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MiR-19a mediates the negative regulation of the NF-κB pathway in lipopolysaccharide-induced endometritis by targeting TBK1

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Abstract

Objective In both humans and animals, endometritis is severe inflammation of the uterus, and it causes great economic losses in dairy cow production. MicroRNAs have been reported to play an important role in various inflammatory diseases. However, the regulatory mechanisms of miR-19a in endometritis remain unclear. Thus, the aims of this study are to investigate the role of miR-19a in a mouse model of lipopolysaccharide (LPS)-induced endometritis and elucidate the possible mechanisms in bovine endometrial epithelial cells (bEECs).

Methods and results Histological analysis showed that LPS induced severe pathological changes, suggesting that the endometritis mouse model was well established. The qPCR assay indicated that miR-19a expression in the uterine tissues of mice with endometritis and in bEECs with LPS stimulation was significantly reduced. The overexpression of miR-19a significantly decreased the expression of inflammatory cytokines (TNF-α, IL-6 and IL-1β) and the phosphorylation of NF-κB p65 and IκBα. Similar results were also obtained following the knockdown of TBK1. Furthermore, a dual luciferase reporter assay further validated that miR-19a inhibited TBK1 expression by binding directly to the 3′-UTR of TBK1.

Conclusion We demonstrated that miR-19a has anti-inflammatory effects and mediates the negative regulation of the NF-κB Pathway in LPS-induced endometritis by targeting TBK1.

Keywords MiR-19a · LPS · TBK1 · Endometritis · NF-κB

Introduction

Endometritis is an inflammatory injury of uterine tissues that often occurs in humans and animals. This disease has a tremendous impact on reproductive function and fertility [1]. Endometritis is becoming a serious problem in high milk-producing cows, triggering losses in milk production and rates of embryo survival [2]. At present, the treatments for endometritis are mainly antibiotics, but the residues of antibiotics and creation of resistant bacteria during treatment will cause serious food safety problems. So far, no effective measures have been found for endometritis, and the main reason for this is that its pathogenesis is not very clear. Studies have shown that the invasion of a variety of pathogenic microorganisms, such as *Escherichia coli*, suppurative plague bacteria, neutrophil Bacteroides or *Prevotella* can cause endometritis [3].

Escherichia coli is a common pathogen for endometritis [4]. Lipopolysaccharide (LPS) are the toxigenic component of *Escherichia coli* and can stimulate a TLR4-related inflammatory response [5], leading to the production of pro-inflammatory cytokines such as tumour necrosis factor (TNF-α), interleukins (IL-6, IL-1β), and interferon (IFN-1β) [6]. The pro-inflammatory cytokines can activate intracellular proteins, particularly the nuclear transcription factor NF-κB [7]. Those cytokines can cause fever by synthesizing PGE_2, which causes an acute phase response by increasing related proteins, leading to classic symptoms including redness, swelling, heat and pain [8]. TBK1 is a member of the IκB kinase (IKK) family and is widely expressed in animals [9]. Previous studies have shown that TBK1 has the ability to regulate the expression of inflammatory cytokines, such

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as TNF-α and IL-6, playing a crucial role in the regulation of immune responses induced by bacterial and viral infections [10, 11]. TBK1 and IKKε are involved in the activation of NF-κB processes [12]. Therefore, there is reason to believe that TBK1 participates in the NF-κB signalling pathway and plays a role in the LPS-induced inflammatory response.

Mature microRNAs (miRNAs), containing approximately 19–23 nucleotide, are a class of evolutionarily conserved endogenously expressed non-coding single-stranded small RNAs that play a critical role in cell differentiation and biological development [13]. The specific relationships between miRNAs and target genes can provide us with new insights on diseases. Growing evidence has shown that miRNAs play a regulatory role in a wide range of biological processes [14], including cell differentiation [15], proliferation [16], apoptosis [17], developmental time [18], and haematopoiesis [19]. An increasing number of studies on the functions of miRNAs have shown the correlation between miRNAs and inflammation. For instance, many studies have reported how miRNAs regulate or affect inflammatory diseases, such as enteritis [20], pneumonia [21], mastitis [22] and endotoxin-induced uveitis [23]. In various studies, the expression levels of some miRNAs have been reported to be significantly changed in bovine clinical and subclinical endometritis [24, 25]. MiR-19a belongs to polycistronic miR-19-72 cluster, which includes miR-17, miR-18a, miR-20a, miR-19b and miR-92a [26]. MiR-19a plays an important role in various cancers, such as colorectal cancer [27], gastric cancer [28] and bladder cancer [29], and affects endothelial cell apoptosis [30] and proliferation [31]. Previous studies have shown that miR-19a was significantly downregulated in various inflammatory diseases, such as colitis, pneumonia and atherosclerosis [32, 33]. However, the function of miR-19a and its relationship with TBK1 in endometritis have not been studied to the best of our knowledge. In this study, we show that miR-19a alleviates LPS-induced endometritis through directly targeting TBK1.

Materials and methods

Reagents

LPS (*E. coli* 055: B5) was obtained from Sigma-Aldrich (St. Louis, United States). The TNF-α, IL-6, and IL-1β enzyme-linked immunosorbent assay (ELISA) kits were purchased from ImmunoWay Biotechnology (Newark, DE, USA). TBK1/NAK (D1B4) rabbit mAb, NF-κB p65 (D14E12) XP rabbit mAb, phospho-NF-κB p65 (Ser536) (93H1) rabbit mAb, IκBα (L35A5) mouse mAb, phospho-IκBα (Ser32) (14D4) rabbit mAb, TLR4 and β-actin were provided by Cell Signalling Technology (Beverly, MA, United States).

