MiRNA-6870-3p regulates LPS-induced epicardial adipose tissue inflammatory genes via targeting Tollip-mediated JNK and NF-κB signaling in coronary artery disease

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Research Article

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Abstract

Objective

MiR-6870-3p acts as a crucial regulator of gene expression at the posttranscriptional level and participates in immune responses. However, the roles of miR-6870-3p and its target genes and their underlying mechanisms in the inflammatory responses of epicardial adipose tissues (EATs) are unknown.

Methods

MiRNA microarray was used to collect miRNA expression profiles in EATs from five patients with coronary artery disease (CAD) and four individuals without CAD (n-CAD). Quantitative real-time polymerase chain reaction (qRT-PCR) was applied to check the expression of miR-6870-3p in CAD and n-CAD EATs. The mRNA and protein expression levels of Tollip and the key genes of the TLR4 signaling pathway were examined by qRT-PCR and Western blot. The levels of inflammatory factors in the cell supernatant were measured by enzyme-linked immunosorbent assay. Bioinformatics algorithms were used to predict the target gene of miR-6870-3p, and the result was validated by dual-luciferase reporter assay. The protein expression levels of JNK and NF-κB were measured by Western blot.

Resules:

Our results showed that miR-6870-3p was remarkably higher in CAD EATs than in n-CAD EATs. MiR-6870-3p was positively correlated with TLR4, IL-6, JNK, NF-κB (p65), and TNF-α in CAD EAT samples. Lipopolysaccharide (LPS) treatment upregulated miR-6870-3p mRNA expression and downregulated Tollip mRNA and protein expression in macrophages. MiR-6870-3p upregulation also aggravated the production of proinflammatory cytokines, such as TNF-α and IL-6, when the macrophages were stimulated with LPS. The result of luciferase reporter assays confirmed that miR-6870-3p directly targets Tollip. Moreover, miR-6870-3p upregulation in macrophages resulted in the activation of the JNK/NF-κB pathway.

Conclusion

Our study concluded that miR-6870-3p regulates human EAT inflammation by targeting Tollip-mediated JNK and NF-κB signaling events.

Introduction

Cardiovascular disease is a common disease and the major cause of death worldwide. Some studies confirmed that atherosclerosis is closely related to coronary artery disease (CAD) [1, 2]. Recent studies have found that visceral adipose tissue (VAT) plays a vital role in CAD pathogenesis through the

paracrine or endocrine pathway to secrete some adipokines, including pro-inflammatory and anti-inflammatory cytokines[3, 4]. In addition, the volume or thickness of cardiac fat is associated with cardiovascular disease[5]. Epicardial adipose tissue (EAT) is a subtype and one of the important components of VAT. EATs are close to the myocardium and warp coronary arteries without fibrous layer separation. EATs can secrete cytokines and chemokines when CAD occurs, which indicates that microcirculation changes take place during the development and progression of CAD[6]. The microcirculation connects EATs and the coronary adventitia via the vasa vasora[7]. A growing body of evidence supports that the inflammatory signaling pathway is activated in the EATs of patients with CAD[8]. According to clinical studies, inflammatory cytokines, particularly T-lymphocytes and proinflammatory M1 macrophages, have higher expression in CAD EATs than in non-CAD (n-CAD) EATs[9]. In addition, some changes, like oxidative stress and gene transcription regulation, have been found in CAD EATs and affect the pathophysiological processes of CAD.Although increasing evidence indicates the correlations of EATs and inflammation with CAD,the underlying molecular mechanisms remain to be further explored.

MiRNAs are a type of small, non-protein-coding RNAs. The function of miRNAs is to interfere with protein translation or reduce transcript levels[10]. MiRNAs can modulate multiple target genes by binding to the complementary sequences of mRNAs at 3' untranslated regions (UTRs)[11]. Many studies found that miRNAs are involved in human diseases, including inflammation[12, 13] Furthermore, some miRNAs play substantial roles in immune responses[14, 15]. MicroRNA-301a and microRNA-101-3p are downregulated to regulate Toll-like receptor (TLR)-triggered inflammatory response in rheumatoid arthritis monocytes or THP-1 cells[16],[17]. The upregulation of miR-92a and miR-146 can enhance LPS-stimulated inflammatory response in mouse macrophages or PC-12 cells[18]. EAT-related CAD physiology and pathology are associated with two types of miRNAs: circulating miRNAs and adipose tissue miRNAs[19]. CAT EATs contain lower miR-103-3p levels than subcutaneous adipose tissues in metabolically healthy cases as revealed by miRNA microarray analysis[19]. Conversely, inflammation-related miR-135b-3p is elevated in CAD EATs according to transcriptomic and miRNome analyses. Despite these strong associations between adipose tissue miRNAs and CAD, whether dysfunctional miRNAs take part in TLR-induced inflammatory responses remains unclear.

The present study aimed to elucidate the underlying target of miR-6870-3p and how Tollip regulates EAT inflammation and verify the potential inflammatory signaling pathway. The expression levels of miR-6870-3p and key genes in inflammatory signaling pathways were first determined. MiR-6870-3p and the key genes in inflammatory signaling pathways had higher expression levels in CAD EATs than in n-CAD EATs, which indicates that miR-6870-3p has a strong association with inflammation. Importantly, the results are consistent with the correlation analysis results. LPS treatment upregulated miR-6870-3p expression and downregulates Tollip mRNA in cells. MiR-6870-3p positively regulated LPS-stimulated inflammatory cytokine production by targeting Tollip-mediated JNK and NF-κB signaling pathways, which can promote inflammation accumulation in EATs.

Methods

Human samples

We collected sample of EAT biopsy samples (average weight of 0.5–1.0 g) from 12 newly diagnosed CAD and n-CAD and 12 individuals with n-CAD according to coronary angiography from January 2019 to December 2019. The patients with CAD who underwent on-pump coronary artery bypass grafting (CABG), and the patients with n-CAD who underwent valvular replacement surgery. The recruitment criteria were as follows: all patients didn't diagnose acute myocardial infarction and severe heart failure, also didn't receive any radiotherapy or chemotherapy therapy before surgery. Patients were excluded from the study if they had other diseases, for example active phase of infectious, hematologic diseases, rheumatologic diseases, steroid or immunomodulatory drug use. After the samples were collected from patients, we used cold PBS to wash blood in samples and putted in liquid nitrogen, and then stored at −80 °C until analysis. The human subject studies were approved and performed according to the standards established by the Ethics Committee on Human Subject Research at Central people's Hospital of Zhanjiang, Guangdong Medical University. We have complied with all relevant ethical regulations. Written informed consent was received from all human participants prior to inclusion in the study.

Cell culture

Human monocytes (THP-1) and human embryonic kidney (HEK) 293T cells were purchased from the Cell Bank of Chinese Academy of Sciences (Shanghai, China). THP-1 cells and HEK 293T cells were grown in RPMI 1640 medium with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 mg/mL) at 37 °C in a humidified atmosphere containing 5% CO2. Before the THP-1 cells were differentiated into macrophages, the cells were seeded in 6-well plates according to protocol and used 100 ng/mL phorbol-12-myristate 13-acetate (Sigma) for 24 h to differentiated into macrophages. After that the cells were incubated with 20 ng/mL IFN-γ (Gibco) and 10 ng/mL LPS(Sigma) for 24 h to further induce M1 macrophages before their use in experiments.

