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LPS induces CXCL16 expression in HUVECs through the miR-146a-mediated TLR4 pathway

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ABSTRACT

Endothelial inflammation characterizes the early stages of atherosclerosis. CXCL16 is a protein that functions as both a chemokine and adhesion molecule, playing a crucial role in the pathogenesis of atherosclerosis. However, it is uncertain if LPS, a major inducer of inflammation, affects CXCL16 expression in endothelial cells and whether miR-146a, a negative regulator of atherosclerosis, participates in this process. The present study showed that exposure of human umbilical vein endothelial cells (HUVECs) to LPS induced the overexpression of CXCL16, TLR4 and NF-κB, and this induction was blocked by the TLR4 inhibitor TAK-242. In addition, LPS induced the upregulation of miR-146a in HUVECs. Overexpression or inhibition of miR-146a either inhibited or increased the LPS-induced expression CXCL16, TLR4 and NF-κB protein production, respectively. Additionally, miR-146a-induced CXCL16 expression was blocked by TAK-242. Thus, in this study, we demonstrate that LPS stimulates CXCL16 expression via the TLR4/NF-κB signaling pathway, and simultaneously, miR-146 negatively regulates LPS-induced CXCL16 expression through a TLR4-dependent mechanism.

1. Introduction

Atherosclerosis is a chronic inflammatory disease that is characterized by endothelial cell dysfunction during its early stages. This dysfunction induces the expression of a series of inflammatory cytokines [1,2]. CXCL16 is expressed in endothelial cells and is known to act as a chemokine and adhesion molecule [3,4]. CXCL16 was shown to be present in the atherosclerotic lesions of both humans and apoE-deficient mice [5,6]. Previous clinical studies in our laboratory demonstrated that the serum levels of CXCL16 increase during atherosclerotic ischemic stroke [7] and are higher in patients who exhibit microembolic signals (MES) relative to MES-negative patients [8,9]. This finding suggests that CXCL16 is closely associated with the inflammatory response in atherosclerosis.

MicroRNAs (miRNA) are small, noncoding RNAs that negatively regulate genes by binding to the 3′ untranslated region (3′ UTR) of target mRNAs at the post-transcriptional level. These transcripts regulate vessel wall inflammation associated with the initiation and development of atherosclerosis [10,11]. Among known miRNAs, miR-146a plays an important role in the inflammatory response. This transcript is an early response gene induced by various inflammatory mediators in the early phase of disease [12,13]. Importantly, a previous study from our laboratory suggested that miR-146a may negatively regulate CXCL16 expression during atherosclerosis in vivo [14].

LPS, a major component of the cell walls of Gram-negative bacteria, is a potent inducer of the inflammatory response. Previous studies have shown that LPS induces the expression of CXCL16 in vivo, in human macrophages and in smooth muscle cells [15–17]. Bacterial LPS is a ligand recognized by toll-like receptor 4 (TLR4) in HUVECs, leading to the activation of the transcription factor NF-κB, which regulates the secretion of various inflammatory cytokines [18]. Additionally, previous research indicates that miR-146a can prevent the inflammatory response via targeting TLR4 [19]. Thus, we used LPS to generate a model of inflammation in endothelial cells to investigate how LPS affects CXCL16 expression and to identify the functional roles of miR-146a in CXCL16 regulation.

