

Research Article

Suppression of lncRNA MALAT1 Reduces LPS- or IL-17A-Induced Inflammatory Response in Human Middle Ear Epithelial Cells via the NF- κ B Signaling Pathway

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Otitis media (OM) is a common inflammatory disease of the middle ear cavity and mainly occurs in children. As a critical regulator of inflammation response, the nuclear factor kappa B (NF- κ B) pathway has been found to play an essential role in the pathogenesis of various human diseases. The aim of this study was to explore the potential mechanism under the inflammatory response of human middle ear epithelial cells (HMEECs). We established *in vitro* models of OM by treating HMEECs with lipopolysaccharide (LPS) or interleukin 17A (IL-17A). Enzyme-linked immunosorbent assay and western blot analysis were used to measure the inflammatory response of HMEECs under LPS or IL-17A stimulation. The results revealed that the concentrations of proinflammatory cytokines ($p < 0.001$) and protein levels of mucin (MUC) (for MUC5AC, $p = 0.002$, $p = 0.004$; for MUC8, $p = 0.004$, $p < 0.001$) were significantly elevated by LPS or IL-17A stimulation in HMEECs. Moreover, we found that LPS or IL-17A treatment promoted the phosphorylation of I κ B α (for p-I κ B α , $p = 0.018$, $p = 0.002$; for I κ B α , $p = 0.238$, $p = 0.057$) and the translocation of p65 from cytoplasm to nucleus in HMEECs (for nucleus p65, $p = 0.01$; for cytoplasm p65, $p < 0.001$). In addition, RT-qPCR analysis revealed that long noncoding RNA (lncRNA) metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was verified to be upregulated in LPS- or IL-17A-stimulated HMEECs ($p < 0.001$). Western blot analysis and immunofluorescence staining assay revealed that MALAT1 knockdown significantly suppressed the activation of the NF- κ B pathway by reducing phosphorylated I κ B α levels and inhibiting the nuclear translocation of p65 ($p < 0.001$) in LPS- or IL-17A-stimulated HMEECs (for p-I κ B α , $p < 0.001$; for I κ B α , $p = 0.242$, $p = 0.647$). Silence of MALAT1 decreased the proinflammatory cytokine production and MUC protein levels ($p < 0.001$). Furthermore, rescue assays revealed that the increase of proinflammatory cytokine production (for TNF- α , $p = 0.002$, $p = 0.015$; for IL-1 β , $p < 0.001$, $p = 0.006$; for IL-6, $p = 0.002$, $p < 0.001$) and MUC protein levels (for MUC5AC, $p = 0.001$, $p < 0.001$; for MUC8, $p < 0.001$, $p = 0.001$) induced by MALAT1 overexpression was neutralized by 4-N-[2-(4-phenoxyphenyl) ethyl] quinazoline-4, 6-diamine (QNZ) treatment in LPS- or IL-17A-stimulated HMEECs. In conclusion, MALAT1 promotes inflammatory response in LPS- or IL-17A- stimulated HMEECs via the NF- κ B signaling pathway, which may provide a potential novel insight for the treatment of OM.

1. Introduction

Otitis media (OM) is a common inflammatory disease occurring in the middle ear cavity among children [1]. OM evolves into a more chronic stage accompanied with persistent inflammation and effusion in the middle ear [2]. OM has become a great threat to children's health, which may lead to conductive hearing loss, otalgia, and even induce behavioral problems [3, 4]. The spectrum of otitis media includes

acute and chronic forms, each of which can be either suppurative or nonsuppurative [5]. Determination of accurate incidence figures is impeded by the unavailability of universally accepted diagnostic criteria [5]. Inflammation in the middle ear, which can be provoked by different primary factors such as bacterial and viral infection, local allergic reactions, and reflux, is the crucial event in the pathogenesis of OM. Unresolved acute inflammatory responses or defective immunoregulation of middle inflammation can promote chronic

