

Circ_KATNAL1 promotes the inflammation and apoptosis in human middle ear epithelial cells induced by lipopolysaccharide by regulating the miR-153-3p / TLR4 axis

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ABSTRACT

The purpose of this experiment was to explore the effects and mechanism of circ_KATNAL1 on inflammatory injury and apoptosis of human middle ear epithelial cells (HMEECs) induced by lipopolysaccharide (LPS). For this aim, the cell inflammatory injury model was established by HMEECs cells induced by LPS. It was divided into a blank control, model, circ_KATNAL1 and circ_KATNAL1 + LPS groups. The cell viability was detected by the MTT method. The apoptosis rate of each group was detected by flow cytometry. The cell migration ability of each group was detected by cell scratch assay. The mRNA expression levels of miR-153-3p and TLR4 in the cells of each group were detected by RT-PCR method. The protein expressions of BCL-2 and TLR4 in the cells of each group were detected by WB method. The levels of IL-6 and TNF- α were detected by ELISA method. Results showed that compared with the control group, the cell viability in the model group was decreased, the cell apoptosis rate was increased, the cell migration ability was weakened, the mRNA expression level of miR-153-3p and protein expression level of BCL-2 in the cells were decreased, and the mRNA and protein expression levels of TLR4 were increased and the levels of IL-6 and TNF- α in the cell supernatant were increased. Compared with the model group, the cell viability in the circ_KATNAL1 group was increased, the cell apoptosis rate was decreased, and the cell migration ability was increased, the mRNA expression level of miR-153-3p and BCL-2 protein expression level in the cells were increased, the mRNA and protein expression levels of TLR4 were decreased, and the contents of IL-6 and TNF- α in the cell supernatant were decreased. Compared with the model group, the cell viability in the circ_KATNAL1 + LPS group was decreased, cell apoptosis rate was increased, cell migration ability was weakened, the mRNA expression level of miR-153-3p and protein expression level of BCL-2 in cells were decreased, mRNA and protein expression levels of TLR4 were increased, and the content of IL-6 and TNF- α in the cell supernatant were increased. The differences were all statistically significant ($P < 0.05$). It showed that LPS could promote cell injury by increasing inflammatory cell pyroptosis, and the abnormal expression of circ_KATNAL1 played an important role in cell inflammation induced by LPS. Up-regulation of circ_KATNAL1 could promote inflammatory pyroptosis in HMEECs induced by LPS. miR-153-5p and TLR4 were downstream targets of circ_KATNAL1. The inhibition of miR- 153-5p or up-regulation of TLR4 could reverse the protective effects of silencing circ_KATNAL1. In conclusion, circ_KATNAL1 can promote an inflammatory role in human middle ear epithelial cells through the miR- 31-5p / TLR4 axis, which may become an important target for the diagnosis and treatment of otitis media.

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Introduction

Otitis media (OM), one of the most common infectious diseases in kids, is an ear inflammation caused by a bacterial or viral infection(1). The pathogenesis of OM is complex, with various clinical symptoms and many complications (2), so there is no unified treatment plan. At present, the conventional clinical treatment mostly adopts combination therapy of antibiotics, antihistamines, anti-

congestive and anti-inflammatory drugs (3).

Circular RNA (circRNA), a type of non-coding RNA without 5' and 3' ribonucleotide terminal structures, is created by the reverse splicing of pre-mRNA(4), which is significant in human inflammatory immunological disorders(5). circ_KATNAL1 can enhance the inflammatory pyroptosis of cells(6), but its function and mechanism of action in otitis media remain to be explored. Existing research has demonstrated that miR-

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153-3p can lessen LPS-induced cell inflammation and death(7), while TLR4 has the opposite effects, and studies indicated that blocking TLR4 can help lessen LPS-induced cell damage by decreasing inflammation and apoptosis(8).

In order to simulate otitis media injury, the aim of this study was to examine the role of circ_KATNAL1 in the proliferation, apoptosis, and inflammatory response of human middle ear epithelial cells (HMEECs) induced by LPS.

Materials and Methods

Materials

Cells

The HMEECs cell line was provided by the Institute of Peking Union Medical College.