2. Methods

2.1. Cell culture

Human umbilical vein endothelial cells (HUVECs) obtained from the

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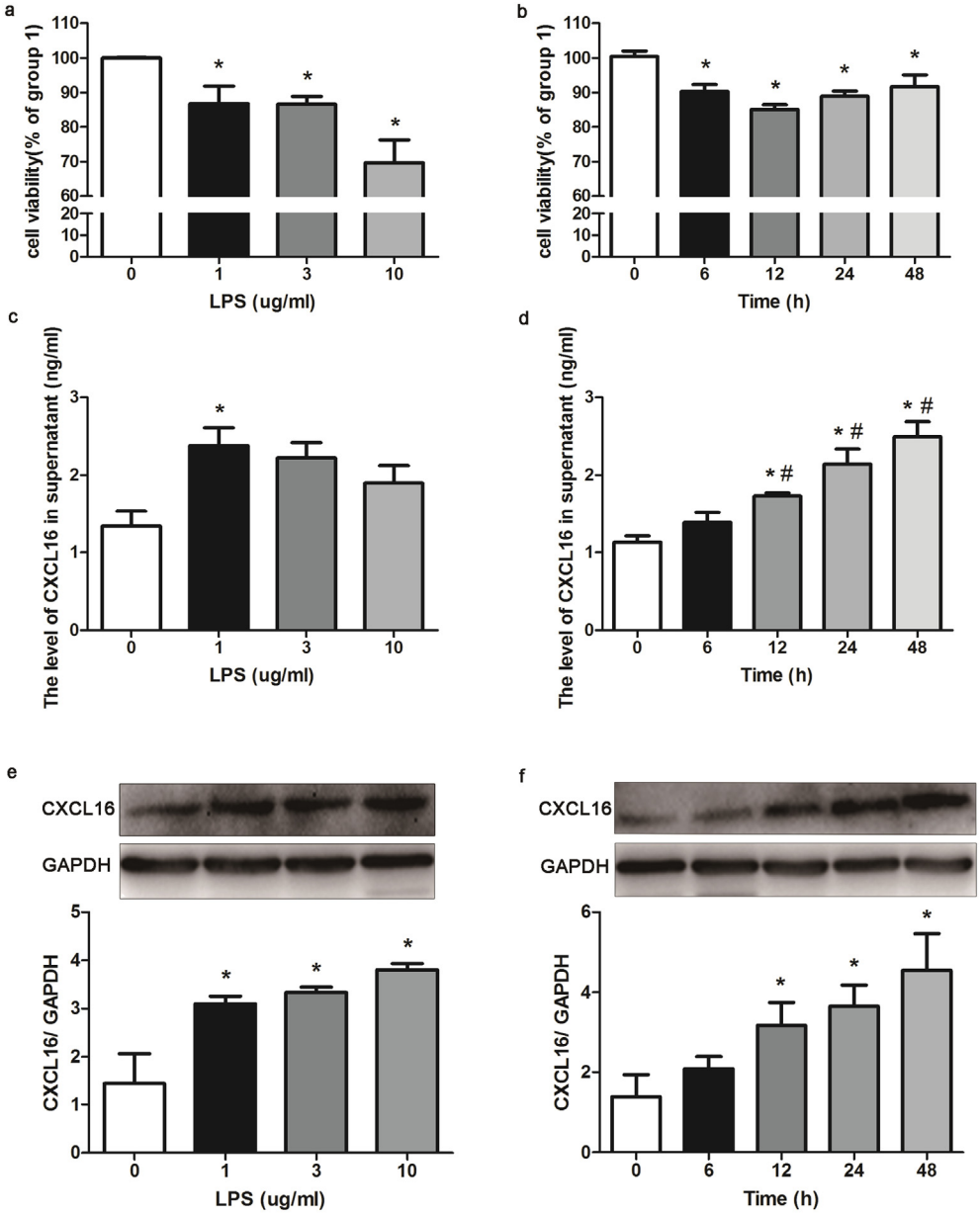

Fig. 1. Effects of LPS on cell viability and CXCL16 expression in HUVECs. a, b, Cell viability in HUVECs after treatment with LPS. c, d, Levels of LPS-induced CXCL16 release into the supernatant were quantified by ELISA. e, f, LPS-induced CXCL16 expression in HUVECs was analyzed by Western blotting. a, c, and e correspond to dose-responses to LPS (0–10 μg/ml for 24 h), while b, d, and f correspond to time course LPS treatment (1 μg/ml for 0–48 h). Data are presented as mean ± S.D of four independent experiments. *, $p < 0.05$ (compared with blank control group); #, $p < 0.05$ (compared between groups).

American Type Culture Collection (ATCC, Manassas, USA) were cultured in high glucose DMEM (HyClone, Logan, Utah, USA) supplemented with 10% fetal bovine serum (FBS) (Biological Industries, Israel) and 1% penicillin-streptomycin (HyClone, USA) and maintained at 37 °C and 5% CO_2. HUVECs were incubated in culture dishes for treatment with LPS (Sigma, St Louis, MO) in subsequent experiments.

2.2. Cell viability assay

Cell viability was detected using the cell counting kit (CCK-8, MCE, Monmouth Junction, USA) assay. First, 0.5–1 × 10^4 cells were seeded into 96-well plates with 100 μl culture medium and incubated overnight. Next, 10 μl of CCK-8 solution was added per well, which was followed by exposure to LPS (either 0–10 μg/ml for 24 h or 1 μg/ml for 0–48 h). The plates were incubated for 3 h in the incubator, and then the absorbance of each well was subsequently measured at a wave length of 450 nm.

2.3. Transfection with miRNA mimics or inhibitors

For the overexpression and inhibition of miR-146a activity, miR-146a mimics and inhibitors were used, respectively. HUVECs were seeded at a density of 0.5–2 × 10^5 cells/ml in a well plate containing growth medium without antibiotics and incubated overnight. Lipofectamine™ 2000 (Invitrogen, Carlsbad, USA) was used according to the manufacturer's instructions for 4–6 h to transfect miR-146a mimics, miR-146a inhibitors, negative control mimics or inhibitors (GenePharma, Shanghai, China). The effects of these interventions were evaluated by FAM fluorescence and real-time PCR. The following experimental treatments were performed 6–24 h after transfection.