Quantitative real-time PCR

Total RNAs from human adipose tissue samples or cells were extracted using Trizol reagent (Invitrogen), then reverse-transcribed into complementary DNA (cDNA) using PrimeScript RT reagent Kit with gDNA Eraser (Takara Bio) according to the manufacturer's protocol. qRT-PCR was performed using Universal SYBR Green qPCR Supermix (US EVERBRIGHT INC) using Bio-Rad PCR System. The primers used for mRNA quantification were follows miR-6870-3p, forward 5'-ACGCTCATCCCCATCTCC-3', reverse 5'-CAGTGCGTGTCGTGGAGT-3'; IL-6, forward 5'-CTCTCCGCAAGAGACTTCCAG-3', reverse 5'-TGTGGGTGGTATCCTCTGTGA-3'; TNF-α, forward 5'-GTGACAAGCCTGTAGCCCAT-3', reverse 5'-TATCTCTCAGCTCCACGCCA-3'; TLR4, forward 5'-AATGCCAGGATGATGCCTCT-3', reverse 5'-AGGAAGTACCTCTATGCAGGG-3'; JNK, forward 5'-ACACCACAGAAATCCCTAGAAG-3', reverse 5'-CACAGCATCTGATAGAGAAGGT-3'; NF-kB (p65), forward 5'-CTGAGTCCTGCTCCTTCCA-3', reverse 5'-CTTCGGTGTAGCCCATTTGT-3'; Tollip, forward 5'-GGACAACGGTCAGCGACGCA-3', reverse 5'-

CATAGCCCAGACGCAGGCGG-3’; β-actin, forward 5’-AGGCCGGCTTCGCGGGCGAC-3’, reverse 5’-CAGGGGAGCACACGCAGCTC-3’; U6, forward 5’- GCTTCGGCAGCACATATACTAAAAT-3’, reverse 5’- CGCTTCACGAATTTGCGTGTCAT-3’. The relative expression levels of the genes were calculated using the $2^{-\Delta\Delta CT}$ method.

Plasmid construction

For Tollip overexpression, Tollip was directly cloned from human genomic DNA into pcDNA3.1 expression vector at the Hind III and Not I sites. The Tollip-3'-UTR was cloned from genomic DNA into psiCHECK-2 luciferase reporter vector at the Not I and XhoI sites. The Tollip-3'-UTR mutant was generated using four primers according to an overlapping PCR. All constructs were verified through plasmid DNA sequencing.

Transfection

MiRNA negative control (miR-con and anti-miR-con), miR-6870-3p mimics, and anti-miR-6870-3p were obtained from GenePharma (Shanghai, China). The macrophage cells were transfected with miR-6870-3p mimics, anti-miR-6870-3p, negative control and lipofectamine 3000 (Invitrogen, Germany) in accordance with the manufacturer’s protocol.

Luciferase reporter assay

HEK 293T cells were cultured in a 24-well plate at a density of 70% and then the cells were co-transfected with miRNA-6870-3p mimics or controls and luciferase reporter plasmids using lipofectamine 3000 reagent (Invitrogen). Cell extracts were prepared 24 h after transfection, and the relative luciferase activities were measured using a Dual Luciferase Reporter Assay Kit (Promega).

Western blotting

Total proteins were extracts from cells and tissue were prepared using PMSF phosphatase inhibitors and RIPA lysis buffer. We used bicinchoninic acid method to detected the protein concentrations. Approximately 30 μg to 50 μg of total protein was separated using SDS-PAGE on 10% gels, and then transferred onto polyvinylidene fluoride membranes. Membrane was blocked with 5% skim milk for 2 h at room temperature and incubated at 4 °C overnight with primary antibodies were followed by incubations with the appropriate horseradish peroxidase-conjugated secondary antibodies. Antibodies used in the study were β-actin (ZSGBBIO, China), Tollip (Abcam, USA), p-IκBα (Cell Signal Technology, USA), JNK (Cell Signal Technology, USA), p-JNK (Cell Signal Technology, USA), p-p65 (Cell Signal Technology, USA), mouse IgG and rabbit IgG (ZSGBBIO, China).

Enzyme-linked immunosorbent assay (ELISA)

After the cells stimulation with LPS at the indicated time points, the cell culture supernatants were collected and then assessed the level of TNF-α and IL-6 using human IL-6 and TNF-α, human IL-6 ELISA and TNF-α kits (Invitrogen). Assays were performed in accordance with the manufacturer’s protocols.

Statistical Analysis

All the data were expressed as mean ± SEM from at least three independent experiments. The differences between two groups were analyzed by Student's *t*-test. Correlations in gene expression was assessed by Spearman's rank correlation coefficient for non-normally distributed data. GraphPad Prism 8 statistical software package (GraphPad Software, USA) and SPSS version 20 were used for data analysis. $P<0.05$ was considered as statistically significant.

Results

Differential profiling of EAT by miRNA microarray analysis

A microarray analysis of EATs was performed to identify the miRNA expression patterns from five patients with CAD and four matched n-CAD individuals (controls). The most diferentially expressed miRNAs were selected according to the following criteria: $-1.5 \geq$ fold change ≥ 1.5 and false discovery rate-adjusted $P \leq 0.05$. Based on this analysis, 12 differentially expressed miRNAs were selected (Fig. 1A). Among them, five (has-miR-6870-3p, has-miR-4429, has-miR-4687-3p, has-miR-5703, and has-miR-630) were obviously upregulated and seven (has-miR-3651, has-miR-664b-3p, has-miR-619-5p, has-miR-564, has-miR-8485, has-miR-125-5p, and has-miR-574-3p) were downregulated in CAD EATs compared with n-CAD EATs ($P<0.05$). Moreover, pathway analysis (Fig. 1B) and Gene Ontology (GO) analysis (Fig. 1C) were also applied to analyze the differentially expressed mRNAs. GO analysis indicated the most substantial biological processes, cellular components, and molecular function. Pathway analysis indicated that the most substantial pathways consisted of the TLR signaling pathway, TGF-beta signaling pathway, and mTOR signaling pathway. According to the pathway analysis results, we selected the classical pathway related to inflammatory response: "TLR signaling pathway." In summary, according to the microarray results and bioinformatics analysis, we identified signal molecules related to the TLR signaling pathway as the target of interest.

Levels of miR-6870-3p and inflammation-related factors in CAD and n-CAD EATs

MiR-6870-3p levels in human EATs from healthy controls (n-CAD, n = 12) and patients with CAD (n = 12) were examined to determine the relationship between EAT and CAD. The mRNA expression level of miR-6870-3p was markedly elevated in CAD relative to controls (Fig. 2A). The mRNA expression levels of TLR4, IL-6, JNK, NF-κB (p65), and TNF-α were also remarkably higher in CAD than in n-CAD (Figs. 2B–F).

Correlation of miR-6870-3p, TLR4, and EATinflammation indices in CAD

The relationship between miR-6870-3p and inflammation indices was investigated. The results of the correlation analysis showed the remarkably positive correlations of miR-6870-3p level with TLR4, IL-6, TNF-α, JNK, and NF-κB (p65) (Figs. 3A–E). These findings suggest that high miR-6870-3p expression plays an important role in EAT inflammation.