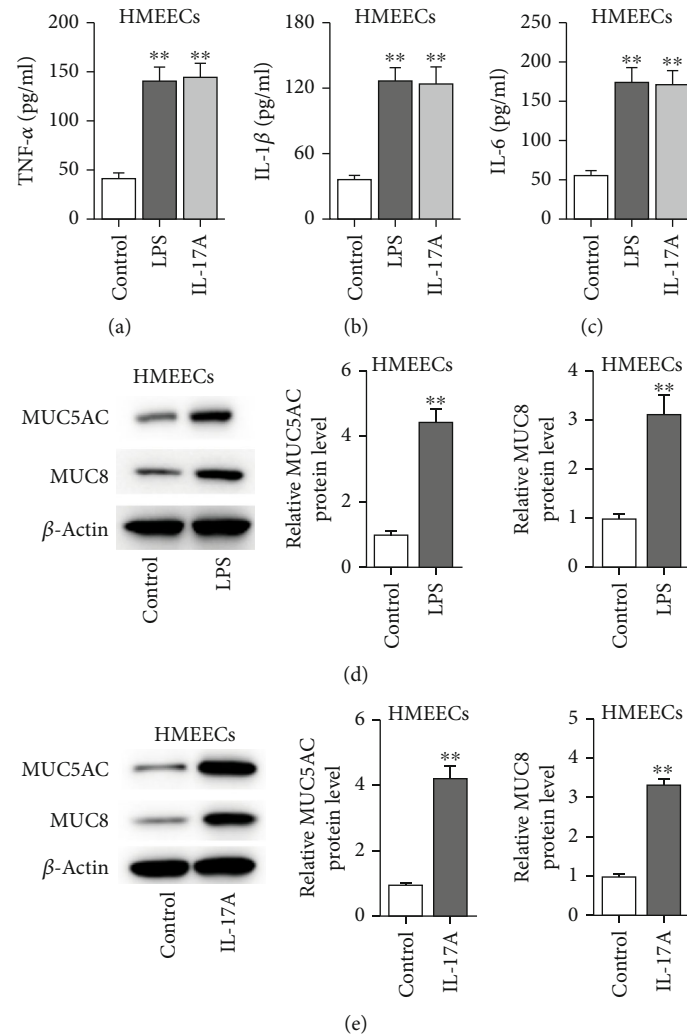


FIGURE 1: The levels of proinflammatory cytokines and MUC proteins in LPS- or IL-17A-stimulated HMEECs. (a–c) ELISA was used to measure concentrations of inflammatory cytokines (TNF- α , IL- β , and IL-6) in HMEECs under LPS or IL-17A treatment. (d, e) The protein levels of MUC5AC and MUC8 were measured by western blot in HMEECs under LPS or IL-17A treatment. ** $p < 0.01$.

inflammatory processes [6]. Cytokines are the central molecular regulators of middle ear inflammation and can switch the acute phase of inflammation in the chronic stage and induce molecular-pathological processes [6, 7]. Therefore, exploring the molecular mechanism underlying inflammatory response in OM progression will be of great significance for improving the therapy for this disease.

The sequence of the human genome was published in 2001, and studies showed that only about 1.2% encodes proteins, the rest being deemed “noncoding” [8]. Long noncoding RNAs (lncRNAs) are longer than 200 nucleotides and lack of open reading framework and protein-coding potential [9–12]. They can control nuclear architecture and transcription in the nucleus and modulates mRNA stability, translation, and posttranslational modifications in the cytoplasm [11]. Increasing studies had proposed that lncRNAs are implicated in various physiological and pathological processes including inflammatory response [13, 14]. For example, AC006129.1 reactivates a SOCS3-induced anti-

inflammatory response via DNA methylation-induced CIC downregulation in schizophrenia [15]. Moreover, Arid2-IR regulates inflammatory response by targeting the TGF- β /smad signaling pathway, which may serve as a novel therapeutic target for the improvement of renal inflammation [16]. Importantly, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) was reported to accelerate inflammatory response in sepsis [17]. Additionally, knock-down of MALAT1 suppresses inflammatory responses by increasing miR-146a level in acute lung injury [18]. However, the effect of MALAT1 on inflammatory response in OM remains unknown.

Nuclear factor-kappa B (NF- κ B), a ubiquitous deoxyribonucleic acid- (DNA-) binding transcription factor, is a key member of NF- κ B signaling proteins, which is closely associated with inflammatory response in OM [19, 20]. For instance, cigarette smoke activates NF- κ B and upregulates Muc5b protein levels in mouse middle ear cells [21]. Moreover, guggulsterone contributes to the inflammation by

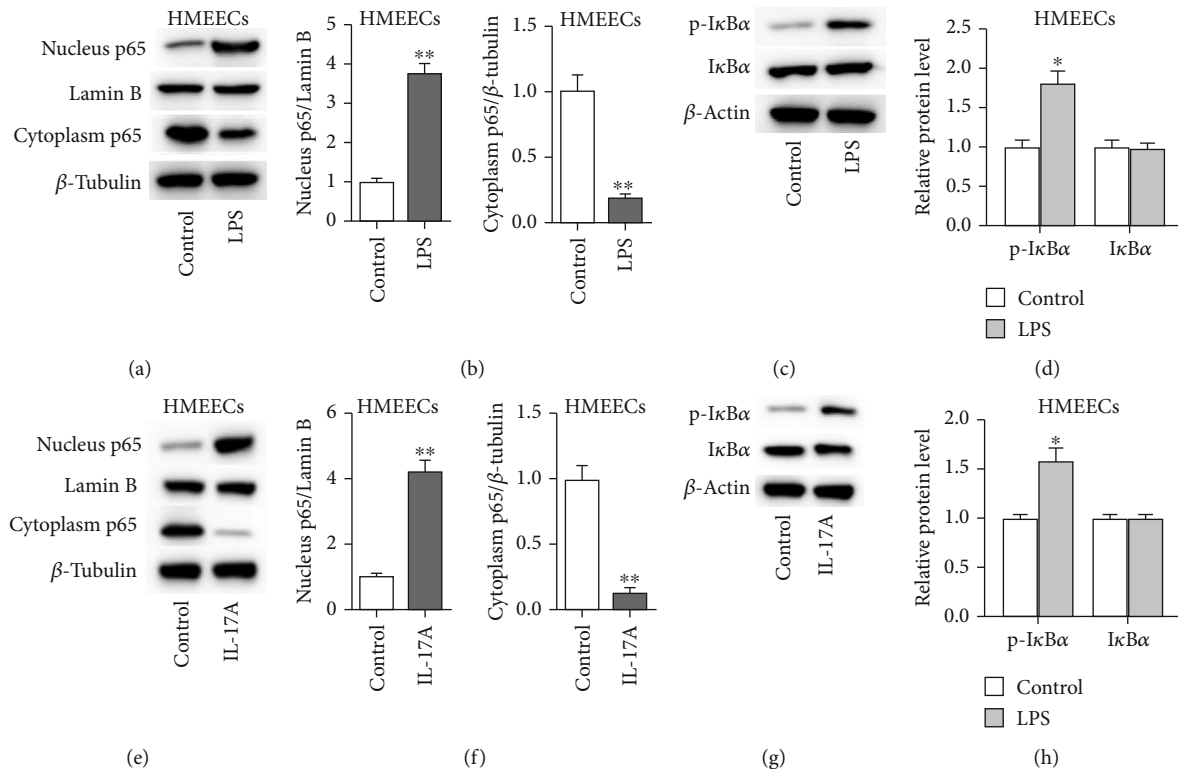


FIGURE 2: The NF- κ B pathway is activated by LPS or IL-17A treatment in HMEECs. (a, b) The effect of LPS on protein levels of cytoplasm p65 and nucleus p65 was determined by western blot analysis. (c, d) The effect of LPS on protein levels p-I κ B α and I κ B α in HMEECs. (e, f) The effect of IL-17A on protein levels of cytoplasm p65 and nucleus p65 was determined by western blot analysis. (g, h) The effect of IL-17A on protein levels p-I κ B α and I κ B α in HMEECs. * $p < 0.05$, ** $p < 0.01$.