Instruments

The instruments included CO₂ cell incubator (Thermo Scientific, USA); Real-Time PCR instrument Q162D (Hebei Sanshi Biotechnology Co., Ltd.); protein and nucleic acid analyzer (Eppendorf, Germany); Odyssey two-color infrared laser scanning developer (Li-Cor company, USA); ELISA (Thermo, USA); RT-6100 Microplate Reader (Rayto); fluorescence microscope (Carl Zeiss); optical microscope (Beijing INVT Technology Co., Ltd.); D3024R table top high-speed refrigerated centrifuge (DRAGONLAB Corporation); MV-100 Vortex Mixer (Wuhan Sevier Biotechnology Co., Ltd.); JY92-11N ultrasonic cell breaker (Ningbo Xinzhi Biotechnology Co., Ltd.); pipette (Eppendorf, Germany).

Drugs and reagents

DMEM high glucose medium and fetal bovine serum (article number: C11995500BT, 16000044) were purchased from Gibco, USA; dimethyl sulfoxide (analytical grade, article number: D2650) was purchased from Sigma, USA; LPS (article number: L2880) was purchased from Sigma company; RIPA lysate was purchased from Wuhan Service Biological Co., Ltd.; qPCR kits: Taq pro Universal SYBR qPCR Master Mix (article number: Q712-02) was purchased from China Novozyme; Trizol reagent (article number: 15596018) was purchased from Invitrogen, USA; PCR primers were provided by Sangon Bioengineering (Shanghai) Co., Ltd.; IL -6 and TNF- α enzyme-linked immunosorbent assay (ELISA) kits (batch numbers CAS148157-34-0, CAS94948 -59-1) were purchased from Shanghai Kemin Biotechnology Co., Ltd.; primary antibodies BCL-2 and TLR4 (article numbers: GB124830 , GB 11519), internal reference primary antibody GAPDH (article number: GB15002), goat anti-mouse secondary antibody, goat anti-rabbit secondary antibodies (article numbers: GB23301, GB23303) were purchased from Wuhan Service Biotechnology Co., Ltd.

Methods

Cell culture, grouping and transfection

HMEEC cells were inoculated in DMEM medium with 10% fetal bovine serum, and placed in an incubator at 37°C and 5% CO₂ for routine culture. The cells in the logarithmic growth phase were taken, and the cell

density was adjusted to 1×10^5 cells/mL with culture medium, and then the cells were inoculated in a 96-well plate with 100 μ L per well. Cells were divided into 4 groups: blank control group, model group, circ_KATNAL1 group and circ_KATNAL1 + LPS group. The blank control group was not treated, the model group was treated with LPS, and the circ_KATNAL1 group was transfected with circ_KATNAL1 and the circ_KATNAL1 + LPS group was treated with LPS after transfection with circ_KATNAL1. circ_KATNAL1 group and circ_KATNAL1 + After the cells in the circ_KATNAL1 group and circ_KATNAL1 + LPS group were cultured in the transfection medium for 6 h, the medium was replaced and the culture was continued for 18 h, and the transfection efficiency was determined by qRT-PCR (9).

Detection of cell viability by MTT method

After the cells were treated in group in the culture solution and continued to culture for 24 h, the culture solution was aspirated. Then 20 μ L MTT solution (5 mg/ml) was added and cultured continuously at the condition of 37°C, 5% CO₂ for 4 h, and then the culture solution was aspirated, and 100 μ L of dimethyl sulfoxide was added. Then blank wells and control wells were set up. The optical density (OD) value was measured at a wavelength of 490 nm with a microplate reader and the cell viability was calculated.

Cell viability = [(optical density of experimental well - optical density of blank well)/(optical density of control well - optical density of blank well)] \times 100%.

Detection of cell apoptosis rate by flow cytometry

Cells in each group were treated accordingly and collected after 24 h of culture, added binding buffer to resuspend to 1×10^6 cells/mL, then added 5 μ L AnnexinV-FITC respectively, mixed well, and reacted in the dark at 4°C for 10 min. Another 200 μ L of binding buffer was added to mix well, and the cell apoptosis rate of each group was detected by flow cytometry. The steps were repeated 3 times for each group.

Cell apoptosis rate = number of apoptotic cells / (number of apoptotic cells + normal cell number) \times 100%.

Detection of cell migration rate by cell scratch assay

Cells were inoculated into a 6-well plate according to the specification of 1×10^5 /well, and after corresponding treatments were performed on them, a clean 100 μ L pipette tip was used to vertically streak the surface of the plate. This timing was 0 h, and the culture plate was placed in the incubator for 24 h after taking pictures and recording under an inverted microscope, and then the culture plate was taken out to take pictures, and the cell migration rate was calculated according to the change of the scratch area. The steps were repeated 3 times for each group.