MiR-6870-3p regulates the production of proinflammatory cytokines in macrophages after TLR4 stimulation

Macrophages were transfected with miR-6870-3p mimics, inhibitors, or their controls, and the production of proinflammatory cytokines was analyzed to evaluate the role of miR-6870-3p in the regulation of immune responses to inflammatory stimulation. MiR-6870-3p overexpression remarkably increased LPS-stimulated TNF-α and IL-6 production at the mRNA and protein levels (Figs. 4A–D). By contrast, the mRNA and protein expression levels of TNF-α and IL-6 decreased when miR-6870-3p expression was inhibited (Figs. 4E–H), indicating the suppression of LPS-induced inflammation. Together, these data suggest that miR-6870-3p is a regulator of the TLR4-induced production of inflammatory cytokines, such as IL-6 and TNF-α, in macrophages.

MiR-6870-3p targets Tollip

TargetScan 7.2 (www.targetscan.org) was used to determine the putative target genes of miR-6870-3p to predict the effective target sites of miR-6870-3p (Fig. 5A). Previous studies have shown that Tollip, a negative regulator of the TLR signaling cascade, directly binds to the IL-1RI complex and is implicated in the suppression of the TLR4 pathways[20]. In the present study, the mRNA and protein expression levels of Tollip were lower in the CAD group than in the n-CAD group (Figs. 5B and C). Tollip protein level in macrophages transfected with miR-6870-3p or anti-miR-6870-3p was examined to assess whether miR-6870-3p regulates Tollip expression. MiR-6870-3p overexpression remarkably reduced Tollip expression (Fig. 5D), whereas anti-miR-6870-3p increased the Tollip protein level (Fig. 5E). Tollip was remarkably negatively correlated with miR-6870-3p in the CAD group (Fig. 5F). Next, a luciferase reporter assay was conducted to obtain a direct evidence that Tollip is targeting the 3' UTR of miR-6870-3p, Luciferase reporter vectors were generated by cloning the wild-type or mutated 3' UTR of Tollip mRNA. The reporter plasmids and miR-6870-3p mimics were co-transfected into HEK 293T cells. The results showed that miR-6870-3p overexpression resulted in a remarkable decrease in the luciferase activity of the luciferase reporter that contains the wild-type 3' UTR of Tollip mRNA compared with the set of cells transfected with miR-NC. However, this effect was abolished in the cells transfected with a luciferase reporter that contains the mutant 3' UTR of Tollip mRNA (Fig. 5G). The findings suggest that miR-6870-3p binds to the 3' UTR of Tollip to inhibit its expression.

Effect of miR-6870-3 on the LPS-induced activation of the JNK/NF-κB pathway

Tollip overexpression impedes the TLR4-triggered JNK and NF-κB signaling pathways[21]. We tested whether LPS stimulation could modulate miR-6870-3p and Tollip levels in macrophages. Interestingly, LPS stimulation suppressed Tollip expression at the mRNA and protein levels in a time-dependent manner (Fig. 6A), whereas the expression of miR-6870-3p increased correspondingly (Figs. 6B and C). The effect of miR-6870-3p mimics on the activation of the JNK/NF-κB pathway was investigated next. The cells were transfected with miR-6870-3p mimic and then stimulated with LPS. JNK, p-JNK, p-IκBα, and p-p65 levels were detected by Western blot analysis. As expected, the cells treated with

miR-6870-3p mimics had markedly increased phosphorylation of JNK, p-IκBα, and p-p65 (Figs. 6D–F), which suggests that miR-6870-3p modulates the LPS-activated JNK/NF-κB pathway that contributes to the inflammatory phenotype.

Blocking Tollip abolishes LPS-induced inflammatory responses

Our results showed that the mechanisms by which miR-6870-3p regulates LPS-triggered proinflammatory cytokine production include targeting Tollip in macrophage and knocking down Tollip expression through lentivirus transfection. Tollip expression was remarkably reduced at the mRNA and protein levels (Figs. 7A and B). Interestingly, Tollip downregulation considerably increased the LPS-triggered expression of IL-6 and TNF-α at the mRNA and protein levels in macrophages (Figs. 7C–F). The result suggests that Tollip plays a vital role in the regulation of inflammatory response. The cells were transfected with control mimics combined with vector, miR-6870-3p mimics combined with vector, or miR-6870-3p mimics combined with Tollip to establish the relationship between miR-6870-3p and Tollip(Figs7.G-H). The results suggest that the proinflammatory cytokines were remarkably increased by miR-6870-3p mimics and that this effect could be blocked by Tollip upregulation.

Discussion

This study demonstrated that miR-6870-3p regulates inflammatory response via targeting Tollip-mediated inflammatory signaling pathways in human macrophages. We found that miR-6870-3p was upregulated after LPS stimulation, which selectively downregulated Tollip in macrophages and resulted in the overactivation of JNK/NF-κB p65 and the consequent inflammatory cytokine production. MiR-6870-3p expression was remarkably higher in EAT from the CAD group compared with the n-CAD group. Moreover, miR-6870-3p upregulation aggravated the production of proinflammatory cytokines, such as TNF-α and IL-6, when the macrophages were stimulated with LPS. Conversely, miR-6870-3p inhibition attenuated the production of proinflammatory cytokines in macrophages after LPS stimulation. Furthermore, Tollip expression was lower in CAD EATs than in n-CAD EATs. The inverse correlation between miR-6870-3p and Tollip in CAD EAT indicates that miR-6870-3p may regulate Tollip expression in EATs from patients with CAD. Moreover, miR-6870-3p overexpression suppressed Tollip expression in macrophages, whereas miR-6870-3p knockdown remarkably increased Tollip expression. The result of luciferase reporter assays confirmed that miR-6870-3p directly targets Tollip. Moreover, miR-6870-3p upregulation in macrophages resulted in the activation of the JNK/NF-κB pathway and the subsequent inflammatory cytokine production. Finally, Tollip overexpression in macrophages remarkably decreased the LPS-triggered production of TNF-α and IL-6. Overall, our study revealed that LPS-induced miR-6870-3p expression regulates inflammatory responses by targeting the Tollip/JNK/ NF-κB signaling axis in macrophages.

The differentially expressed miRNAs, including upregulated miRNAs (miR-6870-3p, miR-4429, miR-4687-3p, miR-5703, and miR-630) and downregulated miRNAs (miR-3651, miR-664b-3p, miR-619-5p, miR-564, miR-8485, miR-125-5p, and miR-574-3p), were identified according to the microarray analysis data of

EATs from the CAD and n-CAD groups. Interestingly, the pathway analysis revealed that miR-6870-3p is involved in the TLR signaling pathway. Expectedly, TLR4 and the key genes of the inflammatory signaling pathway were higher in CAD EATs. This result is similar to the results of previous studies[21],[22, 23]. MiR-6870-3p was positively correlated with TLR4, IL-6, JNK, NF-κB (p65), and TNF-α in EAT samples from CAD patients, which indicated that miR-6870-3p plays a critical role in TLR-induced inflammatory response signaling, especially in the TLR4 signaling pathway. The TLR4 signaling pathway was activated and then triggered the translocation of NF-κB into the nucleus, which induced the overexpression of proinflammatory factors[24]. Faccini et al. applied circulating miRNAs, including miR-155, miR-145, and let-7c, to distinguish patients with CAD from n-CAD control subjects and identified the potential diagnostic value of circulating miRNA with CAD[25]. Inflammation-related miRNAs (miR-155, miR-146a, and miR-124) can regulate the inflammatory response in LPS-treated macrophages[26]. Currently, the roles and functions of miRNAs during disease development and progression remain unknown. In our study, miR-6870-3p overexpression using miR-6870-3p mimics further increased the production of proinflammatory cytokines in macrophages after LPS stimulation. Conversely, miR-6870-3p knockdown eliminated the inflammatory response of macrophages to LPS stimulation. Furthermore, miR-6870-3p expression increased after LPS stimulation in macrophages. Our results suggested that LPS stimulation increased the levels of miR-6870-3p, which resulted in the accumulation of TNF-α and IL-6 in macrophages.