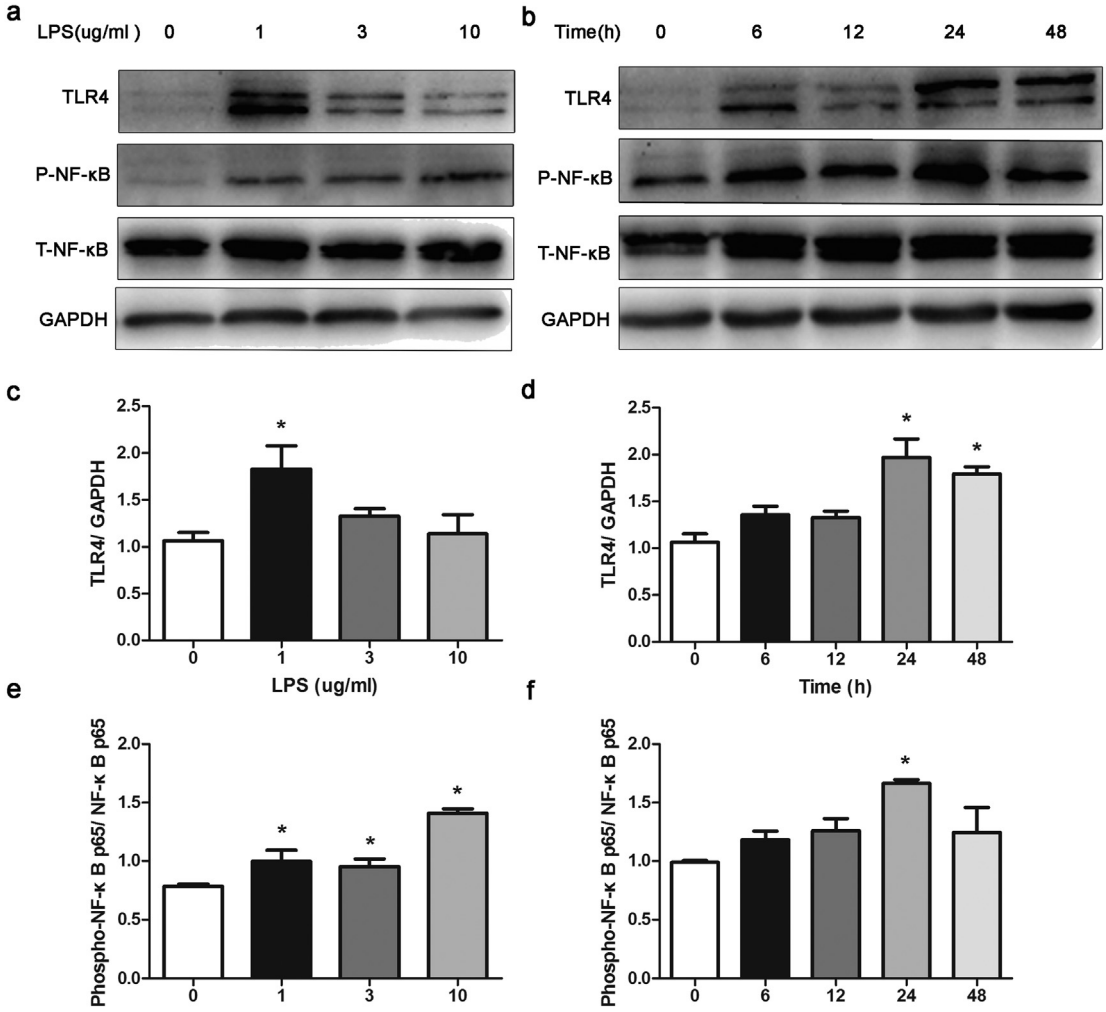

Fig. 2. Effects of LPS on TLR4/NF-κB signaling pathway protein expression in HUVECs. a, b, Expression of TLR4, phospho-NF-κB p65, NF-κB p65 and GAPDH in LPS-treated HUVECs was detected by Western blot analysis. c–f Quantitative analysis of Western blot images (c, d, TLR4/GAPDH, e, f, phospho-NF-κB p65/NF-κB p65). a, c, and e correspond to dose-responses (0–10 μg/ml for 24 h), while b, d, and f, correspond to time course LPS treatment (1 μg/ml for 0–48 h). Representative images are shown, and data are presented as the mean ± S.D of three independent experiments. *, $p < 0.05$ compared with the blank control group.

2.4. miRNA analysis

The expression of miR-146a was quantified using qRT-PCR. Total RNA was extracted from cells using the TRIzol reagent (Invitrogen, USA) and reverse-transcribed into cDNA using Mir-X miRNA qRT-PCR SYBR Kits (Takara, Dalian, China). Next, the cDNA was quantitatively amplified using a miR-146a primer (Takara, China) and SYBR Advantage qPCR Premix. Relative miRNA expression was determined using the Ct method and was normalized to the expression of U6 small nuclear RNA (snRNA).

2.5. Western blotting

Cells were lysed in ice cold standard RIPA buffer with PMSF and phosphatase inhibitor cocktail (MCE, USA) for 30 min. Protein samples were subjected to 10% SDS polyacrylamide gels and then transferred to a PVDF membrane (Millipore Co, NJ, USA). Primary antibodies used were against CXCL16 (R&D Systems, Minneapolis, MN, USA), TLR4 (Novus Biologicals, CO, USA), phospho-NF-κB p65, NF-κB p65 (Cell Signaling Technology, Boston, USA) and GAPDH (Elabscience, Wuhan, China). Antibody binding was detected using HRP-conjugated secondary antibodies (R&D Systems, USA) and ECL reagents. Images were then gathered and analyzed by Quantity One.

2.6. ELISA

For the examination of cytokine production in the supernatant, human CXCL16 ELISA Duoset kits were purchased from Elabscience (Wuhan, China). We seeded 0.1 ml of 10^4 and 0.5 ml of 0.5–1 × 10^5 cells into 96-well and 24-well plates, respectively. The concentration of each plate was detected according to the manufacturer's protocol.