Cell migration rate = (0 h scratch area - 24 h scratch area) / 0 h scratch area \times 100%.

Detection of the mRNA expression levels of miR-153-3p and TLR4 in cells by RT-PCR method

RNA sample was extracted by using Trizol reagent and the total RNA was extracted according to the instructions of the RNA extraction kit, then the reverse

Table 1. RT-PCR primer sequences.

Gene		Primer sequences (5' - 3')
U6	Forward:	GCTTCGGCAGCACATATACTAAAAT
	Reverse:	CGCTTCAGAAATTTGCGTGTTCAT
GAPDH	Forward:	AAGAGGAGGTTCGCGCCCGATCG
	Reverse:	CTGACTGATGGAATTAGGCTAAC
miR-153-3p	Forward:	GGGTTGCATAGTCACAAAAAG
	Reverse:	TTTGGCACTAGCACATT
TLR4	Forward:	GGTAGTGTACGGACACCATGAG
	Reverse:	CCTCGTACAAGG TCCGCTATTCT

transcription kit was used to reverse transcribe the total RNA to obtain the cDNA, and Real-Time PCR reaction was performed by using the Real-Time PCR instrument according to the instructions of the qPCR kit, and the reaction condition was 95°C for 120 s, 95°C for 50 s, and 60°C for 30 s, with a total of 40 cycles, and 72°C for 120 s. The data were recorded, with U6 as the internal reference of miR-153-3p and GAPDH as the internal reference of TLR4, and the mRNA expression levels of miR-153-3p and TLR4 were calculated according to the CT value. Relative expression levels were calculated by using the $2^{-\Delta\Delta C_t}$ method. Primer sequences are shown in Table 1.

Detection of the protein expression levels of BCL-2 and TLR4 in cells by Western blot method

After the HMEECs cells of each group were washed with pre-cooled PBS, RIPA buffer was added for lysis, then tissue protein was extracted after centrifugation, and the protein concentration was determined with a BCA kit. HMEECs cell protein samples in each group were separated with sodium dodecyl sulfate-polyacrylamide gel to perform polyacrylamide gel electrophoresis, transfer to membrane, block, and incubate different primary antibodies respectively, and then BCL-2 (1: 2 000), TLR4 (1: 30 00) and internal reference GAPDH (1: 2 000) were added, kept at 4°C overnight. After rinsing the membrane, the samples were incubated with a secondary antibody (1: 5 000) for 2 h at room temperature. After washing 3 times, a developing solution was added, and the samples were exposed and observed in a chemiluminescence imaging system to obtain protein bands of BCL-2, TLR4 and GAPDH, then Image-J software was used to scan the gray value of protein bands, and the relative quantification of the expression levels of BCL-2 and TLR4 was analyzed.

Detection of the levels of inflammatory factors IL-6 and TNF- α in the cell supernatant by ELISA method

The supernatant of cells in each group was collected, and the contents of IL-6 and TNF- α in the supernatant of cells in each group were detected according to the instructions of the ELISA kit.

Statistical analysis

The experimental data obtained were processed and analyzed by using SPSS 26.0 software. Quantitative data were presented in the form of mean \pm standard deviation (mean \pm standard deviation) and the comparison between two groups was performed by independent sample t-test, and the comparison among multiple

groups was performed by one-way analysis of variance. $P < 0.05$ meant the difference was statistically significant.

Results

Comparison of cell viability in each group

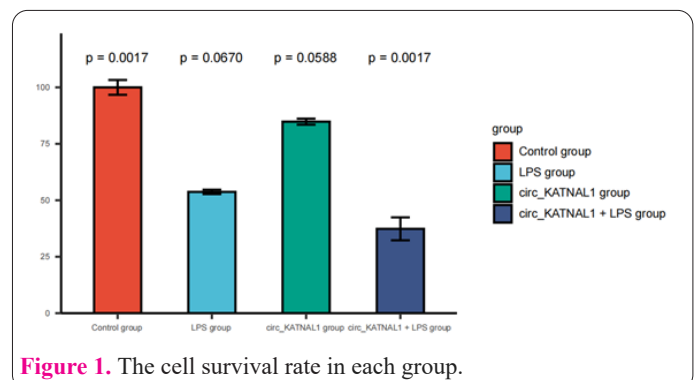
The cell survival rate of the blank control group was (100.00 \pm 3.28) %, the cell survival rate of the model group was (53.71 \pm 0.97) %, the cell survival rate of the circ_KATNAL1 group was (84.82 \pm 1.33) %, and the cell survival rate of the circ_KATNAL1 + LPS group was (37.36 \pm 5.08) %. Compared with the blank control group, the cell survival rate in the model group was significantly decreased; compared with the model group, the cell survival rate in the circ_KATNAL1 group was significantly increased, and the cell survival rate in the circ_KATNAL1 + LPS group was significantly decreased, and the differences were statistically significant ($P < 0.05$). See Table 2 and Figure 1.