inactivating NF- κ B in human middle ear epithelial cells (HMEECs) [22]. However, the relationship between MALAT1 and NF- κ B in HMEECs remains uncharacterized.

In the current study, lipopolysaccharide (LPS) or interleukin 17A (IL-17A) was used to treat HMEECs to induce the inflammatory response. We intended to explore the role and molecular mechanism of MALAT1 in LPS- or IL-17A-stimulated HMEECs. This discovery may be helpful for a better understanding of OM treatment.

2. Materials and Methods

2.1. Cells and Cell Treatment. Human middle ear epithelial cells (HMEECs, provided by Dr. David J. Lim, House Ear Institutes, LA) were immortalized with the E6/E7 genes of human papillomavirus type 16 as reported previously [23, 24]. HMEECs were incubated in a mixture (1 : 1) of Dulbecco's modified Eagle's medium (DMEM; Invitrogen, USA) and bronchial epithelial basal medium (Clonetics, USA) containing hydrocortisone (0.5 mg/ml), bovine pituitary extract (52 mg/ml), epinephrine (0.5 mg/ml), hEGF (0.5 ng/ml), gentamicin (50 mg/ml), transferrin (10 mg/ml), triiodothyronine (6.5 ng/ml), insulin (5 mg/ml), retinoic acid (0.1 ng/ml), and amphotericin B (50 ng/ml). HMEECs were kept at subconfluence at 37°C in a carbon dioxide-enriched humidified atmosphere with 95% air and 5% CO₂. LPS (Sigma Aldrich, USA) or IL-17A (MedChemExpress, Shanghai, China) was used to induce the inflammatory response. HMEECs were cultured

to 80% confluence in six-well plates. LPS or IL-17A was added after starvation for 24 h. In the experimental group, 50 mg/ml of LPS or 100 ng/ml of IL-17A was added in medium. In the control group, LPS or IL-17A was not added in the medium. 4-N-[2-(4-phenoxyphenyl) ethyl] quinazoline-4, 6-diamine (QNZ) was purchased from Selleckchem (Houston, USA). QNZ dissolved in 0.1% dimethyl sulfoxide (DMSO; Sigma, USA; 1 μ M) was added into medium to inhibit NF- κ B signaling. The same dose of DMSO was also added for negative control.

2.2. Cell Transfection. The short hairpin RNA (shRNA) targeting MALAT1 (sh-MALAT1) with negative control (sh-NC) was used to knockdown MALAT1. MALAT1 was subcloned into pcDNA3.1 vector to overexpress MALAT1 in HMEECs with empty pcDNA3.1 as a control. The above-mentioned vectors were synthesized by GenePharma (Shanghai, China) and transfected into HMEECs for 48 h using Lipofectamine 2000 (Invitrogen, USA) under the manufacturer's instructions.

2.3. Enzyme-Linked Immunosorbent Assay (ELISA). The contents of tumor necrosis factor α (TNF- α), interleukin-6 (IL-6), and interleukin-1 β (IL-1 β) in HMEECs cellular supernatants in each group were detected by an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's instructions. Each experiment was repeated three times.