Mouse model of endometritis

A total of 30 8-week-old female BALB/c mice (20–25 g) were obtained from the Experimental Animal Center of Huazhong Agricultural University (Wuhan, China). All animals were maintained under a 12 h light/12 h dark cycle. The animal experiments were performed in accordance with the guidelines provided by the Laboratory Animal Research Center of Hubei Province. The mice were randomly divided into two groups ($n = 15$): control group and LPS group. The method for establishing the endometritis model was performed as previously described [5]. Briefly, the uterus was infused with 20 µl of LPS (2.5 mg/ml) to induce endometritis. After 24 h, all mice were euthanized with CO_2, and the uteri were collected and stored at − 80 °C for further experiments.

Histological assessment of the uterine tissues

Uterine tissues were isolated from the mice, and then they were fixed in 4% paraformaldehyde for 24 h. In addition, the samples were embedded in paraffin, sliced, and then stained with haematoxylin and eosin (H&E).

Cell culture

The bovine endometrial epithelial cells line (bEECs) and a human embryonic kidney cell line (HEK293T cells) were purchased from American Type Culture Collection (ATCC, Manassas, VA, United States), and both of them were cultured in DMEM containing 10% foetal bovine serum (FBS; Sigma, St. Louis, MO, United States), penicillin (50 U/ml), and streptomycin (50 µg/ml) with 5% CO_2 at 37 °C. When the cell density reached 80–90%, the cells were passaged for further culture or passaged to a six-well plate for testing.

CCK-8 assay

Cell viability after treatment with LPS (2 µg/ml) was examined using a cell counting kit-8 (CCK-8) assay kit (Beyotime, Shanghai, China). Briefly, the bEECs (1.5×10^5 cells/ml) were plated in 96-well plates and incubated at 37 °C for 1 h, and then the cells were treated with LPS for 0, 6, 12, or 24 h. After, the cells were incubated with 10 µl of CCK-8 solution for 3 h at 37 °C, and the absorbance was read at 450 nm using a microplate reader (Bio-Rad Instruments, Hercules, CA, United States).

Dual luciferase reporter assay

The bioinformatical algorithms TargetScan (http://www.targetscan.org) and miRDB (http://www.mirdb.org) were used to predict the possible binding sites between miR-19a and the target gene TBK1. We first amplified TBK1 3′-UTR, and then wild-type TBK1 3′-UTR was formed by inserting the amplified TBK1 3′-UTR into a psiCHECK™-2 vector (Promega, Madison, WI, United States). The same procedure was used to synthesize the mutant TBK1 3′-UTR. For the luciferase reporter assays, HEK293T cells were co-transfected with the luciferase reporter plasmid and miR-19a mimics or miR-19a mimic NC using Lipofectamine™ 2000.

Over-expression and inhibition of miR-19a

The miR-19a mimics, inhibitors and their negative controls were synthesized by GenePharma (Shanghai, China). The bEECs were equally distributed into 6-well plates (Corning Inc, Corning, NY, United States). When the confluence reached 50%, we transfected cells with miR-19a mimics, miR-19a mimic negative controls (mimic NC), miR-19a inhibitors and miR-19a inhibitor negative controls (inhibitor NC) with Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instructions. The transfection efficiency was measured by qPCR assay.

Small interfering RNA assay

TBK1 small interfering RNA (si-TBK1) and the negative control RNA were designed and synthesized by GenePharma and transfected into bEECs using Lipofectamine™ 2000 according to the manufacturer's instructions. The si-TBK1 sequence was 5′-GCAGCAGAGUUAGGUGAAATT-3′, 5′-UUUCACCUAACUCUGCUGCTT-3′. After 24 h of transfection, the cells were stimulated with 2 μg/ml LPS for 12 h. The transfection efficiency was evaluated by a qPCR assay.

ELISA assay

The uterine tissues were homogenized in PBS to collect the supernatants. The cell culture media were collected for testing. All the supernatants, including cells and tissues, were used to detect the levels of inflammatory cytokines (TNF-α, IL-1β, and IL-6) by using an ELISA kit according to the manufacturer's directions. The absorbance was read at 450 nm with an automatic enzyme standard instrument (Multiskan MK3, Thermo Fisher Scientific, MA, United States), and all absorbance results were normalized via standard curves.

RNA isolation and qPCR

Total RNA from the bEECs or uterine tissues was extracted using TRIzol (Invitrogen, United States). Quantitative PCR for miRNAs was performed using a miRNA real-time PCR kit (GenePharma, Shanghai, China) according to the manufacturer's instructions. The relevant primer sequences of miR-19a were as follows: stem loop RT primer, 5′-CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGTCAGTTTT-3′; miR-19a forward primer, 5′-GCCGAGTGTGCAAATCTATGC-3′; and miR-19a reverse primer, 5′-GGAGTCGGCAATTCAGTTGAG-3′. For the detection of mRNA levels, a PrimeScript RT reagent kit (Takara, Dalian, China) was used for reverse transcription, followed by qPCR experiments using a SYBR green plus reagent kit (Roche, Basel, Switzerland). The StepOne real-time PCR system (Life Technologies Corp. Waltham, MA, United States) was used. The primer sequences for the inflammatory cytokines for PCR are shown in Table 1. After the reaction, a comparative threshold cycle (ΔCT) method was used to compare the expression levels of each group, and the results were presented as $2^{-\Delta\Delta CT}$. MiR-19a was normalized to U6 snRNA, and the relative expression levels of mRNAs were normalized to GAPDH.