2.7. Identifying potential target genes of miR-146a

Bioinformatic analysis was used to predict the potential target genes of miR-146a, including the web tools Miranda (www.microrna.org), Pictar (pictar.mdc-berlin.de) and Targetscan (www.targetscan.org). To confirm whether miR-146a could regulate CXCL16 by targeting TLR4, the TLR4 inhibitor TAK-242 (10 μM for12 h, MCE, USA) was used with or without miR-146a inhibitor.

2.8. Statistical analysis

Data are presented as the mean ± standard deviation (S.D). Statistical significance was determined using Student's *t*-tests and one-way analysis of variance (ANOVA). Statistical analyses were performed using SPSS 22.0; p-values < 0.05 were considered to be statistically significant.

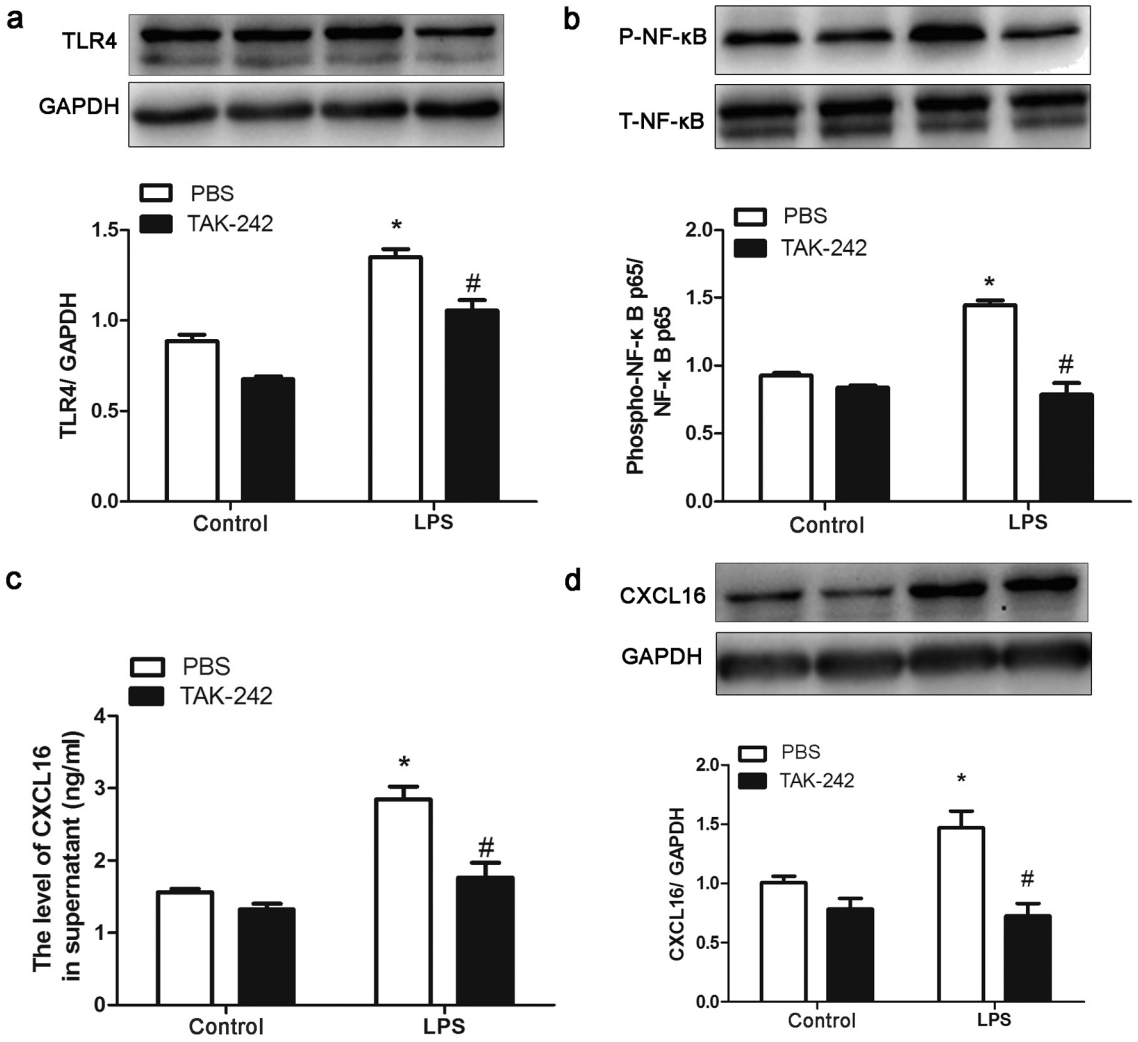

Fig. 3. LPS stimulates CXCL16 expression through the TLR4/NF-κB pathway. HUVECs were treated with 10 μM of the TLR4 inhibitor TAK-242 with or without 1 μg/ml LPS for 24 h. a, b, protein expression of TLR4(a), phospho-NF-κB p65 and NF-κB p65(b). c, d, CXCL16 supernatant levels were quantified by ELISA (c) while expression levels in HUVECs were detected by Western blotting (d). Data are presented as the mean ± S.D of three independent experiments. *, $p < 0.05$ (compared with blank control group); # $p < 0.05$ (compared with pre-incubation with LPS).