Another interesting finding in this study was the identification of Tollip as a direct target of miR-6870-3p. Tollip is a conserved gene that participates in the TLR signaling pathway and is considered an important negative regulator in host innate immunity[27, 28]. Tollip deficiency could resist apoptosis when primary coelomocytes are stimulated with LPS[29]. Importantly, the role of Tollip was also reported in inflammation, endoplasmic reticulum stress, and innate immune responses[30]. In *Salmonella* infection, SE or LPS induces an increase in miR-1306-5p levels, which activates the production of inflammatory cytokines by inhibiting Tollip[31]. Moreover, Tollip protects against NLRP3 inflammasome activation by inhibiting TLR2/4-NF-κB signaling, which provides a potential treatment for acute kidney injury after paraquat intoxication[32]. We found that miR-6870-3p negatively regulates Tollip expression, that is, miR-6870-3p expression increased with the inhibition of Tollip in macrophages. Interestingly, we also found a negative correlation between miR-6870-3p and Tollip in human EATs from patients with CAD. Furthermore, the level of Tollip remarkably decreased in macrophages after LPS stimulation. Indeed, miR-6870-3p overexpression suppressed Tollip expression, whereas miR-6870-3p inhibition increased Tollip expression in macrophages. These results indicated that Tollip levels are regulated by miR-6870-3p.In our present work, we demonstrated that Tollip overexpression negatively regulates the production of TNF-α and IL-6 after LPS treatment in macrophages. The results were consistent with a previous report that Tollip overexpression attenuates PQ-induced lung injury likely via the reduction of inflammation and the suppression of NF-κB signaling pathway activation[33]. However, the role of Tollip in the production of EAT inflammation has remained unclear. In addition, we found that the expression of proinflammatory cytokine was remarkably increased by miR-6870-3p overexpression and that this effect could be

abolished by increasing Tollip. Our results clearly showed that LPS stimulates the proinflammatory mechanisms by stimulating miR-6870-3p expression and inhibiting Tollip-mediated cell signaling, which promote inflammatory production in EATs. Mitogen-activated protein kinases (MAPKs) are key inflammatory pathway signaling molecules associated with cytokines, stress, and inflammation[34]. The MAPK signaling pathway includes extracellular signal-regulated kinase (ERK), c-jun N-terminal kinase (JNK), and p38 MAPK, which regulate inflammatory response in macrophages in LPS stimulation[35]. Earlier investigations proved that NF-κB and JNK are upregulated in CAD EATs compared with n-CAD EATs[36, 37]. In support of these findings, our data clearly indicate that LPS stimulates proinflammatory mechanisms via JNK/NF-κB activation in macrophages. Thus, miR-6870-3p likely promotes the LPS-induced production of inflammatory cytokines by targeting Tollip via the JNK/NF-κB pathway. In summary, our study revealed that miR-6870-3p is upregulated in macrophages after LPS stimulation, which resulted in the overactivation of Tollip/JNK/NF-κB signaling and the increase in the production of proinflammatory cytokines. Thus, our data suggest that miR-6870-3p regulates inflammatory genes by targeting Tollip-mediated JNK and NF-κB signaling events in human EATs.

Declarations

Ethics approval and consent to participate

The human subject studies were approved and performed according to the standards established by the Ethics Committee on Human Subject Research at Central people's Hospital of Zhanjiang, Guangdong Medical University. We have complied with all relevant ethical regulations. Written informed consent was received from all human participants prior to inclusion in the study.All methods were carried out in accordance with relevant guidelines and regulations

