Article

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Role of the TLR-4/NF-κB Signaling in Cochlear Inflammation and Its Therapeutic Potential for Age-Related Hearing Loss

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Background: Age-related hearing loss (ARHL) is a common sensory disorder predominantly affecting older individuals, primarily caused by progressive degeneration of cochlear. While the toll-like receptor 4 (TLR-4)/nuclear factor kappa-B (NF-κB) signaling pathway plays a crucial role in inflammation, its precise role in cochlear inflammation and ARHL remains to be elucidated. Therefore, this study explored the role of TLR-4/NF-κB signaling axis in cochlear inflammation and its impact on ARHL pathogenesis.

Methods: HEI-OC1 cells were treated with lipopolysaccharide (LPS) to establish an inflammatory model, with TLR-4 and NF-κB inhibitors used as interventions. Similarly, male C57BL/6 mice (8 weeks and 24 months old) were treated with LPS to induce an ARHL model. Cellular viability and apoptosis rate were assessed using the cell counting kit-8 (CCK-8) assay. Furthermore, hearing function was determined using distortion product otoacoustic emission (DPOAE) and auditory brainstem response (ABR). Finally, the expression levels of TLR-4, NF-κB, and pro-inflammatory cytokines were assessed using quantitative real-time polymerase chain reaction (qRT-PCR), western blot, and enzyme-linked immunosorbent assay (ELISA).

Results: *In vitro* experiments revealed that TLR-4 and NF-κB inhibitors significantly restored cell viability and reduced LPS-induced apoptosis ($p < 0.05$). LPS treatment activated TLR-4/NF-κB signaling pathway and increased interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6) expression levels, while these effects were suppressed through TLR-4 inhibition ($p < 0.05$). *In vivo* experiments showed that LPS-treated mice exhibited significant hearing impairment, which was alleviated through TLR-4 inhibitor ($p < 0.05$). Histopathological analysis revealed that LPS induced cochlear inflammation and cell damage, whereas TLR-4 inhibition mitigated these pathological changes. Furthermore, suppression of the TLR-4/NF-κB pathway significantly reduced the expression of pro-inflammatory cytokines in cochlear tissues, thereby alleviating inflammatory responses ($p < 0.05$).

Conclusion: The TLR-4/NF-κB pathway plays a crucial role in LPS-induced cochlear inflammation and ARHL progression. Inhibition of this pathway effectively alleviates age-related hearing loss by attenuating cochlear inflammation and protecting cochlear cells from apoptosis.

Keywords: TLR-4; NF-κB; age-related hearing loss; cochlear cells; inflammatory response

Introduction

Presbycusis, also known as age-related hearing loss (ARHL), is a progressive degenerative disorder that impairs auditory function. It is the third most prevalent condition in the elderly population [1]. With the increasing global population aging, ARHL has become a significant public health concern. Epidemiological evidence indicates that about 20% of people over 65 years of age are affected by ARHL, with the prevalence rising to 65% in those over 85 [2]. Beyond diminishing quality of life, this disease leads to social isolation, cognitive decline, and mental health problems [3,4]. Therefore, elucidating ARHL pathophysiology and developing effective treatment approaches are crucial.

Recent studies have underscored the crucial role of cochlear cell inflammatory responses in the onset and progression of ARHL [5]. The cochlea, a key component of the auditory system, is responsible for translating sound waves into neural signals [6,7]. Excessive inflammatory reactions can cause apoptosis and damage to cochlear cells, thereby increasing hearing loss [8]. In response to various pathogenic stimuli, cochlear cells release cytokines, including interleukin-1 beta (IL-1β), tumor necrosis factor alpha (TNF-α), and interleukin-6 (IL-6), and their overexpression is strongly linked to the development of ARHL [9].

Toll-like receptor 4 (TLR-4) serves as a primary sensor of pathogen-associated signals [9], activating nuclear factor kappa-B (NF-κB) and other inflammatory pathways

that are crucial for immune regulation and disease progression [10–12]. Previous studies have reported that TLR-4 activation promotes NF-κB nuclear translocation and increases the synthesis of pro-inflammatory proteins, processes that are crucial for cochlear inflammation triggered by noise exposure [13]. However, the precise role of this signaling pathway in ARHL remains poorly understood.