3. Results

3.1. LPS-induced changes in HUVEC viability and CXCL16 expression

To determine whether LPS affects CXCL16 expression, HUVECs were treated with serial dilutions of LPS (0–10 μg/ml) for 24 h or with constant doses of LPS (1 μg/ml) for 0–48 h. Cell viability was significantly changed with treatment of 1, 3 and 10 μg/ml of LPS (Fig. 1a), and the percentage of viable cells decreased at 6, 12, 24, and 48 h (Fig. 1b). Treatment with 1 μg/ml of LPS resulted in a significant induction of CXCL16 expression in HUVECs and the supernatant (Fig. 1c and e). Furthermore, LPS significantly induced the expression of CXCL16 after 12 h, and the effects were maintained for at least 48 h (Fig. 1d and f).

3.2. LPS-induced CXCL16 expression via the TLR4/NF-κB pathway

We next assessed whether LPS stimulates CXCL16 expression through the TLR4 signaling pathway. First, HUVECs were treated with LPS, as described in Fig. 1. TLR4, NF-κB and phospho-NF-κB protein levels were measured via Western blotting. TLR4 and phospho-NF-κB levels were significantly increased by the 1 μg/ml LPS treatment (Fig. 2a, c, and e), and LPS induced significant increases in TLR4 and phospho-NF-κB expression in the 24-h treatment condition (Fig. 2b, d, and f).

Next, we blocked TLR4 using a 10 μM concentration of the TLR4 inhibitor TAK-242 for 12 h followed by exposure to either vehicle or 1 μg/ml LPS for 24 h. Upon assessing TLR4 and NF-κB protein levels, we determined that knockdown of TLR4 reduced the upregulated TLR4 and NF-κB that occurs with LPS treatment (Fig. 3a and b). Furthermore, TAK-242 inhibited LPS-induced CXCL16 expression in HUVECs and supernatant (Fig. 3c and d), implicating the involvement of the TLR4/NF-κB pathway.

3.3. miR-146a regulation of LPS-induced CXCL16 expression

To further investigate the effect of miR-146a on LPS-stimulated production of CXCL16, we measured miR-146a levels after exposing HUVECs to 1 μg/ml LPS for 24 h. LPS treatment significantly induced in the expression of miR-146a to levels approximately threefold higher than baseline (Fig. 4a). HUVECs were later transfected with miR-146a mimics and inhibitors to either increase or decrease miR-146a expression, respectively. As shown in Fig. 4b, fluorescence micrographs of HUVECs showed that the efficiency of transfection can reach 80%–90%, and qRT-PCR analysis revealed miR-146a mimics and inhibitors significantly altered miR-146a levels after transfection for 48 h compared