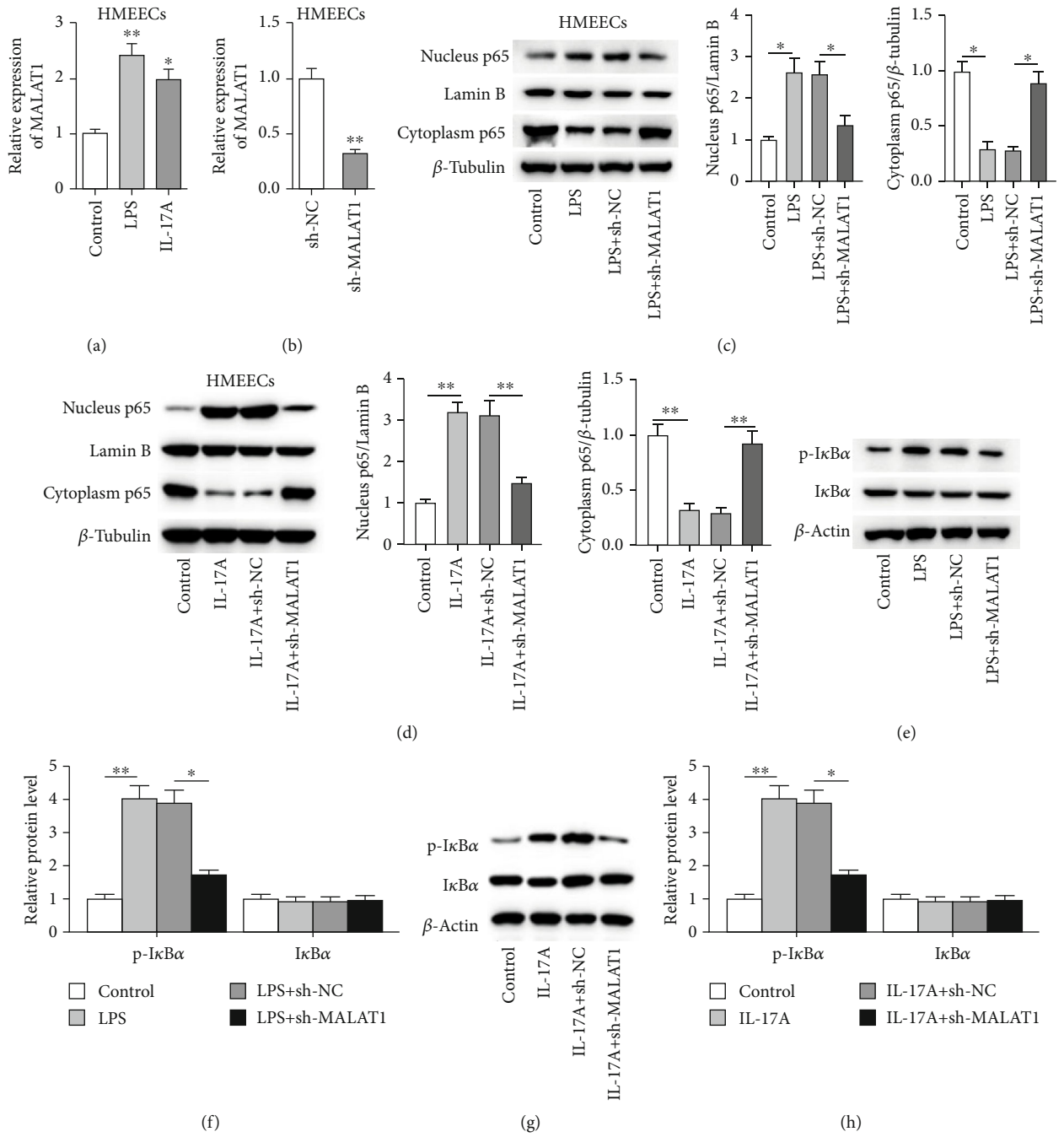


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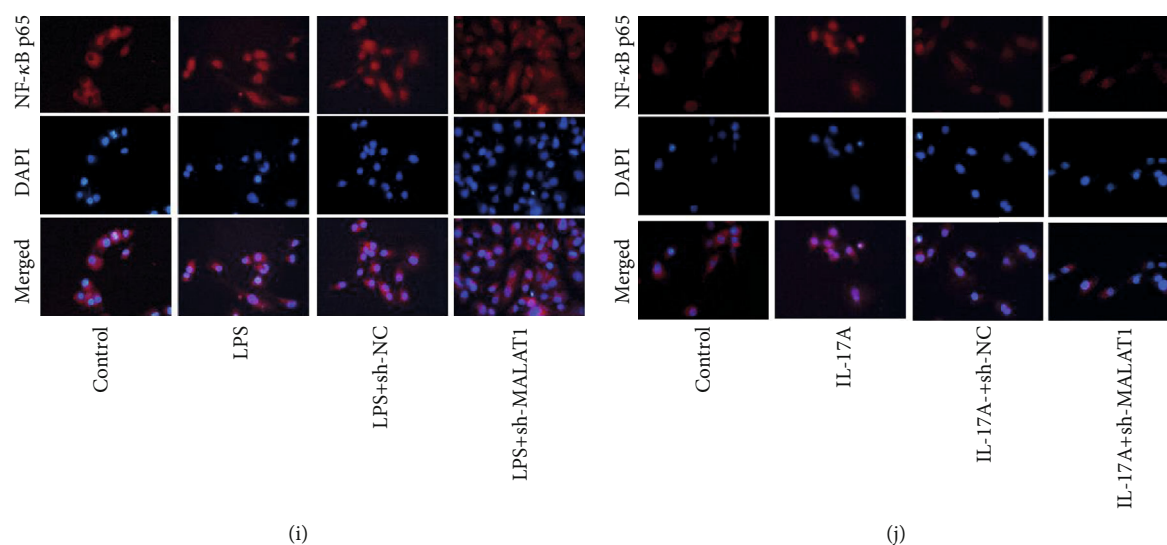


FIGURE 3: Knockdown of MALAT1 inactivates the NF- κ B pathway. (a) The level of MALAT1 was explored by RT-qPCR assay in control, LPS, or IL-17A group. (b) The knockdown efficacy of sh-MALAT1 in HMEECs was explored by RT-qPCR analysis. (c-h) The effect of MALAT1 on protein levels of cytoplasm p65, nucleus p65, p-I κ B α , and I κ B α in HMEECs under LPS or IL-17A treatment. (i, j) Immunofluorescence assay was conducted to assess the nuclear translocation of p-NF- κ B in HMEECs under LPS or IL-17A treatment. * $p < 0.05$, ** $p < 0.01$.

2.4. Reverse-Transcription Quantitative Polymerase Chain Reaction (RT-qPCR). The extraction of total RNA from HMEECs was performed by the TRIzol Reagent (Invitrogen). The RevertAid First Strand cDNA Synthesis Kit (K1622, Thermo Scientific, Waltham, MA) was applied for reverse transcription. The qPCR was carried out in the SYBR-Green PCR Master Mix kit (Applied Biosystems, USA). Each experiment was repeated three times. The relative expression levels of MALAT1 were calculated with the $2^{-\Delta\Delta C_t}$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a control gene. The primer sequences are listed as follows:

MALAT1: 5'-AAT GTT AAG AGA AGC CCA GGG-3' (forward), 5'-AAG GTC AAG AGA AGT GTC AGC-3' (reverse); GAPDH: 5'-TAT GAT GAT ATC AAG AGG GTA GT-3' (forward), 5'-TGT ATC CAA ACT CAT TGT CAT AC-3' (reverse).