Comparison of cell apoptosis rate in each group

The apoptosis rate of the blank control group was (6.01 \pm 0.21) %, the apoptosis rate of the model group was (13.01 \pm 2.25) %, the apoptosis rate of the circ_KATNAL1 group was (8.57 \pm 1.93) %, and the apoptosis rate of the circ_KATNAL1 + LPS group was (14.62 \pm 3.72) %. Compared with the blank control group, the cell apoptosis rate of the model group was significantly increased; compared with the model group, the cell apoptosis rate of the circ_KATNAL1 group was significantly decreased, and the cell

Table 2. Detection results of cell survival rate ($\bar{x} \pm s$, $n = 3$).

Group	Cell survival rate (%)
Control group	100.00 \pm 3.28
LPS group	53.71 \pm 0.97
circ_KATNAL1 group	84.82 \pm 1.33
circ_KATNAL1 + LPS group	37.36 \pm 5.08

**Figure 1.** The cell survival rate in each group.

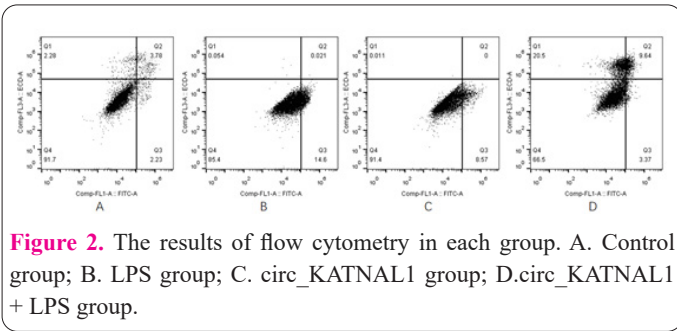


Figure 2. The results of flow cytometry in each group. A. Control group; B. LPS group; C. circ_KATNAL1 group; D. circ_KATNAL1 + LPS group.

Table 3. Detection results of apoptosis rate ($\bar{x} \pm s$, n = 3).

Group	Cell apoptosis rate (%)
Control group	6.01 ± 0.21
LPS group	13.01 ± 2.25
circ_KATNAL1 group	8.57 ± 1.93
circ_KATNAL1 + LPS group	14.62 ± 3.72

Table 4. Calculation results of cell migration rate ($\bar{x} \pm s$, n = 3).

Group	Cell migration rate(%)
Control group	89.77 ± 3.04
LPS group	43.12 ± 2.75
circ_KATNAL1 group	73.51 ± 3.48
circ_KATNAL1 + LPS group	27.36 ± 5.19

Table 5. The mRNA expression levels of miR-153-3p and TLR4 in cells ($\bar{x} \pm s$, n = 3).

Group	miR-153-3p	TLR4
Control group	1.02 ± 0.13	0.99 ± 0.04
LPS group	0.61 ± 0.07	4.23 ± 0.15
circ_KATNAL1 group	0.83 ± 0.04	1.29 ± 0.08
circ_KATNAL1 + LPS group	0.48 ± 0.09	5.12 ± 0.18

apoptosis rate of the circ_KATNAL1 + LPS group was significantly increased, and the differences were statistically significant ($P < 0.05$). See Table 3 and Figure 2.