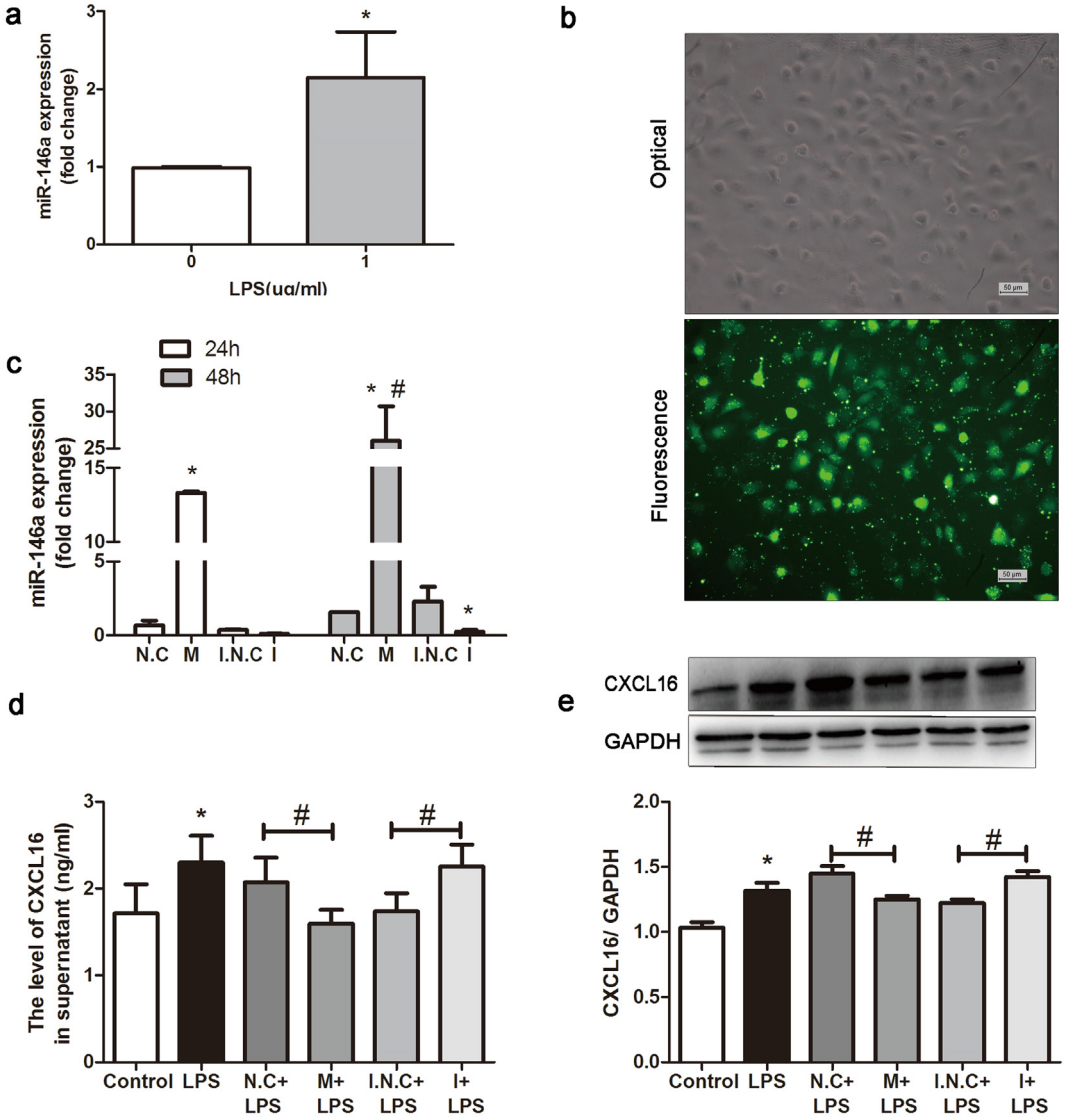

Fig. 4. miR-146a negatively regulates LPS-induced CXCL16 production in HUVECs. a, Expression of miR-146a after treatment with 1 μg/ml LPS for 24 h in HUVECs. *, $p < 0.05$ versus control. b, Fluorescence micrographs of HUVECs after transfection with N.C. FAM (FAM labeled negative control). c, Expression of miR-146a was measured by RT-PCR after transfection with N.C. (Negative control), M (miR-146a mimics), I.N.C. (microRNA inhibitor N.C.) and I (miR-146a inhibitors) at a concentration of 80 nM for either 24 h or 48 h. *, $p < 0.05$ versus control mimic or inhibitor; #, $p < 0.05$ versus miR-146a mimics for 24 h. d, e HUVECs were treated with 1 μg/ml LPS for 24 h after transfection with 80 nM N.C., M, I.N.C., or I for 24 h. CXCL16 levels in the supernatant were quantified by ELISA (c), CXCL16 expression in HUVECs was analyzed by Western blotting (d). *, $p < 0.05$ (compared with blank control group); #, $p < 0.05$ (compared with control mimic or inhibitor). Data is presented as mean ± S.D of 3–6 independent experiments.

to the control (Fig. 4c). Subsequently, cells were exposed to LPS for 24 h, and we assessed both the concentration of CXCL16 in the supernatant and CXCL16 levels in HUVECs. Transfection of miR-146a mimics dramatically reduced the LPS-induced increase in CXCL16 expression, whereas the miR-146a inhibitor further increased its expression level (Fig. 4d and e). Taken together, these results demonstrate that miR-146a negatively regulates LPS-induced CXCL16 expression.

3.4. CXCL16 expression regulation by miR-146a in a TLR4-dependent manner in LPS treated HUVECs

Simultaneously, we determined whether miR-146a regulated TLR4/NF-κB signaling pathway. We transfected miR-146a mimics or inhibitors into HUVECs to investigate their effects on TLR4 protein expression. As shown in Fig. 5, miR-146a overexpression inhibited LPS-induced TLR4 and NF-κB protein expression, whereas the inhibition of miR-146a increased their expression. This finding occurred in a similar manner to the negative regulation of CXCL16 by miR-146a.

Previous work and bioinformatic analysis (Fig. 6a) demonstrated that TLR4 is a target of miR-146a [18]. To further determine whether miR-146a regulated LPS-induced CXCL16 expression in a TLR4-dependent manner, HUVECs were transfected with miR-146a inhibitors for 24 h followed by the addition of the TLR4 inhibitor TAK-242 for 12 h. CXCL16, TLR4 and NF-κB levels were subsequently measured. The results showed that TAK-242 blocked the ability of miR-146a inhibitors to promote CXCL16 expression in LPS treated cells (Fig. 6b and c). Similarly, miR-146a increased TLR4 and NF-κB expression, but the effect was blocked by TLR4 inhibition (Fig. 6d and e).