2.5. Western Blot. After transfection, the treated or control cells were lysed with lysis buffer containing protease inhibitors. The protein samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels and then transferred to polyvinylidene fluoride membranes. After blocking with 5% defatted milk, the membranes were incubated with primary antibodies against MUC8 (Santa Cruz Biotechnology, Beijing, China), MUC5AC (ab198294, Abcam, USA), p65 (ab16502, Abcam), Lamin B (ab194109, Abcam), β -tubulin (ab6046, Abcam), I κ B α (ab32518, Abcam), p-I κ B α (ab133462, Abcam), and β -actin (ab8227, Abcam) at a dilution of 1:1000 at 4°C overnight. Following washing with Tris-Buffered Saline Tween-20 (TBST), the membranes were exposed to HRP-conjugated goat anti-rabbit (1:5000, Abcam) secondary antibodies. At last, protein bands were detected with an ECL detection system and

quantified by ImageJ. Each experiment was repeated three times.

2.6. Preparation of Nuclear Extracts. The HMEEC suspensions were prepared by Trypsin-EDTA treatment. The proteins were extracted from the cytoplasm and nucleus of HMEECs using a kit from Pierce (Rockford, IL) according to the manufacturer's instructions.

2.7. Immunofluorescent Staining. Treated or control HMEECs were transfected with sh-NC or sh-MALAT1 on a cover slide in 12-well plates. Then, HMEECs were washed 3 times with phosphate buffer saline (PBS; Invitrogen), followed by fixation for 10 minutes with 4% paraformaldehyde at room temperature and washed again. Afterward, HMEECs were blocked with 1% bovine serum albumin incubated with primary antibody anti-p65 (ab16502, Abcam). After washing with PBS, the secondary antibody fluorescein isothiocyanate-conjugated IgG was added. At last, the slides were rinsed, mounted, and viewed on a confocal microscope (FV1000, Olympus, Japan) at 488 nm. Each experiment was repeated three times.

2.8. Statistical Analysis. The data were analyzed by SPSS 19.0 software and showed as the means \pm standard deviation. The unpaired Student's *t*-test was employed to compare the differences between the 2 groups. One-way analysis of variance was applied for the comparisons of more than two groups followed by a Tukey post hoc test. $p < 0.05$ had statistical significance. Experiments were operated for 3 times.

3. Results

3.1. The Level of Proinflammatory Cytokines and MUC Proteins in LPS- or IL-17A-Stimulated HMEECs. To determine the effect of LPS or IL-17A on inducing inflammation