Comparison of cell migration rate in each group

The migration rate of the blank control group was (89.77 ± 3.04) %, the cell migration rate of the model group was (43.12 ± 2.75) %, the cell migration rate of the circ_KATNAL1 group was (73.51 ± 3.48) %, and the cell migration rate of the circ_KATNAL1 + LPS group was (27.36 ± 5.19) %. Compared with the blank control group, the cell migration rate in the model group was significantly decreased; compared with the model group, the cell migration rate in the circ_KATNAL1 group was significantly increased, and the cell migration rate in the circ_KATNAL1 + LPS group was significantly decreased, and the differences were statistically significant ($P < 0.05$). See Table 4 and Figure 3.

mRNA expression levels of miR-153-3p and TLR4 in each group of cells

The mRNA expression level of miR-153-3p in the cells of the blank control group was 1.02 ± 0.13, and the mRNA expression level of TLR4 was 0.99 ± 0.04; the mRNA expression level of miR-153-3p in the cells of the model group was 0.61 ± 0.07, and the mRNA expression level of TLR4 was 4.23 ± 0.15; the mRNA expression level of miR-153-3p in the cells of circ_KATNAL1 group was 0.83 ± 0.04, and the mRNA expression level of TLR4 was 1.29 ± 0.08; the mRNA expression level of miR-153-3p in the cells of circ_KATNAL1 + LPS group was 0.48 ± 0.09, and the mRNA expression level of TLR4 was 5.12 ± 0.18. Compared with the blank control group, the mRNA expression level of miR-153-3p in the cells of the model group was significantly decreased, and the mRNA expression level of TLR4 was significantly increased; compared with the model group, the mRNA expression level of miR-153-3p in the cells of the circ_KATNAL1 group was significantly increased, the mRNA expression level of TLR4 was significantly decreased, and the mRNA expression level of miR-153-3p in the cells of the circ_KATNAL1 + LPS group was significantly decreased, the mRNA expression level of TLR4 was significantly increased and the differences were all statistically significant ($P < 0.05$). See Table 5 and Figure 4.

Protein expression levels of BCL-2 and TLR4 in each group of cells

The protein expression level of BCL-2 in the cells of the blank control group was 0.73 ± 0.03, and the protein expression level of TLR4 was 0.15 ± 0.02; the protein expression level of BCL-2 in the cells of the model group was 0.42 ± 0.07, and the protein expression level of TLR4

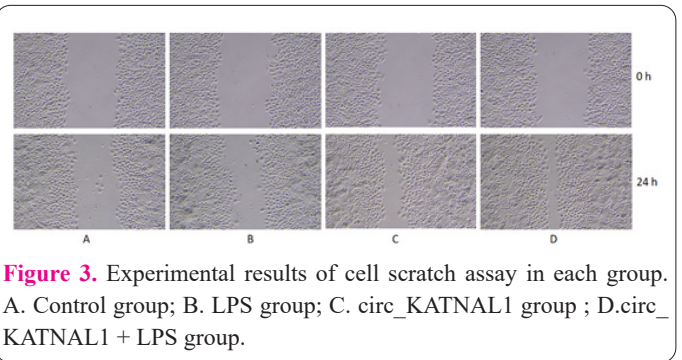


Figure 3. Experimental results of cell scratch assay in each group. A. Control group; B. LPS group; C. circ_KATNAL1 group; D. circ_KATNAL1 + LPS group.

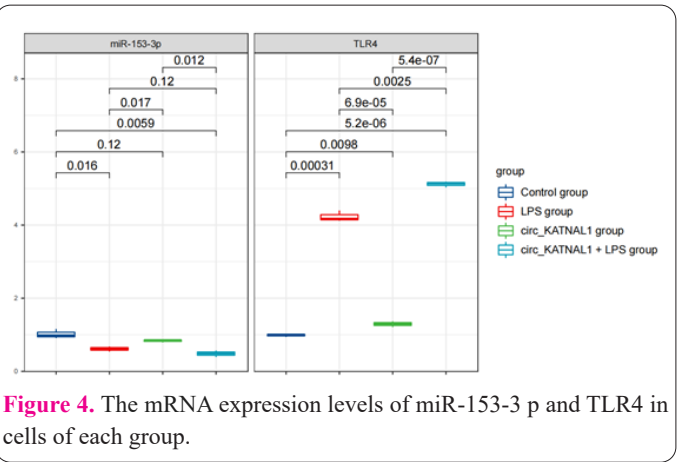


Figure 4. The mRNA expression levels of miR-153-3 p and TLR4 in cells of each group.

was 0.30 ± 0.04; the protein expression level of BCL-2 in the cells of circ_KATNAL1 group was 0.54 ± 0.04, and the protein expression level of TLR4 was 0.2 ± 0.03; the BCL-2 expression level in the cells of the circ_KATNAL1 + LPS group was 0.35 ± 0.06, and the protein expression level of TLR4 was 0.47 ± 0.05. Compared with the blank control group, the protein expression level of BCL-2 in the cells of the model group was significantly decreased, and the protein expression level of TLR4 was significantly increased; compared with the model group, the protein expression level of BCL-2 in the cells of the circ_KATNAL1 group was significantly increased, the protein ex-

Table 6. The protein expression levels of BCL-2 and TLR4 in cells ($\bar{x} \pm s$, n = 3).