Consent for publication

Not Applicable

Availability of data and materials

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

Competing interests

All authors have no competition interests in this article

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Figures

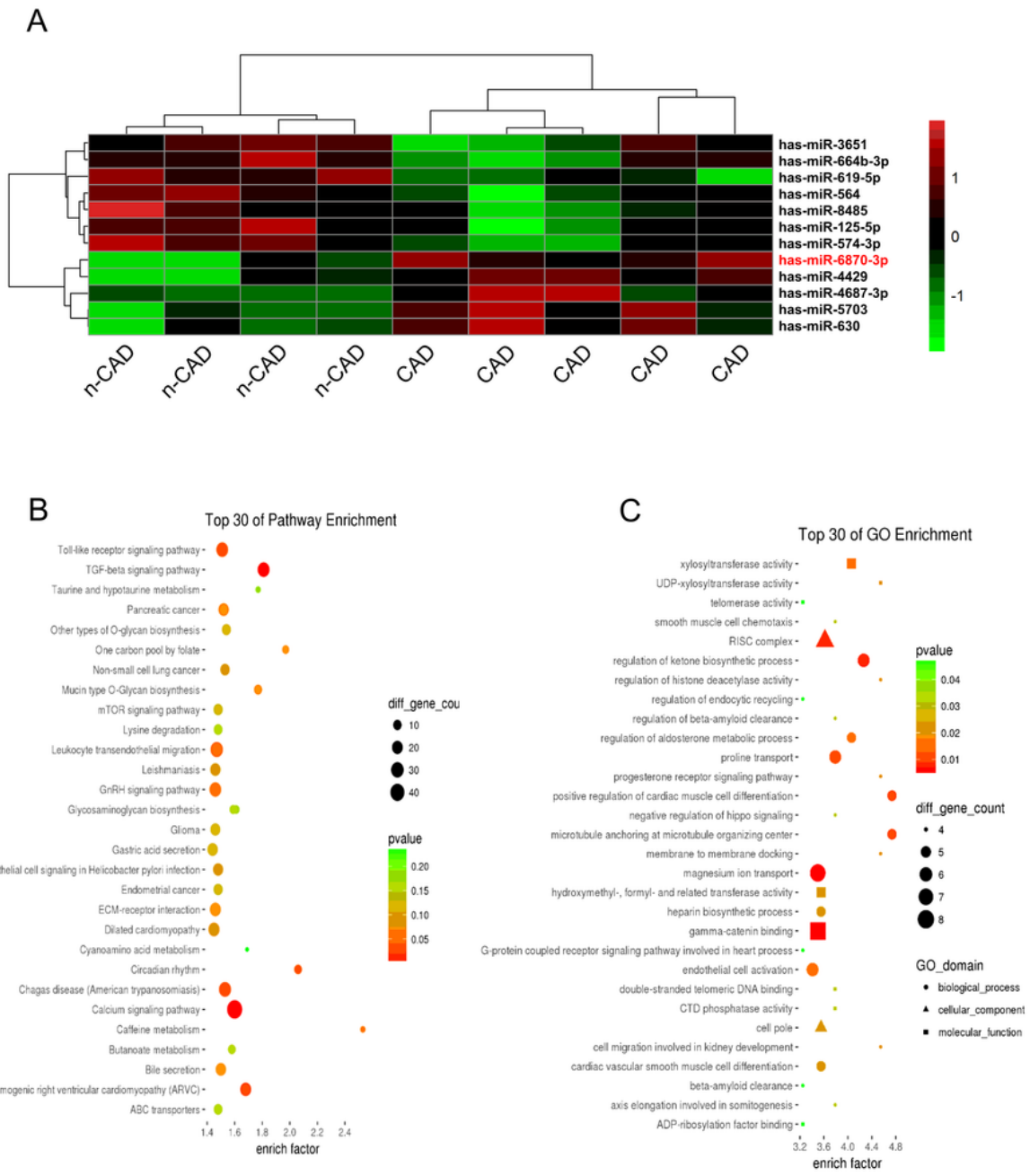

Figure 1

Identification of differentially expressed miRNAs in human EATs.

(A) MiRNA expression profiles in EATs from patients with or without CAD. Red color denotes higher expression in the CAD group, and green color denotes lower expression relative to the CAD group. (B) KEGG pathway analysis for the identification of the pathways enriched for differentially expressed genes

in CAD and n-CAD EATs. (C) GO analysis for the identification of the biological processes enriched for differentially expressed genes in CAD and n-CAD EATs.

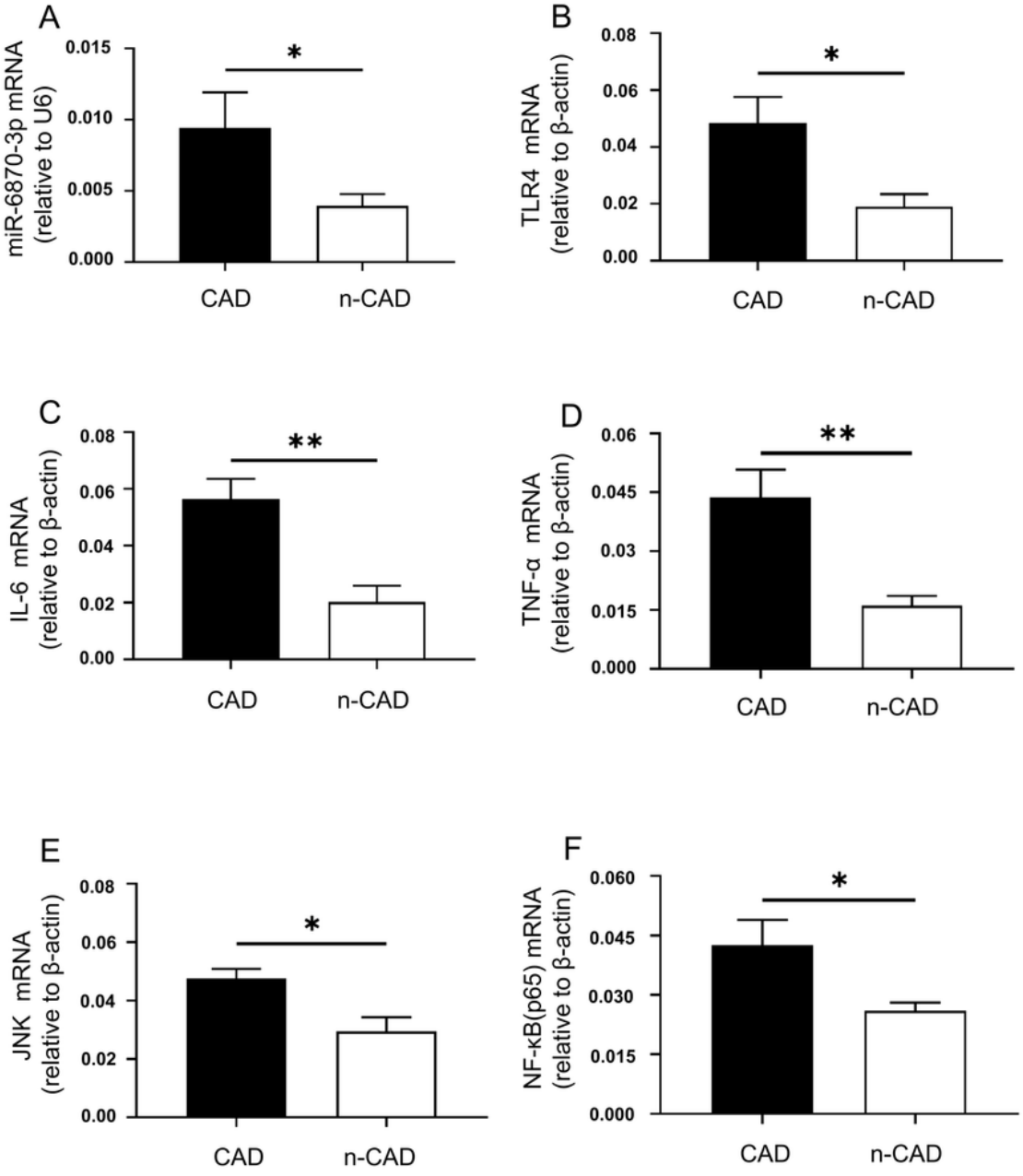

Figure 2

Expression of miR-6870-3p and key genes related to inflammatory signaling pathways in CAD and n-CAD EATs.

(A–F) MRNA expression levels of (A) miR-6870-3p, (B) TLR4, (C) IL-6, (D) TNF-α, (E), JNK, and (F) NF-κB (p65) in CAD and n-CAD EATs determined by qRT-PCR. Data are the mean ± SEM of one representative experiment. *$P \leq 0.05$ and **$P \leq 0.01$.