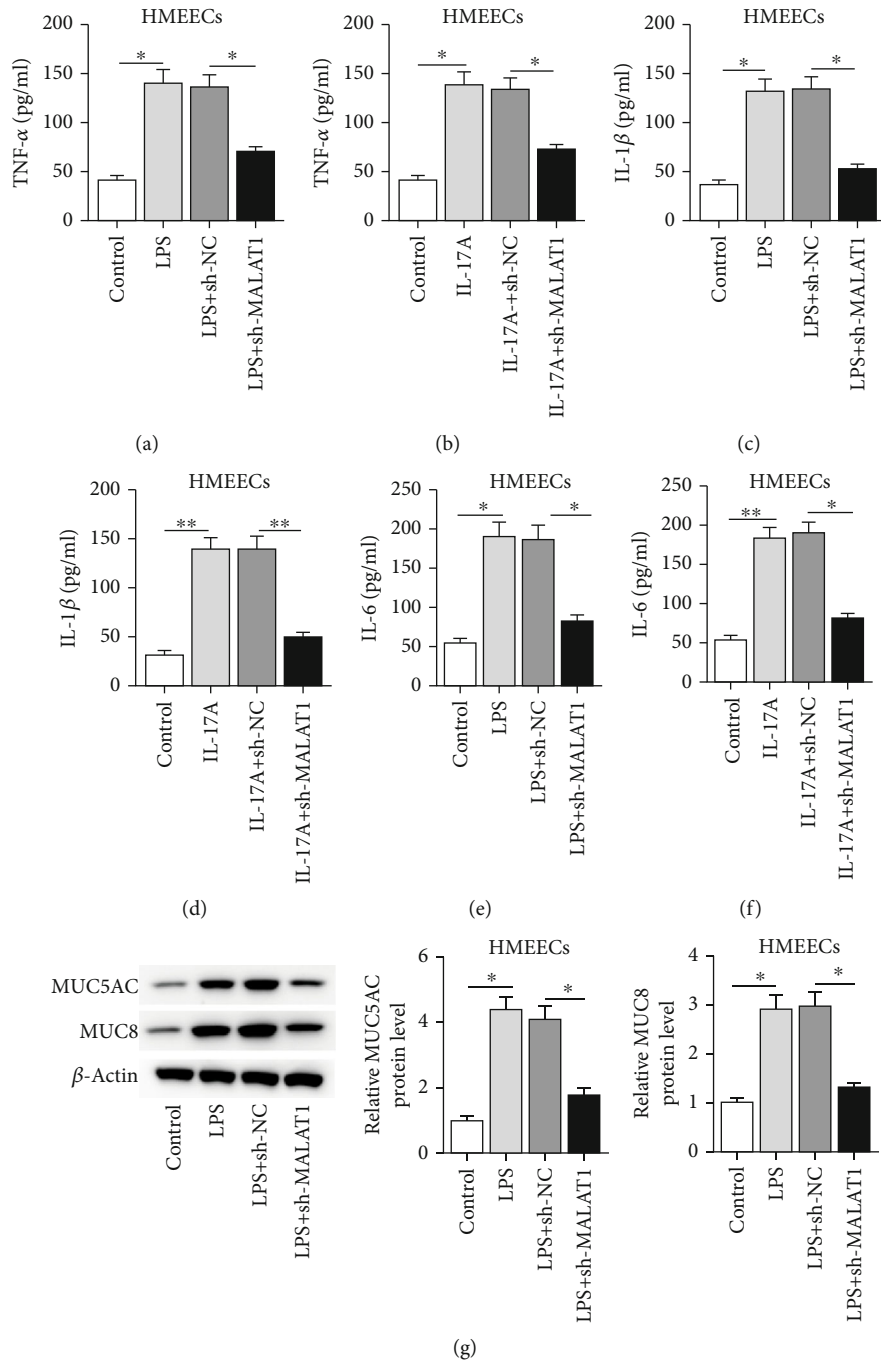


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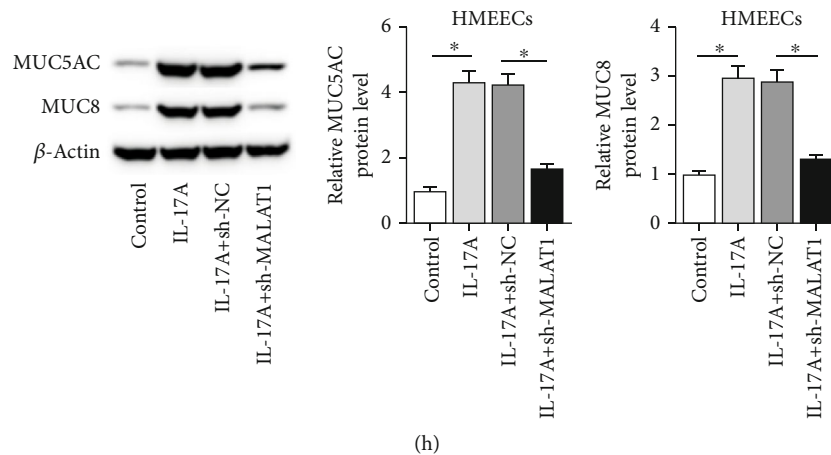


FIGURE 4: Role of MALAT1 in proinflammatory cytokine production and MUC protein levels. (a–f) The effect of MALAT1 on concentrations of inflammatory cytokines (TNF- α , IL- β , and IL-6) was evaluated by ELISA assay in HMEECs under LPS or IL-17A treatment. (g, h) The effect of MALAT1 on protein levels of MUC5AC and MUC8 was measured by western blot in HMEECs under LPS or IL-17A treatment. * $p < 0.05$, ** $p < 0.01$.

in HMEECs, ELISA kits were applied to detect the concentration of proinflammatory cytokines. As presented in Figures 1(a)–1(c), the levels of TNF- α , IL-1 β , and IL-6 were significantly increased in HMEECs treated with LPS or IL-17A (Figure 1(a), $p < 0.001$; Figure 1(b), $p < 0.001$; Figure 1(c), $p < 0.001$). Next, we examined MUC5AC and MUC8 protein levels and found that the MUC5AC and MUC8 protein levels were elevated in HMEECs exposed to LPS or IL-17A (Figures 1(d) and 1(e)) (Figure 1(d), $p = 0.002$, $p = 0.004$; Figure 1(e), $p = 0.004$, $p < 0.001$).

3.2. The NF- κ B Pathway Is Activated by LPS or IL-17A Treatment in HMEECs. We then aimed to explore the NF- κ B signaling in HMEECs. As presented in Figures 2(a) and 2(b), western blot analysis elucidated that LPS treatment induced the rise of nucleus p65 protein level but a decline of cytoplasm p65 protein level (Figure 2(b), for nucleus p65, $p < 0.001$; for cytoplasm p65, $p < 0.001$). In addition, the phosphorylated I κ B α protein level was significantly enhanced by LPS or IL-17A treatment in HMEECs (Figures 2(c) and 2(d)) (Figure 2(d), for p-I κ B α , $p = 0.018$; for I κ B α , $p = 0.238$). Similarly, IL-17A stimulated the NF- κ B signaling in HMEECs (Figures 2(e)–2(h)) (Figure 2(f), for nucleus p65, $p = 0.01$; for cytoplasm p65, $p < 0.001$; Figure 2(h), for p-I κ B α , $p = 0.002$; for p-I κ B α , $p = 0.057$).

3.3. Knockdown of MALAT1 Inactivates the NF- κ B Pathway. RT-qPCR was applied to measure the expression of MALAT1 in HMEECs. The result indicated that MALAT1 level was increased in LPS- or IL-17A-stimulated HMEECs (Figure 3(a), $p < 0.001$). As shown in Figure 3(b), MALAT1 expression was decreased by transfection of sh-MALAT1 into HMEECs (Figure 3(b), $p < 0.001$). Furthermore, we found that MALAT1 knockdown significantly decreased the nuclear p65 protein level but increased cytoplasmic p65 protein level (Figures 3(c) and 3(d)) (Figure 3(c), for nucleus p65, $p < 0.001$; for cytoplasm p65, $p < 0.001$; Figure 3(d), for nucleus p65, $p < 0.001$; for cytoplasm p65, $p < 0.001$), as well as inhibited the phosphorylation of I κ B α and in LPS-

or IL-17A-treated HMEECs (Figures 3(e)–3(h)) (Figure 3(f), for p-I κ B α , $p < 0.001$; for p-I κ B α , $p = 0.242$; Figure 3(h), for p-I κ B α , $p < 0.001$; for p-I κ B α , $p = 0.647$). Moreover, the immunofluorescence staining assay directly revealed that the nuclear translocation of p65 was significantly inhibited by MALAT1 knockdown in LPS- or IL-17A-stimulated HMEECs (Figures 3(i) and 3(j)).