4. Discussion

In this study, we first discovered that LPS treatment induced CXCL16 expression in HUVECs and the secretion of a soluble form of CXCL16 in the supernatant. Further experiments revealed that LPS

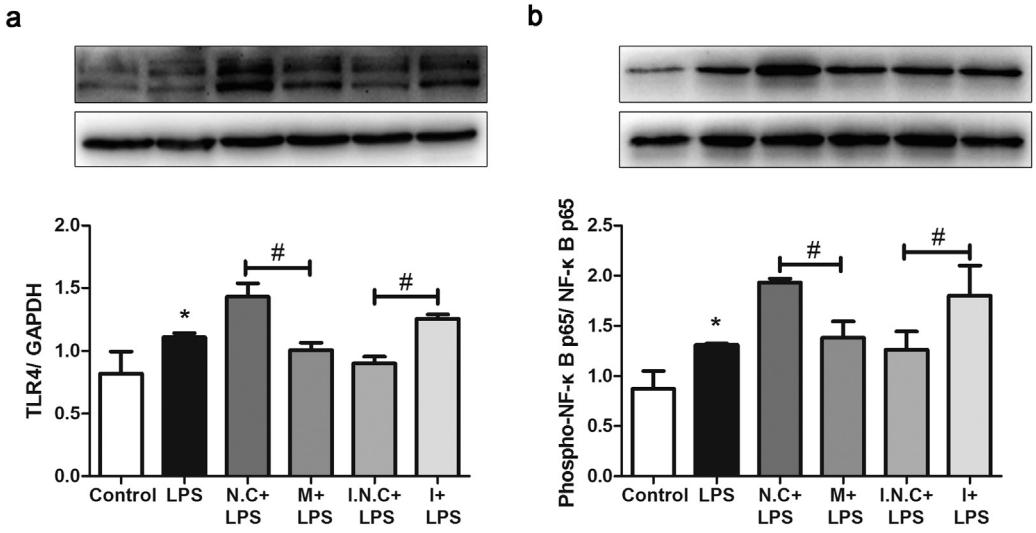

Fig. 5. Regulation of the TLR4/NF-κB signaling pathway by miR-146a. HUVECs were treated as described in Fig. 4d, e. TLR4(a) and NF-κB(b) proteins level were measured by Western blotting. Data is presented as the mean ± S.D of three independent experiments. *, $p < 0.05$(compared with blank control group); #, $p < 0.05$ (compared with control mimic or inhibitor).