Western blot analysis

RIPA buffer (Biosharp, China) was used to lyse uterine tissues and bEECs, and then the mixtures were centrifuged at 12,000 rpm at 4 °C for 15 min. The concentrations of total protein were detected by a Pierce BCA assay protein assay kit (Thermo Fisher Scientific). The protein samples (80 μg) were separated by 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes. After being soaked for 2 h in the blocking buffer, the membranes were incubated overnight for approximately 12 h with a specific primary antibody (1:1000) at 4 °C. Then, the membranes were incubated with a 1:4000 dilution of the secondary antibody for 2 h at room temperature and visualized using enhanced chemiluminescence.

Immunofluorescence staining

BEECs were transfected with miR-19a mimics or si-TBK1 and then stimulated with 2 μg/ml LPS for 12 h. Subsequently, immunofluorescence staining was performed. Briefly, the cells were incubated with special primary antibodies for p-p65 (1:100) for 12 h at 4 °C and then incubated with FITC-labelled secondary antibodies (1:200) in the

Table 1 Primers used for qRCR

Name	Accession number	Primer sequence (5′→3′)	Product size (bp)
Mus-TLR4	NM_021297.2	Forward: ATTCAGAGCCGTTGGTGTATC Reverse: GGGACTTCTCAACCTTCTCAAG	109
Mus-TNF-α	NM_013693.3	Forward: CTTCTCATTCCTGCTTGTG Reverse: ACTTGGTGGTTTGCTACG	198
Mus-IL-1β	NM_008361.4	Forward: CCTGGGCTGTCCTGATGAGAG Reverse: TCCACGGGAAAGACACAGGTA	131
Mus-IL-6	NM_031168.1	Forward: GGCGGATCGGATGTTGTGAT Reverse: GGACCCCAGACAATCGGTTG	199
Mus-TBK1	NM_019786.4	Forward: CCCTGCTGCTCTATCAAGAATTA Reverse: TGATCACTACCTCCGTCTTCT	108
Mus-GAPDH	NM_001289726.1	Forward: GGAGAAACCTGCCAAGTATGA Reverse: TCCTCAGTGTAGCCCAAGA	90
Bta-TLR4	NM_174198.6	Forward: TTTCAGCTCTGCCTTCACTAC Reverse: TGGGACACCACGACAATAAC	110
Bta-TNF-α	NM_173966.3	Forward: GGTGTGAAGCTGGAAGACAA Reverse: CTGAAGAGGACCTGTGAGTAGA	77
Bta-IL-1β	NM_174093.1	Forward: TGAGTCTGTCCTGTACCCTAAC Reverse: TAGGGAGAGAGGGTTTCCATTC	126
Bta-IL-6	NM_173923.2	Forward: AGACTACTTCTGACCACTCCA Reverse: GCTGCTTTCACACTCATCATTC	114
Bta-TBK1	NM_001192755.1	Forward: GATGTGGTGGGTGGAATGAA Reverse: AGACTGTCCGTCTTCTCCTATC	102
Bta-GAPDH	NM_001034034.2	Forward: AAGGTCGGAGTGAACGGATT Reverse: ATGACGAGCTTCCCGTTCTC	194

Mus mouse, *Bta* bovine

dark for 1 h at 25 °C. The nuclei were stained using DAPI for 10 min, and fluorescent images were captured with an inverted fluorescence microscope (IX81, Olympus, Japan). The IOD and area of cells were measured by Image Pro Plus software, and the fluorescence intensity of p-p65 was expressed as IOD/area.

Statistical analyses

The data were expressed as the mean ± SEM. A Student's *t* test was employed to analyse the results, and $P < 0.05$ indicated statistical significance. All results had been collected from three independent experiments.

Results

Changes in the uterus and miR-19a stimulated by LPS

An LPS-induced endometritis mouse model was created to determine whether miR-19a had been changed in endometritis. Oedema and haemorrhages were found in the endometritis mouse model when comparing it with the control group (Fig. 1a). Extensive inflammatory cell infiltration occurred in the mouse uterine tissues (Fig. 1b). The expression levels of TLR4 mRNA and related pro-inflammatory cytokines (IL-1β, IL-6, and TNF-α) were also detected. These results revealed that TLR4 and the pro-inflammatory cytokines were significantly upregulated in the LPS-stimulated group compared with that in the control group (Fig. 1c, d). This result demonstrated that the mouse endometritis model was successful. In addition, as shown in Fig. 1e, miR-19a expression was significantly reduced in the LPS group compared with that in the control group.

Changes in miR-19a and cell viability in bEECs stimulated by LPS

Then, we tested whether miR-19a plays a role in the LPS-induced inflammation response. BEECs were given different concentrations of LPS or were stimulated by LPS for different amounts time, and we found miR-19a showed a dose- and time-dependent downregulation (Fig. 2a, b). Additionally, a CCK-8 assay was used to examine whether cell viability is affected by the administration of LPS, thus identifying the optimal time for stimulation. The results showed that cell viability is not affected by LPS (2 μg/ml), indicating that LPS stimulation has no significant effect on cell growth and proliferation (Fig. 2c). Taken all together, these results indicated that miR-19a was likely involved in LPS-mediated immune responses.

