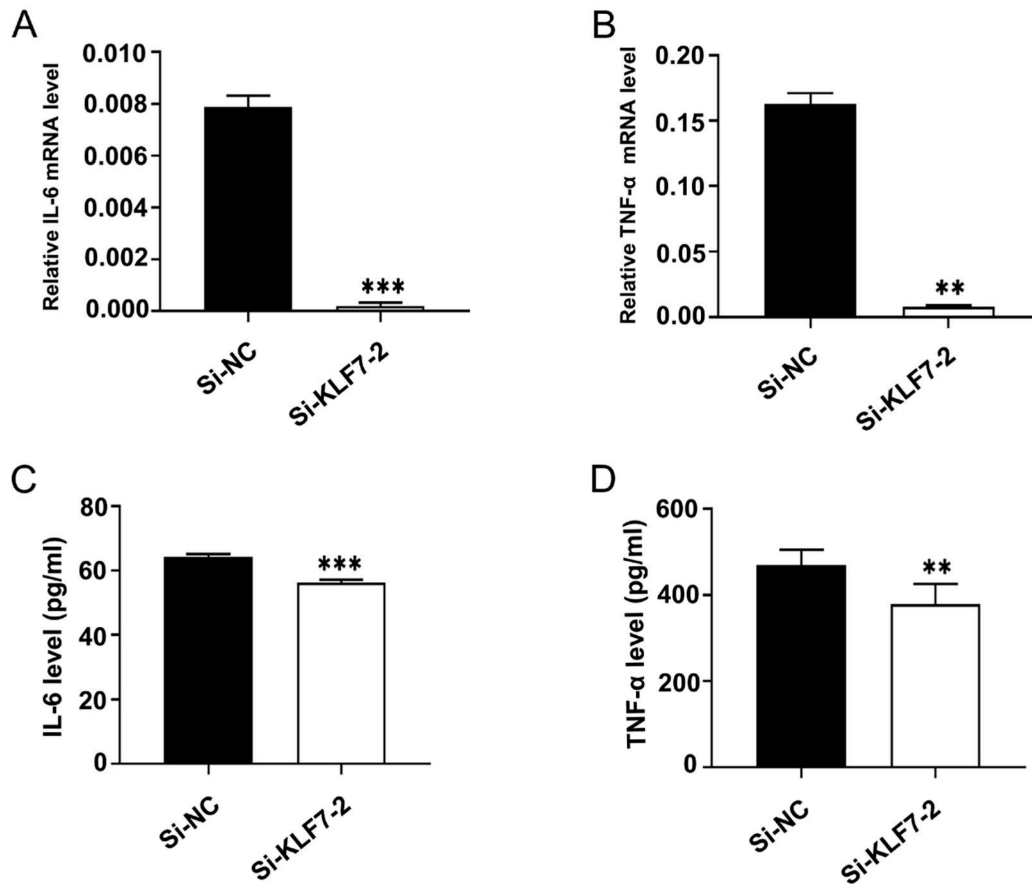

Figure 7. Effects of KLF7 on the LPS-induced IL-6 and TNF-α expressions in human THP-1-derived macrophage. Human THP-1-derived macrophage were transiently transfected with si-KLF7-2 or si-NC for 24 h and then treated with or without 100 ng/mL LPS for 6 h. The mRNA levels of IL-6 (**A**) and TNF-α (**B**) were evaluated by qRT-PCR, and the protein of IL-6 (**C**) and TNF-α (**D**) in the supernatants was determined by ELISA. Data are presented as mean ± S.D. $**p<0.01$, $***p<0.001$ by Student's *t*-test.

of patients without CAD, and protein levels of p-JNK and p-p65 were also higher than in the control. These results showed that TLR4, JNK, and NF-kB might play a key role in the inflammation of EAT in patients with CAD. EAT has a low level of adiponectin, which can inhibit the TLR4-mediated signaling pathway and suppress the pro-inflammatory cytokine secretion. This finding explains the high expression levels of TLR4 and the activation of the TLR4 signaling pathway in patients with CAD[20].

We, then, addressed the potential contribution of macrophages toward the increased inflammatory gene expression profile in the EAT of patients with CAD and patients without CAD. By utilizing CD68 mRNA expression as a marker for macrophages, we discovered that the EAT of patients with CAD showed high levels of CD68 mRNA expression compared with those of patients without CAD. These high levels might also influence the inflammatory status of EAT. Previous studies[3,24,27] have similarly highlighted increased macrophage infiltration in epicardial fat. The high level of macrophage infiltration/activation in this depot indicates that local inflammation occurs, and that macrophages play a crucial role in the inflammatory response in EAT[28]. Furthermore, our results showed that CD68 gene expression was closely correlated with the gene expressions of TLR4, IL-6, and TNF-α, suggesting that macrophages are the cause of this increased inflammation activation. The increased infiltration of macrophages induces local inflammation in EAT that eventually aggravates CAD progression in patients[20].

KLF7 is an ubiquitous Krüppel-like factor and has a wide range of effects on various tissues, including bone tissue, liver tissue, and pancreatic tissue. KLF7 also plays a role in cell differentiation[19]. KLF7 overexpression can regulate the expression of several adipocytokine genes in human adipose tissues and promote the release of IL-6[29]. The knockdown of KLF7 diminishes low-level TGF-β[30], which in turn induces the secretion of pro-inflammatory cytokines, including monocyte chemoattractant protein-1 and macrophage inflammatory protein-3α[31]. These findings indicate that KLF7 plays an important role in inflammatory responses. Furthermore, bioinformatics analysis showed that the promoter region of multiple CAD marker genes contains a KLF7 binding site; thus, KLF7 may influence the occurrence of cardiovascular diseases by regulating the expression of these marker genes[32]. In some conditions, the activation of TLR4-NF-κB pathways leads to the promotion of KLF7 expression[33]. This study's results found that in the EAT of the CAD group, the mRNA expression level of KLF7 was higher than that in the non-CAD group, and KLF7 was significantly positively correlated with TNF-α, TLR4, IL-6, JNK, and NF-kB. *In vitro* cell experiments showed that the THP-1 cells transfected with si-KLF7 had substantially decreased mRNA and protein expression levels of TNF-α and IL-6. Our results showed that KLF7 expression and the correlation of TNF-α, TLR4, IL-6, JNK, and NF-kB indices may contribute to inflammation in CAD EAT.

Notably, KLF7 appears to prevent LPS-induced inflammation *via* JNK-NF-κB, as evidenced by a dramatic decrease in the phosphorylation of p65, JNK, and IkBα. The question of how KLF7 regulates the NF-κB signal pathway remains to be answered. Clarifying the molecular mechanisms that regulate the production of inflammatory cytokines is crucial for understanding how KLF7 promotes phosphorylation in the NF-κB signaling cascade after LPS exposure. This suggests an avenue for future research.

Conclusions

The present study identified a new transcription factor, KLF7, which may be involved in chronic inflammatory responses in CAD EAT. The study found that high concentrations of LPS play an important role in the activation of MAPKs/NF-κB inflammatory signaling pathways by promoting the expression of KLF7 in human THP-1-derived macrophages. Exploring the specific molecular mechanism of KLF7 involvement in this process would provide a new target for the treatment of EAT inflammation.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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