This study aimed to investigate the role of the TLR-4/NF-κB axis in the development of ARHL and its effects on inflammatory responses within cochlear cells. Using *in vivo* C57BL/6 mice models and *in vitro* investigations with HEI-OC1 cells, we aimed to elucidate the molecular mechanisms linking TLR-4/NF-κB activation to ARHL pathogenesis. The findings of this study may offer novel therapeutic insights and identify potential targets to improve auditory function and enhance overall well-being in the elderly population.

Materials and Methods

Experimental Animal and Treatment

Male C57BL/6 mice, aged 6 months, were obtained from Chinese Vital River Laboratory Animal Technology Co., Ltd. The mice were housed under standard conditions with a 12-hour light/dark cycle, a temperature of 22 $\pm$ 2 °C, a humidity of 50–60%, and free access to food and water. All animal procedures were conducted in accordance with IACUC-approved guidelines (Jiangsu Hanjiang Biotechnology Co., Ltd.; Approval No. HJSW24070801, 8 July 2024). Euthanasia was performed through CO_2 inhalation following AVMA recommendations.

C57BL/6 mice were randomly assigned to four groups (n = 10/group): (1) young control (Y-Control) : 8-week-old mice receiving intraperitoneal (i.p.) injection of saline; (2) aged control (A-Control): 24-month-old mice receiving i.p. saline; (3) A- lipopolysaccharide (LPS): 24-month-old mice treated with LPS (i.p., 5 mg/kg) daily for 7 days; and (4) A-LPS-TLR4i: 24-month-old mice co-treated with LPS (5 mg/kg) and TAK-242 (3 mg/kg) through daily i.p. injections for 7 days.

On Day 7, auditory tests were performed, after which mice were euthanized by CO_2 inhalation following AVMA guidelines. Cochleae were collected, with one ear used for histological examination, and the other for RNA and protein analyses.

Cell Culture and Treatment

HEI-OC1 cochlear cells were obtained from Ori-Cell, China (M8-0401). Under light microscopy, the cells showed characteristic epithelial-like morphology, including polygonal or fusiform shape, dense monolayer growth, and tightly organized intercellular arrangement, consistent with typical descriptions of cochlear cell lines. Mycoplasma testing confirmed that the cells were free of contamination.

HEI-OC1 cells were cultured in a DMEM/F-12 medium (11320033, Gibco, USA) containing 10% fetal bovine serum (FBS) (A5670701, Gibco, USA) and 1% (v/v) penicillin-streptomycin (100×) (15140122, Gibco, USA). Cells were maintained at 37 °C and 5% CO_2 until reaching 80% confluence. The cell cultures were then divided into four experimental groups and treated for 24 hours as follows: (1) Control group: no treatment; (2) LPS group: treated with 1 µg/mL LPS (L2630, Sigma, USA) [13]; (3) LPS + TLR-4 inhibitor group: treated with 1 µg/mL LPS alongside 10 µM TAK-242 (614316, Sigma, USA) [14]; (4) LPS + NF-κB inhibitor group: treated with 1 µg/mL LPS and 10 µM BAY 11-7082 (B5556, Sigma, USA) [15].

Auditory Testing in Mice

Auditory function was evaluated using auditory brainstem response (ABR) and distortion product otoacoustic emission (DPOAE) approaches.

ABR testing was performed on Day 0 (before treatment) and Day 7 (post-treatment) using the TDT System 3 device (System 3, Tucker-Davis Technologies, USA). Mice were anesthetized with sodium pentobarbital (50 mg/kg) and placed in a sound-attenuated chamber. Electrodes were inserted into the mice's ears, and auditory stimuli consisting of click sounds (10–90 dB SPL) were delivered to examine ABR thresholds. The stimulus intensity gradually decreased from high to low until no discernible waveform could be detected. The minimum sound pressure level (dB SPL) that elicited a reliable response was recorded as the hearing threshold for each group. Threshold shifts between Day 0 and Day 7 were compared across groups to determine the impact of the interventions on auditory performance. Auditory responses were recorded from both ears, and the average threshold of the left and right ears was used for analysis.