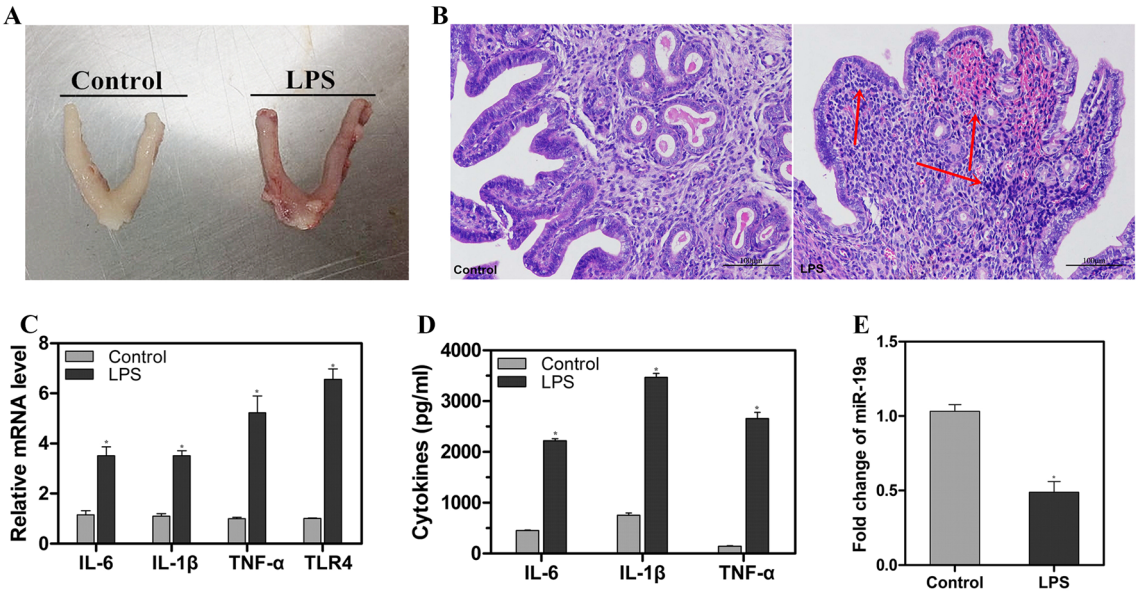

Fig. 1 Changes in the uterus and miR-19a stimulated by LPS. **a** Morphology changes in the uterus of LPS-treated mice. **b** Histopathologic features of the control group and inflammatory cell infiltration in the uterus of the NC group and LPS group by H&E staining of harvested uterine tissues. **c** The mRNA levels of TLR4 and pro-inflammatory factors were measured by quantitative RT-PCR. **d** The proteins level of pro-inflammatory cytokines in uterine tissue were analysed by ELISA assays. **e** MiR-19a expression was detected by qPCR in the uterine tissue of LPS-treated mice. The red arrows indicate the tissue lesion area. The data are presented as the mean ± SEM of three independent experiments. $^*P < 0.05$. (Color figure online)

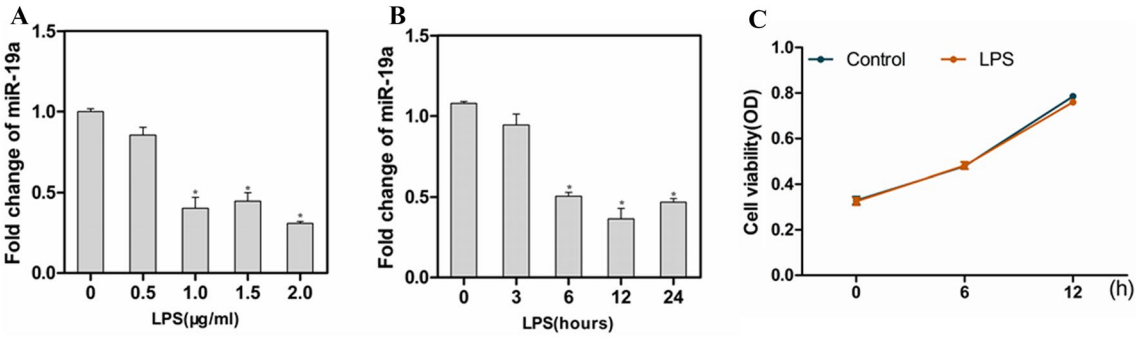

Fig. 2 Changes in miR-19a and cell viability in bEECs stimulated by LPS. **a** BEECs were stimulated with different concentrations of LPS for 12 h, and the expression of miR-19a was measured by qPCR. **b** The bEECs were stimulated with 2 μg/ml LPS for different time periods, and the miR-19a expression level was measured by qPCR. The relative expression of miR-19a was normalized to U6 snRNA. **c** BEECs were stimulated with LPS (2 μg/ml). A CCK-8 assay was used to test cell viability. The data are presented as the mean ± SEM of three independent experiments. $^*P < 0.05$

Effect of miR-19a on the TLR4/NF-κB pathway and cytokine expression

BEECs were transfected with miR-19a mimics or miR-19a mimic NC for 24 h, and they were stimulated with LPS (2 μg/ml) for 12 h. From the results of qPCR, miR-19a was expressed at a much higher level when miR-19a mimics were used compared to that when miR-19a mimic NC was used (Fig. 3a). Inflammatory cytokines were analysed by ELISA and qPCR as well. The over-expression of miR-19a significantly reduced the mRNA and protein levels of inflammatory cytokines (Fig. 3b, c).