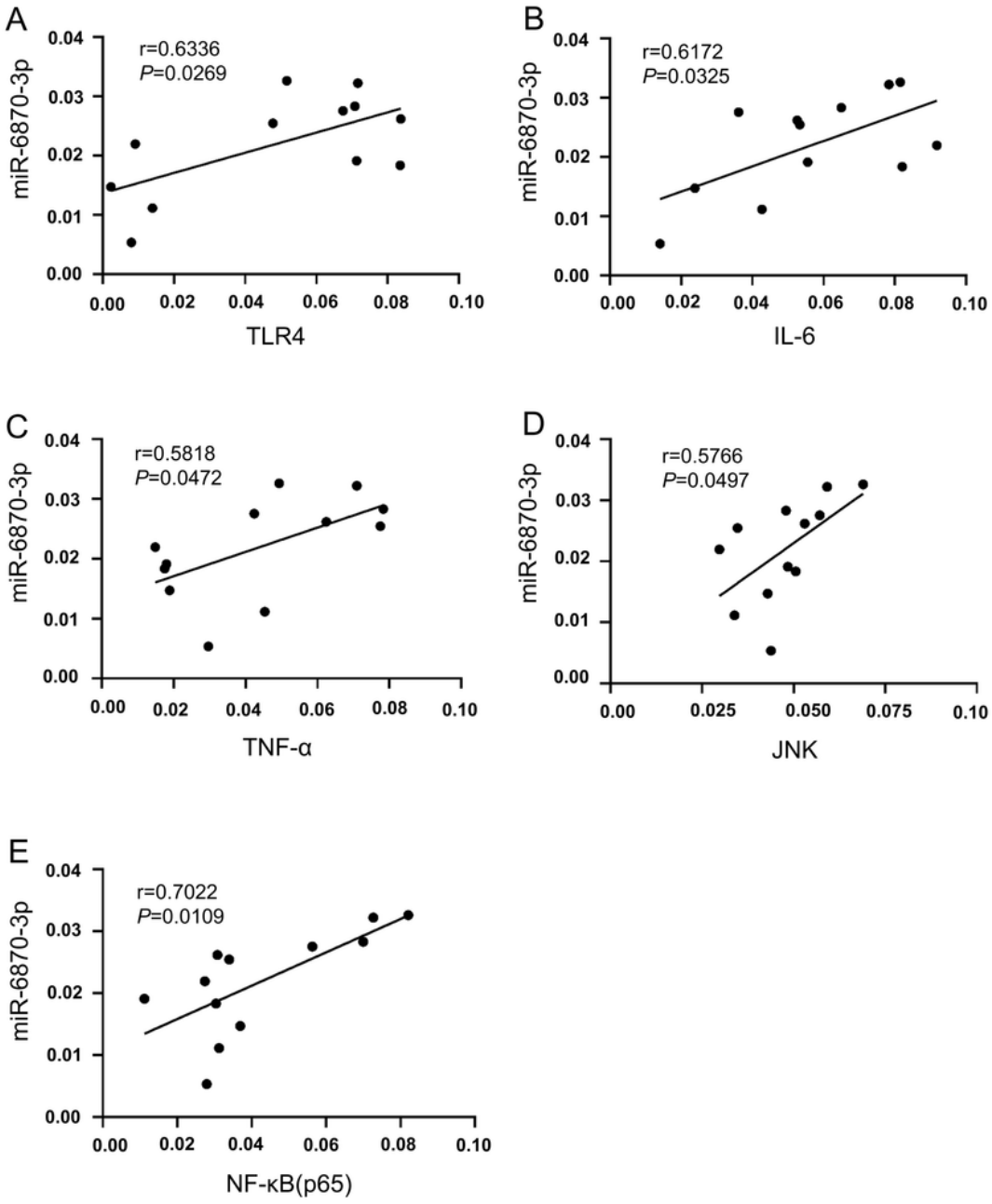

Figure 3

Association of miR-6870-3p with key genes related to inflammatory signaling pathways in CAD EATs.

(A–E) Linear correlation patterns of the significantly positive relationships of miR-6870-3p expression with (A) TLR4, (B) IL-6, (C) TNF-α, (D) JNK, and (E) NF-κB (p65). Person correlation analysis, correlation coefficients, and associated *P* values are shown on each plot. *$P \leq 0.05$ indicates that the correlation between two factors has statistical significance.

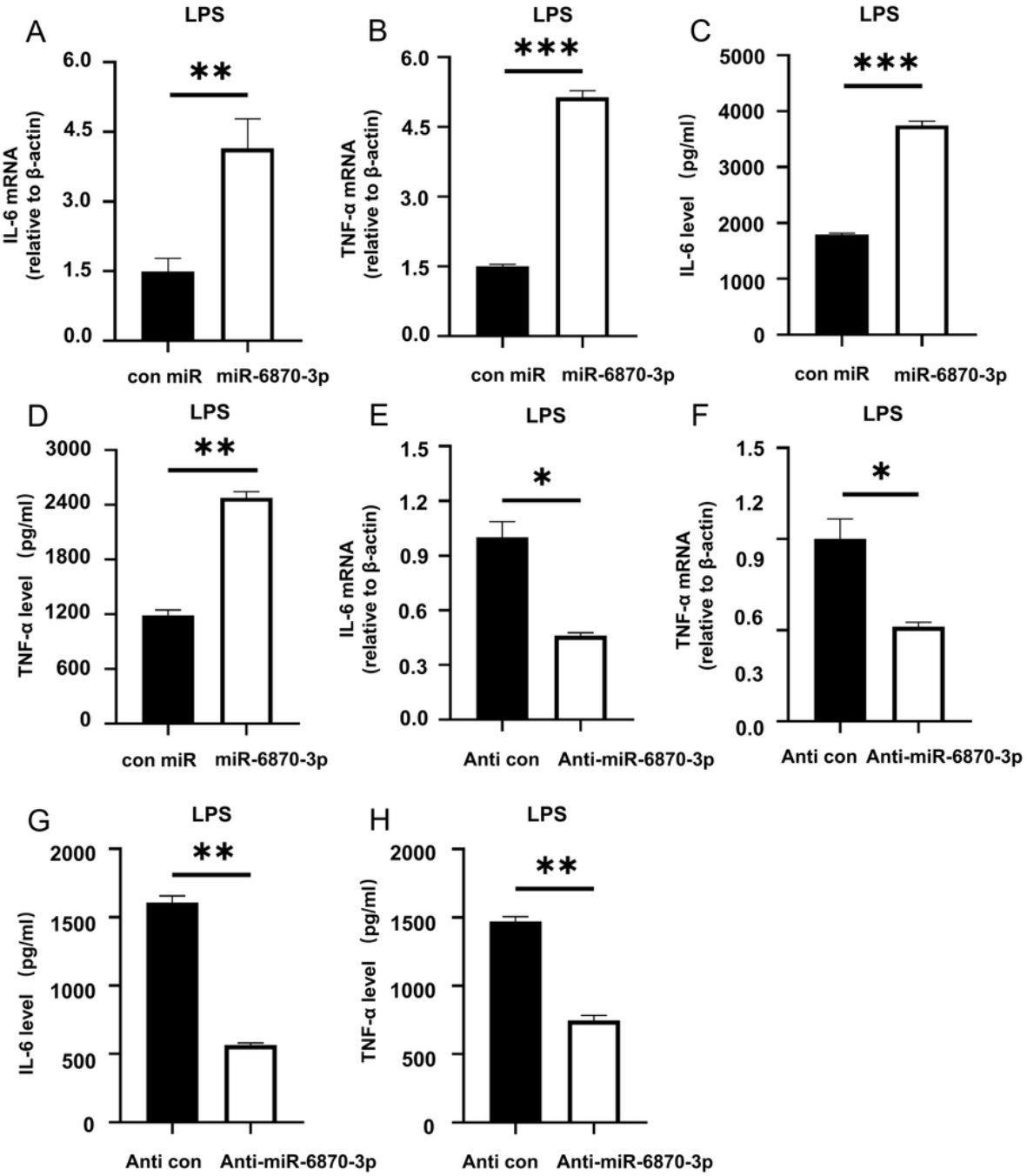

Figure 4

MiR-6870-3p is an important regulator of LPS-stimulated inflammatory responses in macrophages.