At the same time points, as the ABR assessments (Day 0 and Day 7), DPOAE testing was performed in all mouse groups. Using an otoacoustic emission measurement system in a quiet environment, ear emissions were recorded through a miniature probe inserted into the ear canal, which delivered two pure-tone stimuli of different frequencies (f1 and f2). DPOAE responses were measured across the 2–16 kHz frequency range, focusing on the 2f1–f2 distortion product generated by the f1 and f2 tones. Changes in DPOAE signal intensity reflect alterations in outer hair cell function, providing an assessment of the extent and severity of cochlear outer hair cell dysfunction or damage in each group. DPOAE measurements were performed bilaterally, and the mean values of both ears were used to evaluate cochlear function.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from HEI-OC1 cells and cochlear tissues using TRIzol (15596018CN, Thermo

Table 1. Primer sequences used in qPCR.

Gene	Primer direction	Sequence (5′ → 3′)
IL-1β	F	TGGACCTTCCAGGATGAGGACA
	R	GTTCATCTCGGAGCCTGTAGTG
TNF-α	F	GGTGCCTATGTCTCAGCCTCTT
	R	GCCATAGAACTGATGAGAGGGAG
IL-6	F	TACCACTTCACAAGTCGGAGGC
	R	CAAAGTGCATCATCGTTGTTCATACA
TLR-4	F	AGCTTCTCCAATTTTTCAGAACTTC
	R	TGAGAGGTGGTGTAAGCCATGC
NF-κB p65	F	GCTGCCAAAGAAGGACACGACA
	R	GGCAGGCTATTGCTCATCACAG
β-actin	F	GGCTGTATTCCCCTCCATCG
	R	CCAGTTGGTAACAATGCCATGT

F, Forward; R, Reverse; *IL-1β*, interleukin-1 beta; *TNF-α*, tumor necrosis factor alpha; *IL-6*, interleukin-6; *TLR-4*, toll-like receptor 4; *NF-κB p65*, nuclear factor kappa-B p65.

Fisher Scientific, USA). RNA quality was assessed using a NanoDrop One spectrophotometer (ND-ONE-W, Thermo Fisher Scientific, USA). Complementary DNA (cDNA) was synthesized using the PrimeScript™ RT Kit (RR037A, Takara, Japan), followed by qPCR on a qTOWER^3 G real-time PCR system (Analytik Jena, Germany) using TB Green (RR430A, Takara, Japan) and gene-specific primers (Table 1). *β-actin* was used as the internal control, and relative expression was determined using the $2^{-\Delta\Delta Ct}$ method.

Enzyme-Linked Immunosorbent Assay (ELISA)

Protein levels were evaluated in cochlear lysates, tissue homogenates, and cell culture supernatants. Total protein was isolated using RIPA buffer (P0013B, Beyotime, China), followed by centrifugation. For cell culture experiments, conditioned media were collected separately. Cytokine concentrations were determined using ELISA at 450 nm on a microplate reader (Multiskan FC, Thermo Fisher Scientific, USA) with mouse ELISA kits for IL-1β (88-7013-88, Thermo Fisher eBioscience, USA), TNF-α (88-7324-88, Thermo Fisher eBioscience, USA), and IL-6 (88-7064-88, Thermo Fisher eBioscience, USA). Standard curves were generated for each assay to determine cytokine levels, and findings were reported in pg/mL.

Cell Proliferation Assay

Proliferation capability of HEI-OC1 cells was assessed using the CCK-8 (CK04, Dojindo, Japan). The cells were seeded in 96-well plates at a density of 5×10^3 cells per well, incubated for six hours, and then treated with either BAY 11-7082 (NF-κB inhibitor), TAK-242 (TLR-4 inhibitor), or LPS. Following a 24-hour induction, 10 µL of CCK-8 reagent was added to each well, followed by an additional 2-hour incubation. Finally, absorbance was measured at 450 nm using a microplate reader (Multiskan FC, Thermo Fisher Scientific, USA), and cell viability was calculated using the following formula: Cell viability (%) = $(OD_{treatment} - OD_{blank}) / (OD_{control} - OD_{blank}) \times 100\%$.

Cell Apoptosis Assay

Cellular apoptosis rate was assessed using Annexin V-FITC/PI staining (APOAF-20TST, Sigma, USA). HEI-OC1 cells underwent 24-hour treatment with control, LPS, LPS+TAK-242, or LPS+BAY 11-7082, then harvested and resuspended in binding buffer containing Annexin V-FITC and PI. After a 15-minute incubation in the dark, samples were analyzed by flow cytometry using a FACSAria™ II system (BD Biosciences, USA).