It is widely acknowledged that inflammatory cytokines can be regulated by various signalling pathways and that the NF-κB signalling pathway is the most important one. NF-κB plays an important role in LPS-induced endometritis [5]. To further understand the mechanism that miR-19a suppressed inflammatory cytokines, we tested the effect of miR-19a on the NF-κB pathway.

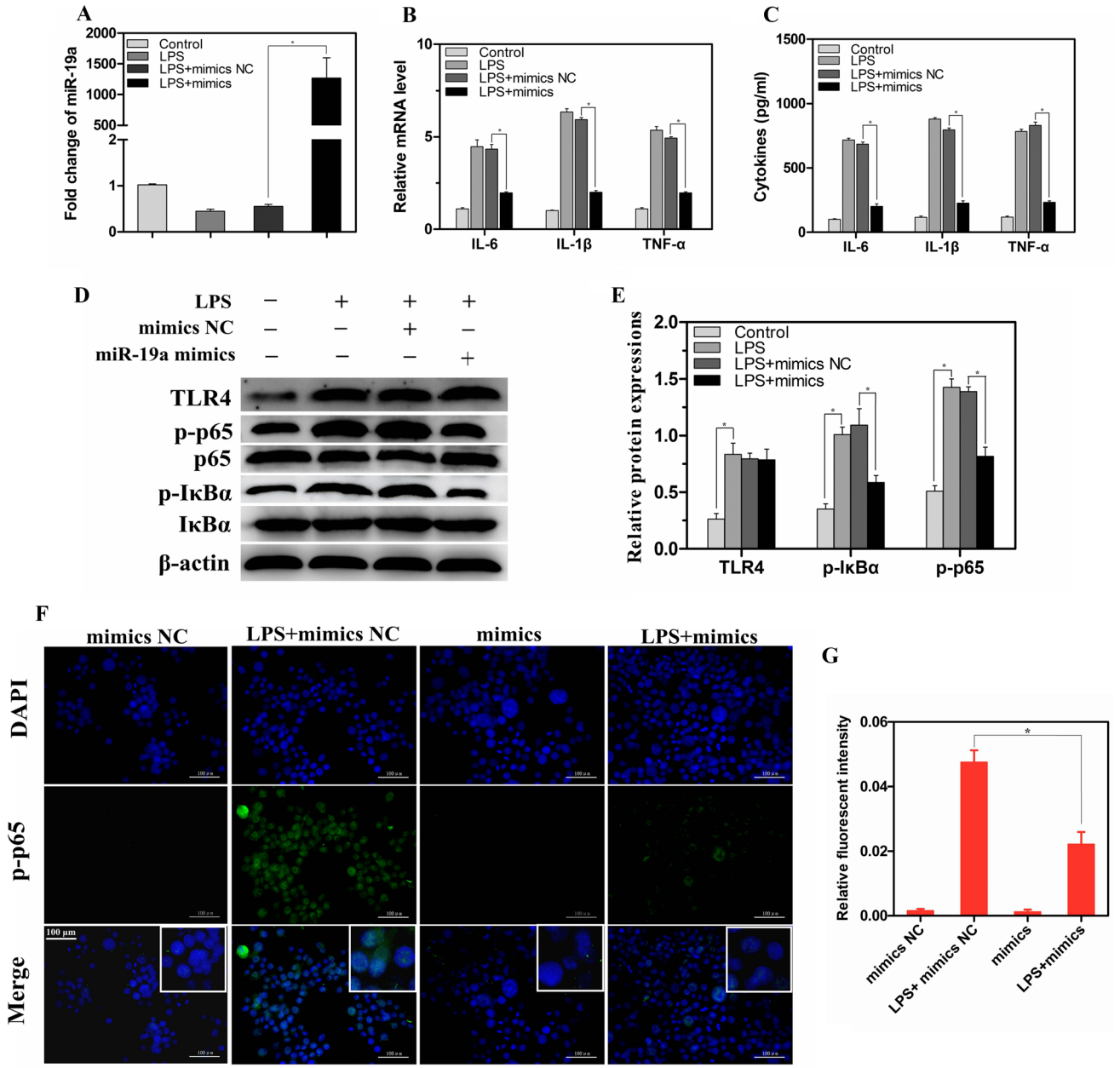

Fig. 3 Effect of miR-19a on the TLR4/NF-κB pathway and cytokine expression. **a** BEECs were transfected with miR-19a mimics or miR-19a mimic NC for 24 h, followed by exposure to LPS for another 12 h. The relative expression of miR-19a was normalized to U6 snRNA. **b, c** The expressions levels of pro-inflammatory cytokines were obtained from qPCR and ELISA assays. **d** The expression levels of upstream and downstream proteins of NF-κB in the different subgroups were detected by western blotting. β-actin was used as an internal control. **e** The IOD of these proteins had been measured when significance was noted. **f** The translocation of the p65 subunit from the cytoplasm to the nucleus was assessed by immunofluorescence staining (×400), scale bar 100 μm. Blue spots represent cell nuclei, and green spots indicate p-p65 staining. **g** The IOD and area of cells were measured by Image Pro Plus software, and the fluorescence intensity of p-p65 was expressed as IOD/area. The data are expressed as the mean ± SEM of three independent experiments. $^{*}P < 0.05$. (Color figure online)

The protein level was measured by western blotting (Fig. 3d), and IOD (Fig. 3e) was used to analyse the results from the images. LPS significantly increased the protein levels of phosphorylated p65 and IκBα compared with those in the control group. However, the phosphorylated p65 and IκBα protein levels were significantly reduced in the miR-19a mimics group compared with those in the mimic NC group. The protein level of TLR4 was not significantly decreased in the miR-19a mimics group and mimic NC group. In addition, immunofluorescence experiments

were also used to examine the effect of miR-19a on NF-κB translocation.