3.4. Role of MALAT1 in Proinflammatory Cytokines Production and MUC Protein Levels. We then aimed to explore the effects of MALAT1 on productions of proinflammatory cytokines in HMEECs. As indicated in Figures 4(a)–4(f), MALAT1 knockdown attenuated the promotive effect of LPS or IL-17A on the concentrations of proinflammatory cytokines (TNF- α , IL-1 β , and IL-6) (Figures 4(a)–4(f), $p < 0.001$). Moreover, LPS- or IL-17A-induced the increase of MUC5AC and MUC8 protein levels was recovered by knockdown of MALAT1 (Figures 4(g) and 4(h), $p < 0.001$).

3.5. MALAT1 Promotes LPS- or IL-17A-Induced Inflammatory Response through the NF- κ B Pathway. Next, a series of rescue assays were conducted to verify that MALAT1 participated in LPS- or IL-17A-induced inflammatory response via the NF- κ B pathway in HMEECs. As revealed in Figure 5(a), the overexpression efficacy of MALAT1 was confirmed by RT-qPCR (Figure 5(a), $p = 0.012$). Moreover, we observed that the contents of proinflammatory cytokines (TNF- α , IL-1 β , and IL-6) were increased by MALAT1 overexpression in LPS- or IL-17A-treated HMEECs. QNZ, an inhibitor of NF- κ B signaling, was widely used to suppress NF- κ B signaling [25–27]. In addition, the effects of MALAT1 on proinflammatory cytokines were significantly reversed by QNZ (Figures 5(b)–5(g)) (Figure 5(b), $p = 0.002$; Figure 5(c), $p = 0.015$; Figure 5(d), $p < 0.001$; Figure 5(e), $p = 0.006$; Figure 5(f), $p = 0.002$; Figure 5(g), $p < 0.001$). In addition, the increase of MUC5AC and MUC8 protein levels induced by MALAT1 overexpression was neutralized by QNZ treatment in LPS- or IL-17A-treated HMEECs (Figures 5(h) and 5(i))