Western Blot Analysis

Total proteins were isolated from HEI-OC1 cells and cochlear tissues using RIPA buffer (P0013B, Beyotime, China) and quantified with a BCA (P0010S, Beyotime, China). Equal amounts of proteins were resolved by SDS-PAGE and then transferred onto PVDF membranes (IPVH00010, Millipore, USA). After blocking, the membranes were incubated overnight with primary antibodies, including anti-TLR-4 (14358, CST, USA); anti-NF-κB p65 (8242, CST, USA); anti-p-NF-κB p65 (3033, CST, USA); anti-β-actin (3700, CST, USA). The next day, membranes were incubated with HRP-conjugated secondary antibodies (A0208/A0216, Beyotime, China) for two hours. Finally, protein bands were visualized using the ECL system and analyzed with ImageJ software (version 1.54f; National Institutes of Health, Bethesda, MD, USA), with β-actin serving as the internal control.

Hematoxylin and Eosin (HE) Staining for Pathological Analysis

Cochlear tissues were fixed in 4% paraformaldehyde (P0099, Beyotime, China) for 24 hours, dehydrated through a gradient of ethanol, cleared in xylene, and em-

bedded in paraffin. Paraffin-embedded tissues were sectioned into 5 µm thick slices, dewaxed, rehydrated, and stained with H&E staining kit (C0105, Beyotime, China)—haematoxylin for 5 minutes and eosin for 2 minutes. After dehydration and mounting, tissue sections were observed under a light microscope (BX53, Olympus, Japan) to examine hair cell morphology and inflammatory infiltration.

Histopathological damage was scored on a 0–3 scale and reported as follows:

0: Normal morphology;

1: Mild disorganization/limited inflammation;

2: Moderate disarray/visible infiltration;

3: Severe structural disruption/widespread inflammation.

Three randomly selected sections per mouse were independently evaluated by two blinded pathologists, and the mean scores were used for further analysis.

Statistical Analysis

Graphical representations were performed using GraphPad Prism 8.0 (GraphPad Software, San Diego, California USA). Western blot were analyzed by grayscale densitometry using ImageJ. Each experiment was independently repeated three times. Between-group comparisons were performed using two-tailed unpaired Student's *t*-tests in IBM SPSS Statistics 29.0 (IBM Corp., Armonk, NY, USA). Data were presented as mean ± standard deviation (SD), and differences were considered statistically significant at $p < 0.05$.

Results

Inhibiting the TLR-4/NF-κB Pathway Improves Cell Viability and Reduces Apoptosis

To investigate the functional role of the TLR-4/NF-κB pathway in cochlear inflammation, an *in vitro* model was established using HEI-OC1 cells treated with LPS, a widely recognized TLR-4 agonist that elicits strong inflammatory responses. The results showed that cell viability was significantly higher in the control group, whereas LPS treatment substantially reduced cell survival, resulting in a decrease in cell viability ($p < 0.05$). Following exposure to a TLR-4 inhibitor, cell viability was dramatically restored ($p < 0.05$), and an NF-κB inhibitor similarly enhanced cell viability (p