The immunofluorescence assay showed that the over-expression of miR-19a significantly reduced the nuclear translocation of NF-κB p65 induced by LPS (Fig. 3f). The results indicated that miR-19a inhibits the activation of NF-κB and production of inflammatory cytokines under the stimulation of LPS. Further studies are needed to confirm that TBK1 is the target gene of miR-19a.

TBK1 was a target of miR-19a in the NF-κB pathway

Two computational bioinformatical algorithms were used to identify the putative target miRNAs for TBK1, which contributes to the effect of the TLR4/ NF-κB signalling pathway. The putative binding sites for TBK1 3′-UTR and miR-19a are indicated in Fig. 4a, and the mutated binding sites were also designed. The seed sequence was highly homologous across species. Further verification tests were conducted. MiR-19a mimics, inhibitors and negative controls were transfected into bEECs. Quantitative PCR was used to determine the efficiency of transfection (Fig. 4b).

As shown in Fig. 4c–e, the over-expression of miR-19a significantly inhibited TBK1 mRNA and protein, while the transfection of the miR-19a inhibitors increased the expression of TBK1. This result suggested that miR-19a might regulate TBK1 expression at the transcriptional level. Next, a luciferase reporter assay was performed to verify the miR-19a and TBK1 binding site. In the TBK 3′-UTR wild-type group, the relative luciferase activity was significantly reduced in the miR-19a mimics group compared with that in the miR-19a mimic NC group. When the target binding site was mutated, there was no change when the cells were transfected with miR-19a mimics or miR-19a mimic NC. These results indicated that TBK1 is a direct target of miR-19a.