(A) IL-6 and (B) TNF-α mRNA levels evaluated by qRT-PCR and (C) IL-6 and (D) TNF-α protein levels determined by ELISA in cells transfected with control mimics or miR-6870-3p mimics for 48 h and cultured with LPS for 6 h. (E) IL-6 and (F) TNF-α mRNA levels evaluated by qRT-PCR and (G) IL-6 and (H) TNF-α protein levels determined by ELISA in cells transfected with control inhibitor or miR-6870-3p inhibitor for 48 h and cultured with LPS for 6 h. Data are the mean ± SEM of one representative experiment. Similar results were obtained in at least three independent experiments. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$.

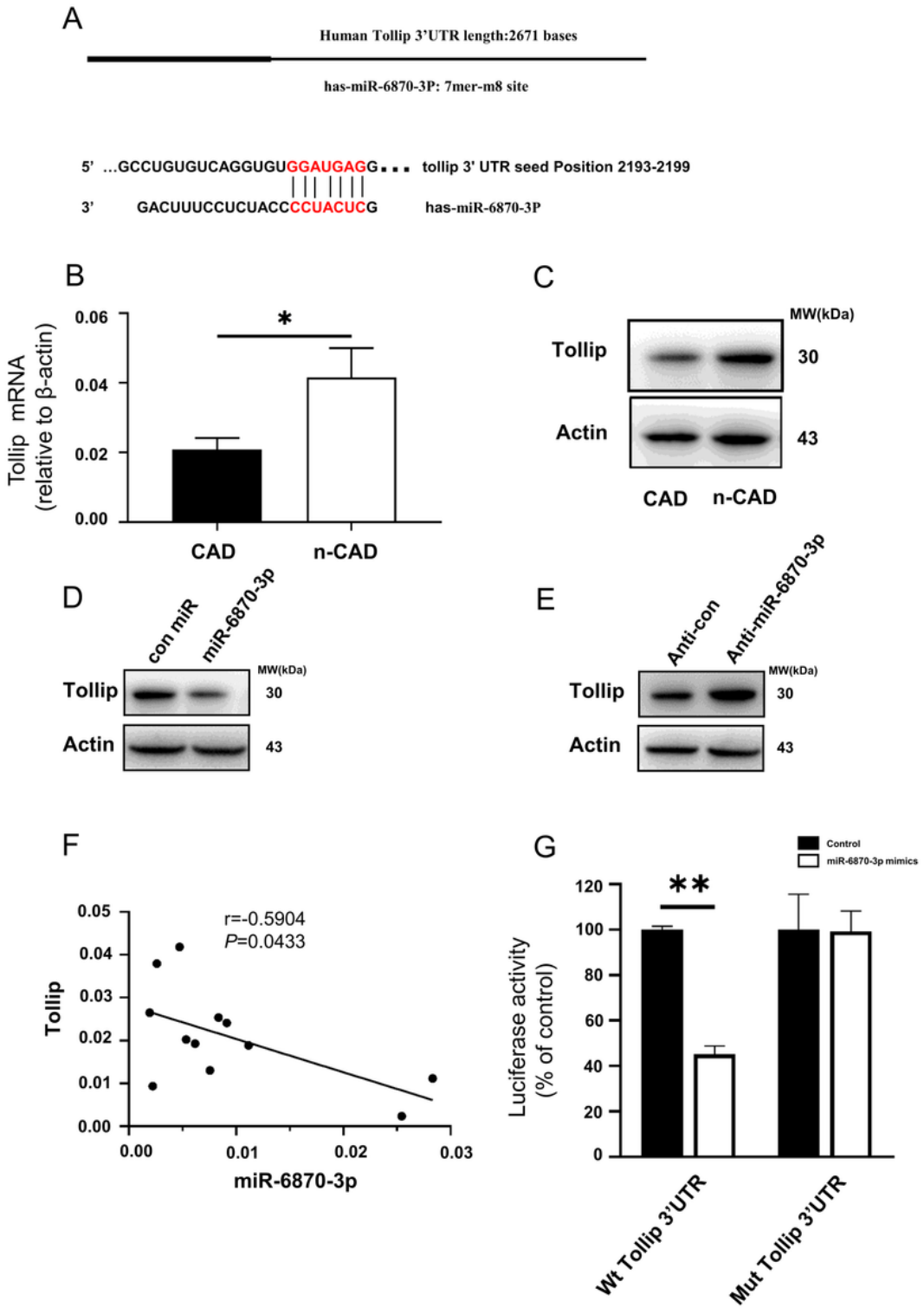

Figure 5

Tollip is a target of miR-6870-3p.

(A) Tollip mRNA was identified as a potential miR-6870-3p target by TargetScan. Putative miR-6870-3p and Tollip mRNA 3' UTR alignments are highlighted with red boxes. (B) Gene expression level of Tollip in CAD and n-CAD EATs. (C) Protein expression level of Tollip in CAD and n-CAD EATs. (D) Protein

expression of Tollip in cells transfected with control or miR-6870-3p mimics. (E) Protein expression of Tollip in cells transfected with control or anti-miR-6870-3p. (F) Correlation analysis between miR-6870-3p and Tollip expression in CAD EATs. (G) Luciferase activity in HEK293 cells co-transfected with the vector carrying the wild-type or mutant portion of the 3' UTR of Tollip and the control or miR-6870-3p mimics. Data are the mean ± SEM of one representative experiment. Similar results were obtained in at least three independent experiments. *$P \leq 0.05$ and **$P \leq 0.01$.