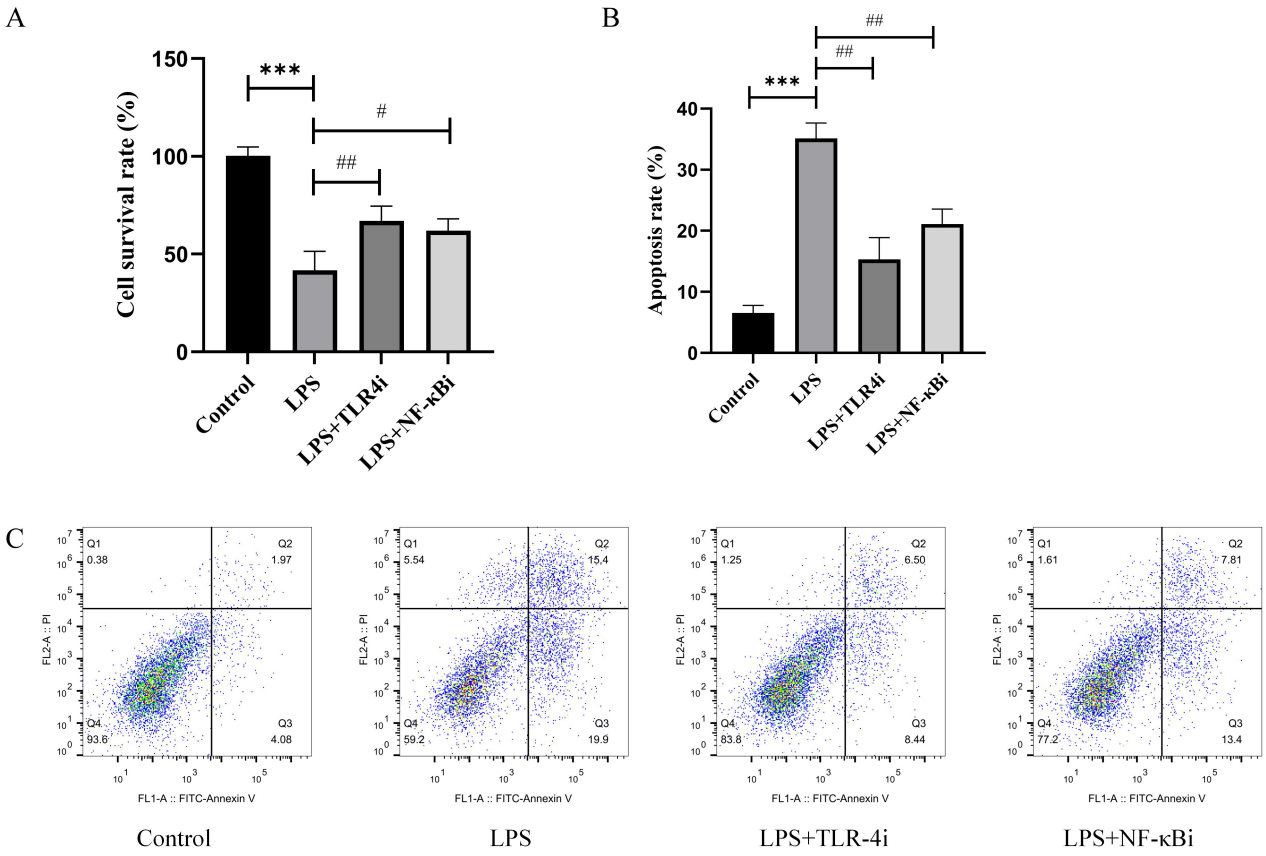

Fig. 1. Impact of TLR-4/NF-κB signaling pathway on cell viability and apoptosis rate. (A) Cell viability assessed after 24-hour treatment. (B) Flow cytometry analysis of apoptosis rate in each group. (C) Representative flow cytometry plots of apoptosis in HEI-OC1 cells under different treatments. Bars: mean ± SD (n = 3); ***$p < 0.01$ vs the Control group; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs the LPS group. LPS, lipopolysaccharide; TLR4i, toll-like receptor 4 inhibitor; NF-κBi, nuclear factor kappa-B inhibitor.