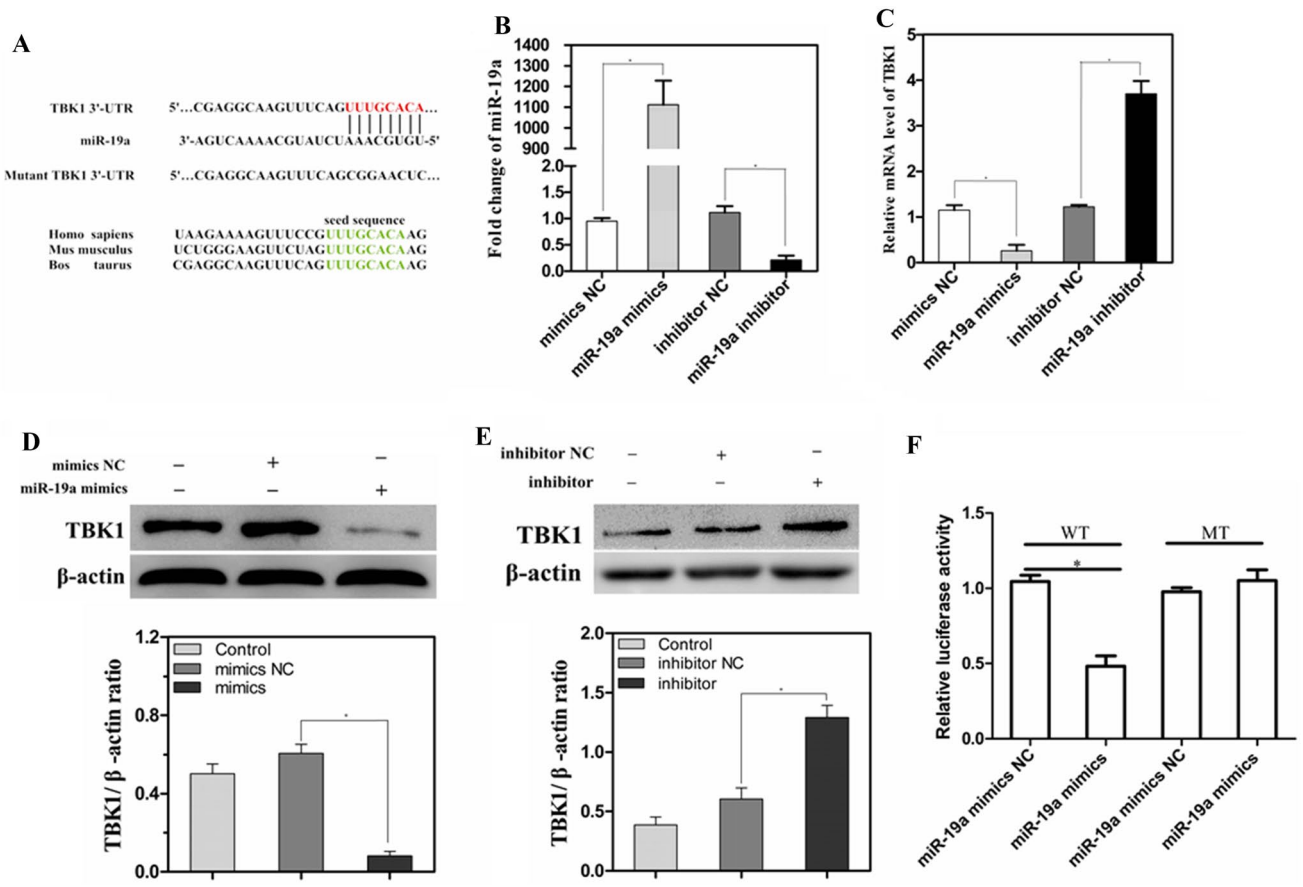

Fig. 4 TBK1 was a target of miR-19a in the NF-κB pathway. **a** TargetScan and miRDB algorithms were used to predict the possible binding sites between the target gene and miR-19a. **b** miR-19a mimics or inhibitors and their negative controls were transfected into bEECs. After 24 h of transfection, miR-19a levels were detected by qPCR. **c** The mRNA level of TBK1 was also detected by qPCR. **d, e** The TBK1 protein levels in the different groups were detected by western blotting. **f** A dual luciferase reporter assay was performed in 293T cells. The ratio of Renilla activity/firefly activity represents luciferase activity. The data are expressed as the mean ± SEM of three independent experiments. $^{*}P < 0.05$