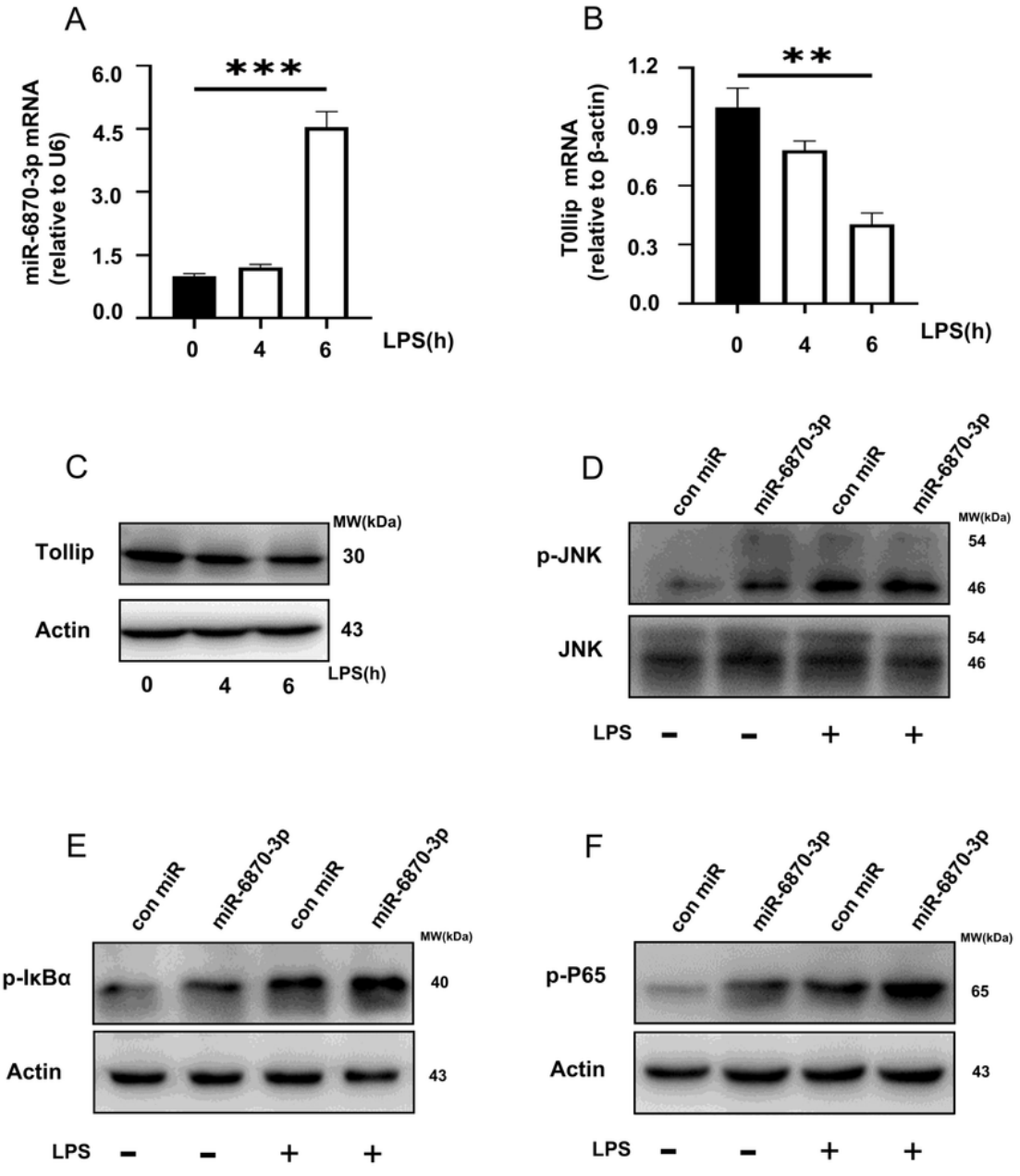

Figure 6

MiR-6870-3p regulates LPS-induced JNK activation

(A) MiR-6870-3p and (B) Tollip mRNA levels measured by qRT-PCR and (C) Tollip protein levels determined by Western blot in macrophages stimulated with the same LPS dose for the indicated length of time. Protein levels of (D) p-JNK and JNK, (E) p-IκBα, and (F) p-p65 determined by Western blot in macrophages transfected with control mimics or miR-6870-3p mimics for 48 h and treated with LPS for 6 h. Data are the mean ± SEM of one representative experiment. Similar results were obtained in at least three independent experiments. *$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$.

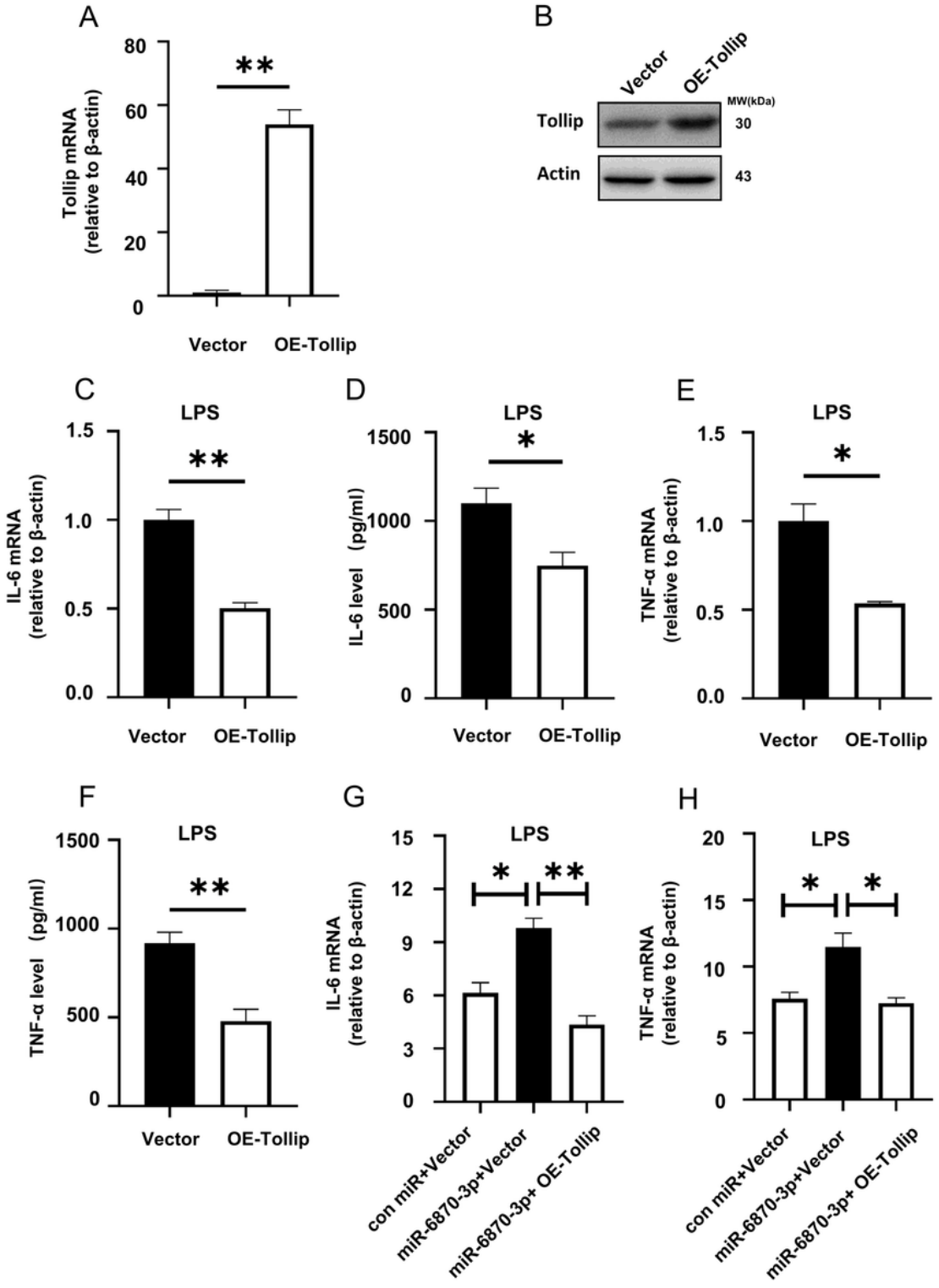

Figure 7

Downregulation of Tollip enhances inflammatory response to LPS stimulation in THP-1 macrophage.

(A) MRNA expression of Tollip measured by qRT-PCR and (B) protein levels of Tollip determined by Western blot in cells transfected with vector or OE-Tollip for 48 h. (C) IL-6 and (E) TNF-α mRNA levels evaluated by qRT-PCR and (D) IL-6 and (F) TNF-α protein levels determined by ELISA in cells transfected

with vector or OE-Tollip for 48 h and treated with LPS for 6 h. (G) IL-6 and (H) TNF-α mRNA levels evaluated by qRT-PCR in cells transfected with control miRNA combined with vector, miR-6870-3p mimics combined with vector, or miR-6870-3p mimics combined with OE-Tollip for 4 8h and cultured with LPS for 6 h. Data are the mean ± SEM of one representative experiment. Similar results were obtained in at least three independent experiments. *$P \leq 0.05$ and **$P \leq 0.01$.