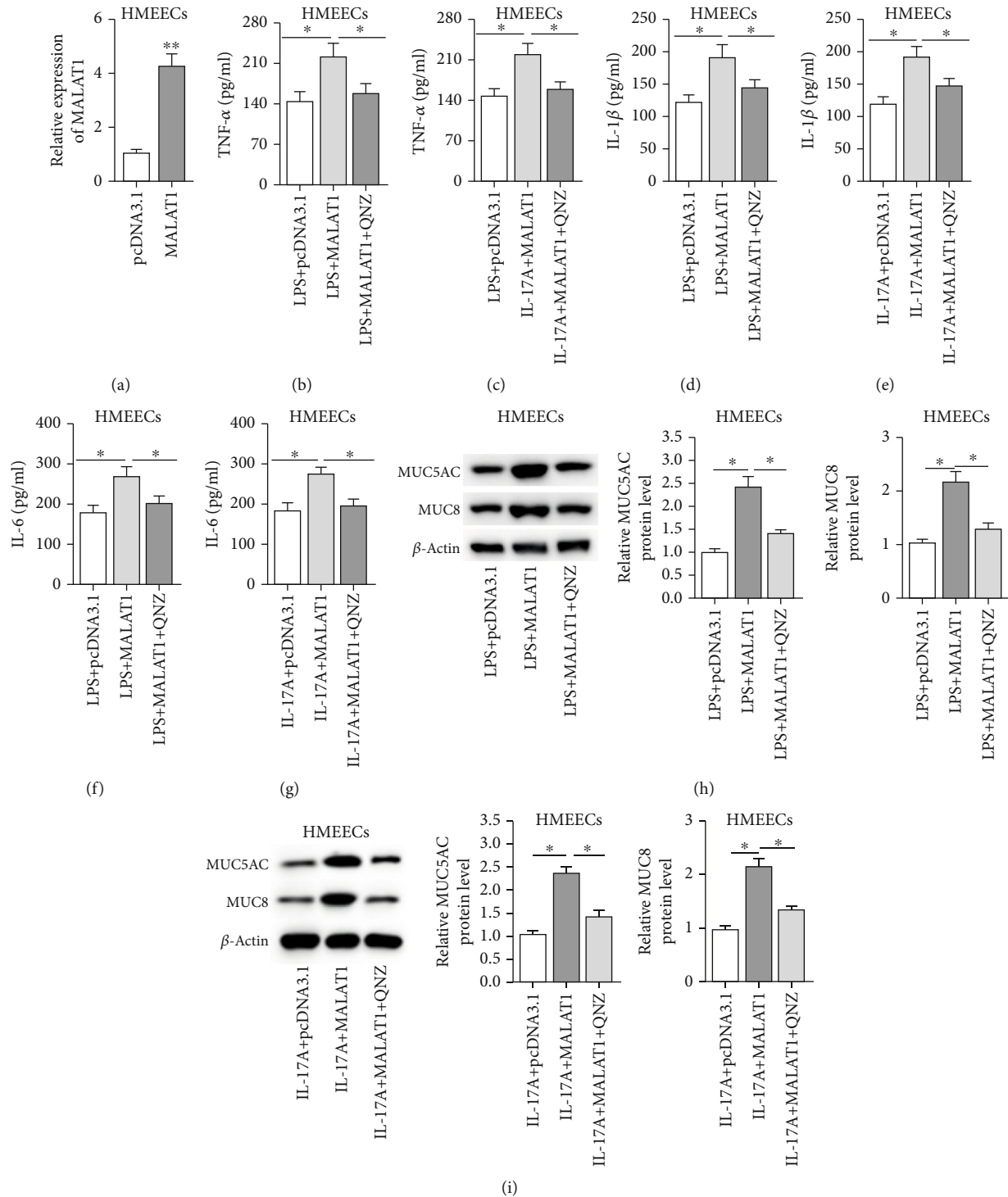


FIGURE 5: MALAT1 promotes LPS- or IL-17A-induced inflammatory response through the NF-κB pathway. (a) The overexpression efficacy of pcDNA3.1/MALAT1 in HMEECs was detected by RT-qPCR analysis. (b-g) The effects of MALAT1 and QNZ on concentrations of inflammatory cytokines (TNF-α, IL-β, and IL-6) were evaluated by ELISA assay in HMEECs under LPS or IL-17A treatment. (h, i) The effects of MALAT1 and QNZ on protein levels of MUC5AC and MUC8 were measured by western blot in LPS- or IL-17A-induced HMEECs. **p* < 0.05, ***p* < 0.01.

(Figure 5(h), for MUC5AC, $p = 0.001$; for MUC 8, $p < 0.001$; Figure 5(i), for MUC5AC, $p < 0.001$, for MUC 8, $p = 0.001$).

4. Discussion

LPS, a component of gram-negative bacteria cell wall, is widely used to induce inflammatory responses in the host cells [28, 29]. In our research, the treatment of LPS increased the concentrations of proinflammatory cytokines (TNF- α , IL-1 β , and IL-6) in HMEECs. Additionally, LPS induced the secretion of MUC protein (MUC5AC and MUC8) levels. IL-17 is known as a proinflammatory cytokine in cellular processes [30, 31]. The IL-17A reagent is used as an activator of inflammatory responses in previous studies [32, 33]. In our report, IL-17A stimulation elevated the concentrations of proinflammatory cytokines (TNF- α , IL-1 β , and IL-6) in HMEECs. Moreover, IL-17A treatment promoted the secretion of MUC protein (MUC5AC and MUC8) levels. These findings indicated the successful establishment of *in vitro* models of OM.

lncRNA MALAT1 has been reported to play an essential role in the regulation of inflammatory response in a variety of diseases. Specifically, MALAT1 impedes inflammation response of in fibroblast-like synoviocytes via methylation of CTNBN1 promoter in rheumatoid arthritis [34]. Additionally, MALAT1 ameliorates LPS-induced inflammatory injury in murine chondrogenic ATDC5 cells by upregulating microRNA-19b [35]. Thus, we speculated that MALAT1 might regulate the inflammatory response in HMEECs. Herein, MALAT1 level was upregulated in LPS or IL-17A-stimulated HMEECs. Functionally, the knockdown of MALAT1 decreased the concentrations of proinflammatory cytokines (TNF- α , IL-1 β , and IL-6) and MUC protein (MUC5AC and MUC8) levels in HMEECs treated with LPS or IL-17A. These findings suggested that MALAT1 can induce the inflammatory response in LPS- or IL-17A-treated HMEECs.

The NF- κ B signaling pathway is a well-known regulator of inflammatory response [19, 20]. When the NF- κ B signaling pathway is activated, I κ B α is phosphorylated. The phosphorylated I κ B α can be ubiquitinated by ubiquitin ligase P-TrCP and then degraded by the 26S proteasome. As a result, NF- κ B is released from the cytoplasmic NF- κ B/I κ B α complex. The cytoplasmic NF- κ B is translocated into the nucleus rapidly and ultimately initiates target gene levels such as TNF- α , IL-1 β , and IL-6 [36, 37]. In our study, the treatment of LPS or IL-17A activated the phosphorylation of I κ B α and the nuclear translocation of NF- κ B. The abnormal activation of NF- κ B signaling has been widely reported to be related to the inflammatory response in OM. For instance, pneumococcal peptidoglycan-polysaccharides regulate inflammatory response in the mouse middle ear epithelial cells by targeting Toll-like receptor 2 [38]. Additionally, guggulsterone suppresses the inflammation of HMEECs by inhibiting I κ B α degradation [22].

In the current research, the knockdown of MALAT1 decreased the protein level of phosphorylated I κ B α and inhibited the nuclear translocation of NF- κ B in LPS- or IL-17A-treated HMEECs. QNZ, an inhibitor of NF- κ B signaling

[39], was used in rescue assays. The results depicted that the promotive effects of MALAT1 on concentrations of proinflammatory cytokines (TNF- α , IL-1 β , and IL-6) and MUC protein (MUC5AC and MUC8) levels in LPS- or IL-17A-treated HMEECs were partially rescued by treatment of QNZ. These findings suggested that MALAT1 can promote inflammatory response in LPS- or IL-17A-stimulated HMEECs via the activation of the NF- κ B signaling pathway. Moreover, silencing of MALAT1 protects human microvascular endothelial cells against inflammatory injury by inhibiting activation of NF- κ B [40]. MALAT1 contributes to the inflammatory response of microglia via the NF- κ B signaling pathway [41].

In conclusion, for the first time, we confirmed that the suppression of lncRNA MALAT1 reduced LPS- or IL-17A-stimulated inflammatory response in HMEECs via inhibiting the NF- κ B signaling pathway. This novel discovery may be helpful for a clinical treatment of OM. However, our study had limitations. First, the effects of MALAT1 on other cellular processes (such as cell apoptosis) or in other cells were not evaluated. Second, the role of MALAT1 in animal models was not explored. Finally, other mechanisms of MALAT1 remain to be investigated in OM models.

Data Availability

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

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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