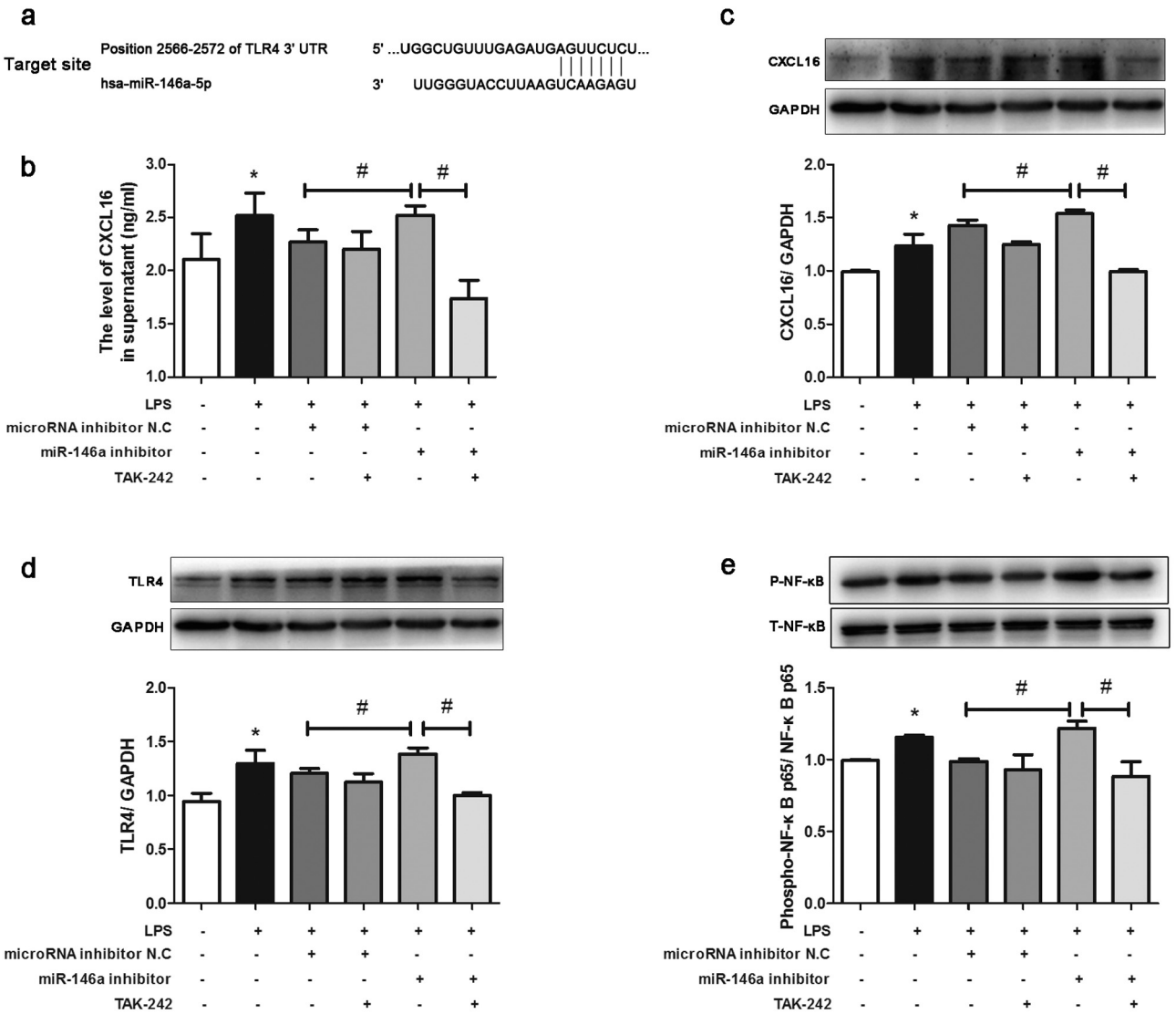

Fig. 6. CXCL16 expression is regulated by miR-146a in a TLR4-dependent manner in LPS treated HUVECs. a, Shown is a sequence alignment of miR-146a and its target site in 3′-UTR of TLR4, which was predicted from Targetscan (www.targetscan.org). b, c, d, e, Downregulation of TLR4 using its inhibitor, TAK-242, blocked the ability of miR-146a to inhibit CXCL16, TLR4 and NF-κB expression in LPS treated HUVECs. TLR4 was blocked using 10 μM of TAK-242 for 12 h. CXCL16 expression was analyzed by ELISA (b) and Western blot analysis (c). TLR4 (d) and NF-κB (e) expression were detected by Western blotting. Data are presented as the mean ± S.D of three independent experiments. * $p < 0.05$ (compared with blank control group); #, $p < 0.05$ (compared between groups).

induced CXCL16 expression through the TLR4/NF-κB signaling pathway. Finally, we identified that LPS triggers significant upregulation of miR-146a, and miR-146a inhibits LPS-induced CXCL16 expression in a TLR4-dependent manner.

A series of previous studies described the significance of CXCL16 in endothelial cells in the pathogenesis of atherosclerosis [15]. These studies suggested that CXCL16 in endothelial acts as both an adhesion molecule that mediates T-cell adhesion to the endothelium and as a chemokine that drives T-cell migration and stimulates cell proliferation. LPS results in the activation of the inflammatory response and has been postulated to play a key role in atherosclerosis plaque formation [20]. LPS increases CXCL16 expression in macrophages and smooth muscle cells [16,17]. In these cells, CXCL16 can also function as a scavenger receptor that binds and internalizes oxidized low-density lipoprotein. In this study, we further demonstrate that CXCL16 is induced by LPS in HUVECs and secreted into the supernatant.

Next, we found that LPS stimulated both TLR4 and NF-κB activation in HUVECs. TLR4 has been identified as the primary receptor that recognizes LPS, which is followed by the recruitment of specific adaptor molecules, such as MyD800, leading to the activation of NF-κB, which regulates the expression of cytokines and chemokines [20,21]. It has been reported that LPS mediates NF-κB activation in ECs [22], suggesting that LPS stimulates CXCL16 expression through the TLR4/NF-κB signaling pathway. For further verification, we used the TLR4 inhibitor TAK-242 and discovered that inhibition of TLR4 blocks LPS-mediated NF-κB and CXCL16 upregulation, indicating that LPS induces CXCL16 expression through the TLR4/NF-κB signaling pathway.

According to Raw et al., LPS induced miR-146a upregulation in HUVECs [23]. However, to the best of our knowledge, no studies have explored whether miR-146a regulates CXCL16 expression through the TLR4/NF-κB pathway. Similar to these findings, our results demonstrated higher miR-146a levels in LPS treated HUVECs compared to controls. Transfection with miR-146a mimics or inhibitors either decreased or increased miR-146a expression, suggesting that miR-146a negatively regulates LPS-induced CXCL16 expression. We identify the potential target genes of miR-146a via bioinformatic analysis, such as Miranda and Pictar. Additionally, Yang et al. demonstrated that miR-146a directly targets TLR4 using luciferase reporter assays in macrophages for the inflammatory response [24]. Therefore, we further assessed whether miR-146a stimulates CXCL16 expression by targeting TLR4. Our results showed that miR-146a negatively regulates the TLR4/NF-κB signaling pathway. On this basis, we used the TLR4 inhibitor TAK-242 and found that the ability of miR-146a inhibitors to promote CXCL16 expression was blocked by it. Thus, we confirmed that miR-146a negatively regulates CXCL16 expression in a TLR4-dependent manner.

In this study, we determined that LPS could enhance CXCL16 and miR-146a production. However, we also found that miR-146a negatively regulates CXCL16 expression. Previous studies suggested that NF-κB, a key activator of inflammation, also orchestrates a self-limiting host response for anti-inflammatory activity [24]. Moreover, the attenuating mechanisms of inflammation involve feedback loops of gene regulation, like miRNAs [25,26]. MiR-146a was found to be transcriptionally regulated by NF-κB with promoter analysis, and the miR-146a expression was blocked by NF-κB inhibitor [12,23]. Here, we also found that up-regulation of miR-146a negatively inhibited NF-κB activation via TLR4/NF-κB signaling pathway. Together, these results suggested that miR-146a form a negative feedback loop controlling NF-κB acitivity.

In summary, this study demonstrated that LPS induces CXCL16 expression via the TLR4/NF-κB signaling pathway, and miR-146a acts as a potential negative regulator of inflammation, which could lead to the downregulation of TLR4, thereby reducing the activity of NF-κB and CXCL16.

Declarations of interest

The authors declare no conflicts of interest.

Acknowledgments

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