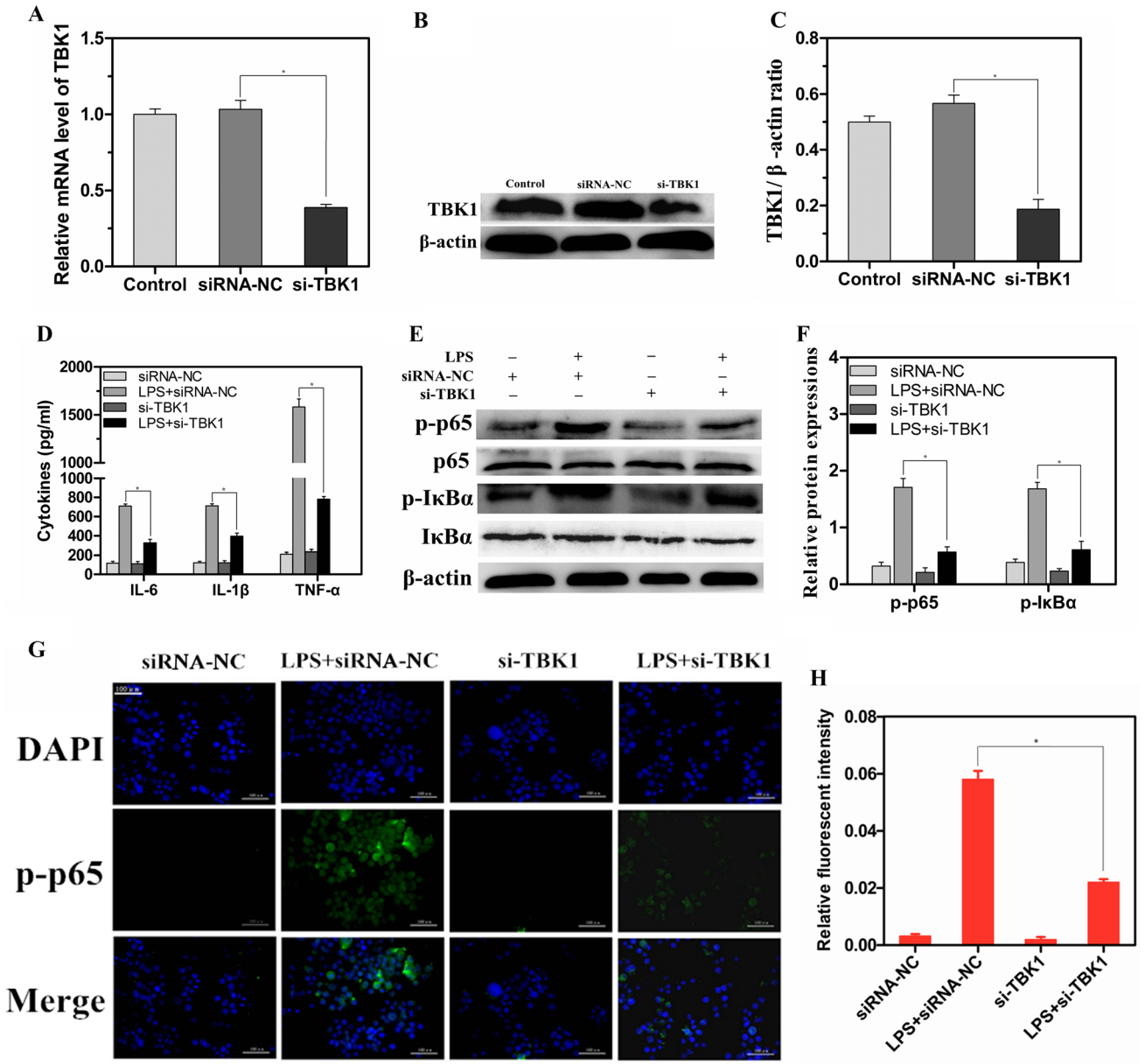

Fig. 5 Effect of TBK1 silencing on NF-κB activation in bEECs. **a** Si-TBK1 or siRNA-NC was transfected into bEECs for 24 or 48 h, and then, the mRNA level of TBK1 was measured by qPCR. **b** The relevant proteins were detected by western blotting. **d** Cells were transfected with si-TBK1 or siRNA-NC for 24 h and then stimulated with 2 μg/ml LPS for 12 h. The levels of cytokines were detected by ELISA. **e** The protein levels of NF-κB p65 and IκBa were measured by western blotting. **c, f** Grey values of the indicated proteins were measured by Image Pro Plus software. **g** The translocation of the p65 subunit from the cytoplasm to the nucleus was assessed by immunofluorescence staining (×400), scale bar 100 μm. **h** The fluorescence intensity of p-p65 was analysed by Image Pro Plus software. The data are expressed as the mean ± SEM of three independent experiments. $^{*}P < 0.05$

Effect of TBK1 silencing on NF-κB activation in bEECs

We already knew that TBK1 can induce NF-κB activation and participate in inflammatory reactions [34]. To fully understand the mechanism of how miR-19a regulates inflammatory responses, si-TBK1 was used to silence the expression of TBK1 in bEECs. Si-TBK1 and siRNA-NC were transfected into bEECs, and then TBK1 was detected by qPCR and western blotting. As shown in Fig. 5a–c, the transfection with si-TBK1 significantly reduced the expression level of TBK1. Furthermore, we found that the levels of pro-inflammatory cytokines were also significantly reduced by silencing the TBK1 gene (Fig. 5d). Western blotting was used to detect the phosphorylation of IκBα and p65, and it was found that si-TBK1 reduced the production of phosphorylated IκBα and p65 that was stimulated by LPS

(Fig. 5e, f). Immunofluorescence staining showed the same results as well (Fig. 5g). In summary, all of these results strongly demonstrated that TBK1 somehow participates in the inflammatory responses induced by LPS, triggering the NF-κB activation and then regulating the expression of pro-inflammatory cytokines.

Discussion

Endometritis impairs reproductive performance and reduces the profits of dairy farms, drawing increasing attention in the dairy industry [35]. After analysing previous studies, we found that the expressions of several microRNAs, including miR-19a, significantly changed with endometritis [36]. These results caused us to become interested in research on the interaction between miR-19a and endometritis. Therefore, the predominant purpose of the study was to reveal the underlying mechanisms of miR-19a in endometritis.

MiR-19a changed significantly after LPS stimulation, and the anti-inflammatory effect of miR-19a was observed in other studies [37, 38]; however, some of the underlying mechanisms are still unknown. In this study, we successfully established a mouse endometritis model and found that the expression of miR-19a significantly decreased in the endometritis mice. MiR-19a expression was also tested in cells, which showed a time- and dose-dependent downregulation in bEECs stimulated by LPS. These results inferred that the miR-19a functions during inflammatory processes that are induced by LPS. Then, we determined the role that miR-19a plays in LPS-induced inflammation and its related mechanism.

MiR-19a mimics were used to increase the expression level of endogenous miR-19a to further investigate the function of miR-19a. The over-expression of miR-19a significantly reduced the expression levels of inflammatory cytokines, including TNF-α, IL-1β and IL-6. NF-κB is an essential nuclear transcription factor that regulates the production of pro-inflammatory cytokines, promoting inflammatory responses. Therefore, we investigated the effect of miR-19a on the NF-κB pathway. The activation of proteins in the NF-κB pathway induced by LPS was significantly attenuated by transfection with miR-19a mimics. All these results indicated that miR-19a exhibits an anti-inflammatory effect on LPS-induced inflammation.

TBK1 does affect inflammatory responses through the NF-κB pathway [39]. TBK1 is involved in a variety of inflammatory processes, and mice lacking TBK1 exhibit a significant reduction in NF-κB transcription given certain stimuli [40]. A previous study showed that in a LPS-induced hepatitis model, TBK1 was expressed at a high level [9]. In this study, through bioinformatics analyses, we predicted that TBK1 was the target gene of miR-19a in the NF-κB pathway. Base pairing between TBK1 3′-UTR and miR-19a was observed, and the murine and bovine binding sites had a high level of homology. Then, cells were transfected with miR-19a mimics or inhibitors under the stimulation of LPS, and the results we acquired demonstrated the correlation between TBK1 and miR-19a. Finally, with a dual luciferase assay, this hypothesis was further verified. In the TBK1 3′-UTR wild-type group, the relative luciferase activity was significantly reduced in the miR-19a mimics group compared with that in the miR-19a mimic NC group. However, changes in fluorescence activity were not observed in the mutant group. These results fully indicated that miR-19a could inhibit the expression of TBK1 by binding to TBK1 3′-UTR. In addition, si-TBK1 was used to silence TBK1 expression in bEECs to verify how much TBK1 would affect the anti-inflammatory process of miR-19a. The results showed that silencing TBK1 significantly alleviated LPS-induced inflammatory responses and the NF-κB signalling pathway. Thus, we can say that miR-19a modulates LPS-induced inflammatory responses by targeting TBK1.

In summary, we showed that TBK1 is a target gene of miR-19a in the NF-κB pathway and that miR-19a can attenuate the activation of the NF-κB signalling pathway by targeting TBK1, thereby reducing the production of pro-inflammatory cytokines and alleviating LPS-induced inflammation. Therefore, miR-19a might possibly be used as a target for the treatment of endometritis.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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