Group	BCL-2 /GAPDH	TLR4 /GAPDH
Control group	0.73 ± 0.03	0.15 ± 0.02
LPS group	0.42 ± 0.07	0.30 ± 0.04
circ_KATNAL1 group	0.54 ± 0.04	0.22 ± 0.03
circ_KATNAL1 + LPS group	0.35 ± 0.06	0.47 ± 0.05

Table 7. The levels of inflammatory factors IL-6 and TNF- α in the cell supernatant ($\bar{x} \pm s$, n = 3).

Group	IL-6	TNF- α
Control group	63.78 ± 9.36	29.87 ± 10.29
LPS group	207.82 ± 18.19	96.84 ± 11.04
circ_KATNAL1 group	105.39 ± 18.72	58.27 ± 13.66
circ_KATNAL1 + LPS group	268.47 ± 15.32	132.25 ± 19.31

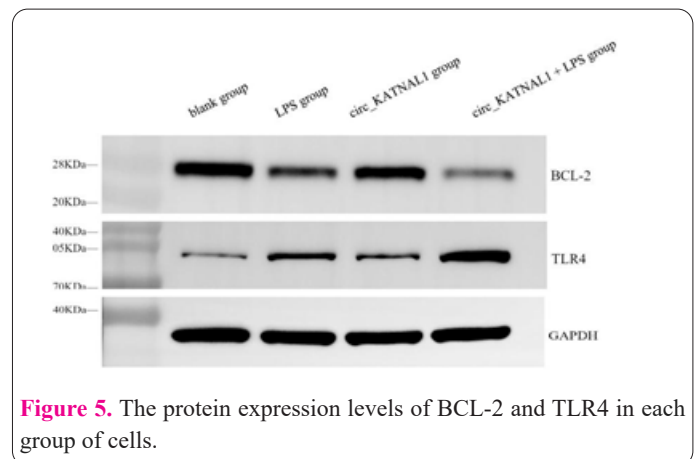
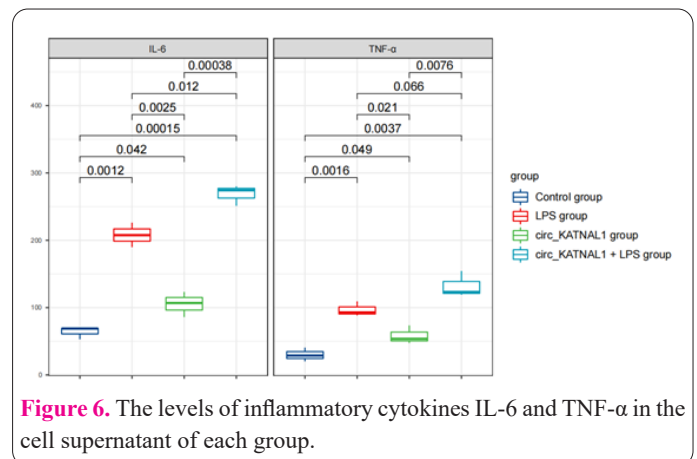
pression level of TLR4 were significantly decreased, and the protein expression level of BCL-2 in circ_KATNAL1 + LPS group cells was significantly decreased, the protein expression level of TLR4 was significantly increased, and the differences were all statistically significant ($P < 0.05$). See Table 6 and Figure 5.

Levels of inflammatory cytokines IL-6 and TNF- α in cell supernatant of each group

The expression level of the inflammatory factor IL-6 in the cell supernatant of the blank control group was 63.78 ± 9.36 , and the expression level of TNF- α was 29.87 ± 10.29 ; the expression level of inflammatory factor IL-6 in the model group cell supernatant was 207.82 ± 18.19 , and the expression level of TNF- α was 96.84 ± 11.04 ; the expression level of the inflammatory factor IL-6 in the cell supernatant in the circ_KATNAL1 group was 105.39 ± 18.72 , and the expression level of TNF- α was 58.27 ± 13.66 ; the expression level of the inflammatory factor IL-6 in the cell supernatant of the circ_KATNAL1 + LPS group was 268.47 ± 15.32 , and the expression level of TNF- α was 132.25 ± 19.31 . Compared with the blank control group, the expression levels of inflammatory factors IL-6 and TNF- α in the cell supernatant of the model group were significantly increased; compared with the model group, the expression levels of the inflammatory factors IL-6 and TNF- α in the cell supernatant of the circ_KATNAL1 group were significantly decreased in the circ_KATNAL1 + LPS group, and the expression levels of inflammatory factors IL-6 and TNF- α in the cell supernatant of the circ_KATNAL1 + LPS group were significantly increased, and the differences were all statistically significant ($P < 0.05$). See Table 7 and Figure 6.

Discussion

Otitis media (OM) is one of the most common diseases in children worldwide (10-13) and one of the most common reasons for outpatient visits, antibiotic prescriptions, and surgery in the United States (14). Clinically, it can be divided into acute otitis media, recurrent acute otitis media, exudative otitis media, chronic suppurative otitis media and chronic supratympanic otitis media (15). Due to the complex pathogenesis of the disease, the results of drug treatment including antibiotics and steroids are inconsistent (16), so it is necessary to further explore its pathogenesis and treatment methods.

**Figure 5.** The protein expression levels of BCL-2 and TLR4 in each group of cells.**Figure 6.** The levels of inflammatory cytokines IL-6 and TNF- α in the cell supernatant of each group.

Studies have shown that lipopolysaccharide (LPS) is closely related to the occurrence and development of otitis media (17). LPS is a substance composed of lipids and polysaccharides and is an important component of the outer wall of Gram-negative bacteria (18-19). LPS can stimulate mononuclear macrophages to release tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), nitric oxide (NO) and other inflammatory factors, triggering inflammatory response (20-21). In this study, the levels of inflammatory factors IL-6 and TNF- α in the cell supernatant of each group were detected, and the results showed that compared with the blank control group, the expression level of inflammatory factors IL-6 and TNF- α in the cell supernatant of the model group were significantly increased ($P < 0.05$); compared with the model group, the expression levels of inflammatory factors IL-6 and TNF- α in the cell supernatant

of the circ_KATNAL1 group were significantly reduced, while the expression levels of inflammatory factors IL-6 and TNF- α in the cell supernatant of the circ_KATNAL1 + LPS group were significantly increased ($P < 0.05$). In addition, the cell viability, apoptosis rate and migration ability of each group were detected, and the results showed that compared with the blank control group, the cell viability and the apoptosis rate of the model group were decreased, while the cell migration ability was weakened ($P < 0.05$); compared with the model group, the cell viability was increased, the apoptosis rate was decreased, and the cell migration ability was increased in the circ_KATNAL1 group, and the cell viability was decreased, the apoptosis rate was increased, and the cell migration ability was decreased in the circ_KATNAL1 + LPS group ($P < 0.05$).

MicroRNA (miRNA) is a non-protein-coding endogenous small RNA molecule that plays an important role in many biological processes (22). MiR-153-3p has been found to play an active role in the treatment of various diseases (23-27). Toll-like receptor 4 (TLR4) is a receptor that mediates LPS to induce innate immunity. The stimulation of TLR4 by LPS can induce the release of key pro-inflammatory cytokines necessary to activate an effective immune response (28), and the expression level of TLR4 detected in inflammatory cells induced by LPS is usually up-regulated (29-30), but the regulatory role of TLR4 in otitis media induced by LPS has not been well studied. In this study, the mRNA expression levels of miR-153-3p and TLR4 and the protein expression levels of BCL-2 and TLR4 in the cells of each group were detected respectively. The results showed that compared with the blank control group, the mRNA expression level of miR-153-3p and protein expression level of BCL-2 in the cells of the model group were significantly decreased, and the mRNA and protein expression levels of TLR4 were significantly increased ($P < 0.05$); compared with the model group, the mRNA expression level of miR-153-3p and protein expression levels of BCL-2 in the cells of circ_KATNAL1 group were significantly increased, and the mRNA and protein expression levels of TLR4 were significantly decreased ($P < 0.05$).

In summary, LPS can induce an inflammatory response and promote apoptosis in HMEECs cells, and the over-expression of circ_KATNAL1 can promote the above effects of LPS, which may be related to the down-regulation of miR-153-3p expression and up-regulation of TLR4 expression.

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