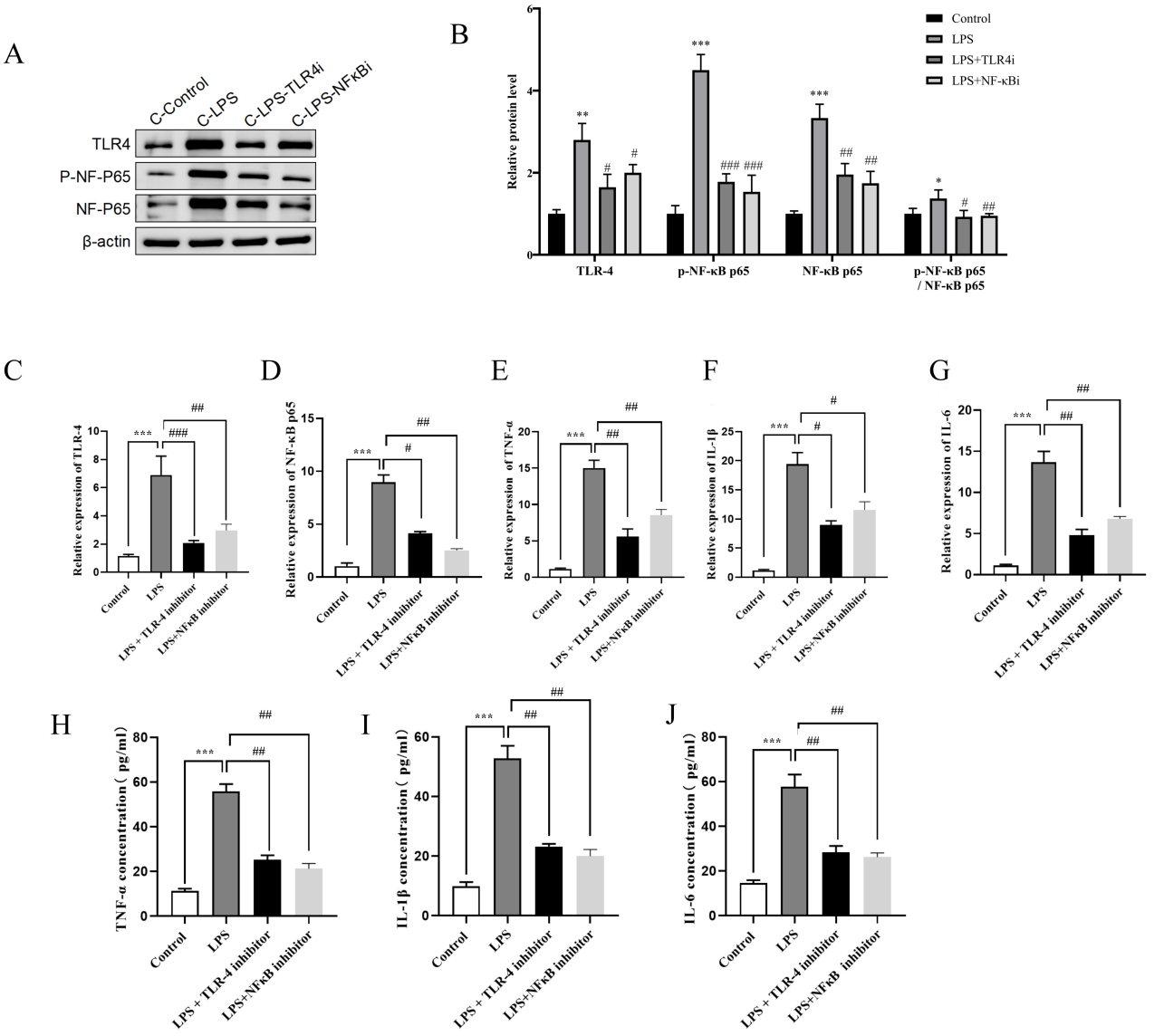

Fig. 2. The expression of TLR-4/NF-κB signaling pathway and inflammatory factors in cochlear cells. (A) Representative Western blot of TLR-4, p-NF-κB p65, NF-κB p65, and β-actin. (B) Densitometric quantification of the p-NF-κB p65/NF-κB p65 ratio (β-actin–normalized; Control = 1.0). (C–G) Relative mRNA expression levels of *TLR-4* (C), *NF-κB p65* (D), *TNF-α* (E), *IL-1β* (F), and *IL-6* (G) assessed using qRT-PCR. (H–J) Concentrations of TNF-α (H), IL-1β (I), and IL-6 (J) in cell supernatants determined employing ELISA. Bars: mean $\pm$ SD (n = 3). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs the Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs the LPS group. TLR-4, toll-like receptor 4; p-NF-κB p65, phospho-nuclear factor kappa-B p65; β-actin , beta-actin; *TNF-α,* tumor necrosis factor alpha; *IL-1β,* interleukin-1 beta; *IL-6,* interleukin-6; qRT-PCR, quantitative real-time polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

< 0.05), though to a slightly smaller degree than the TLR-4 inhibitor (Fig. 1A).

Flow cytometry analysis demonstrated that LPS treatment significantly elevated apoptosis rate ($p < 0.05$), with the control group exhibiting the lowest level of apoptosis. Furthermore, treatment with TLR-4 substantially alleviated apoptosis ($p < 0.05$), whereas NF-κB inhibitor treatment also reduced apoptosis ($p < 0.05$), albeit to a slightly lesser extent than the TLR-4 inhibitor (Fig. 1B,C). Both inhibitors effectively attenuated the LPS-induced decrease in cell viability and increase in apoptosis, suggesting that the TLR4/NF-κB signaling pathway plays a key role in LPS-mediated cochlear cell injury, and that its inhibition can alleviate this injury.

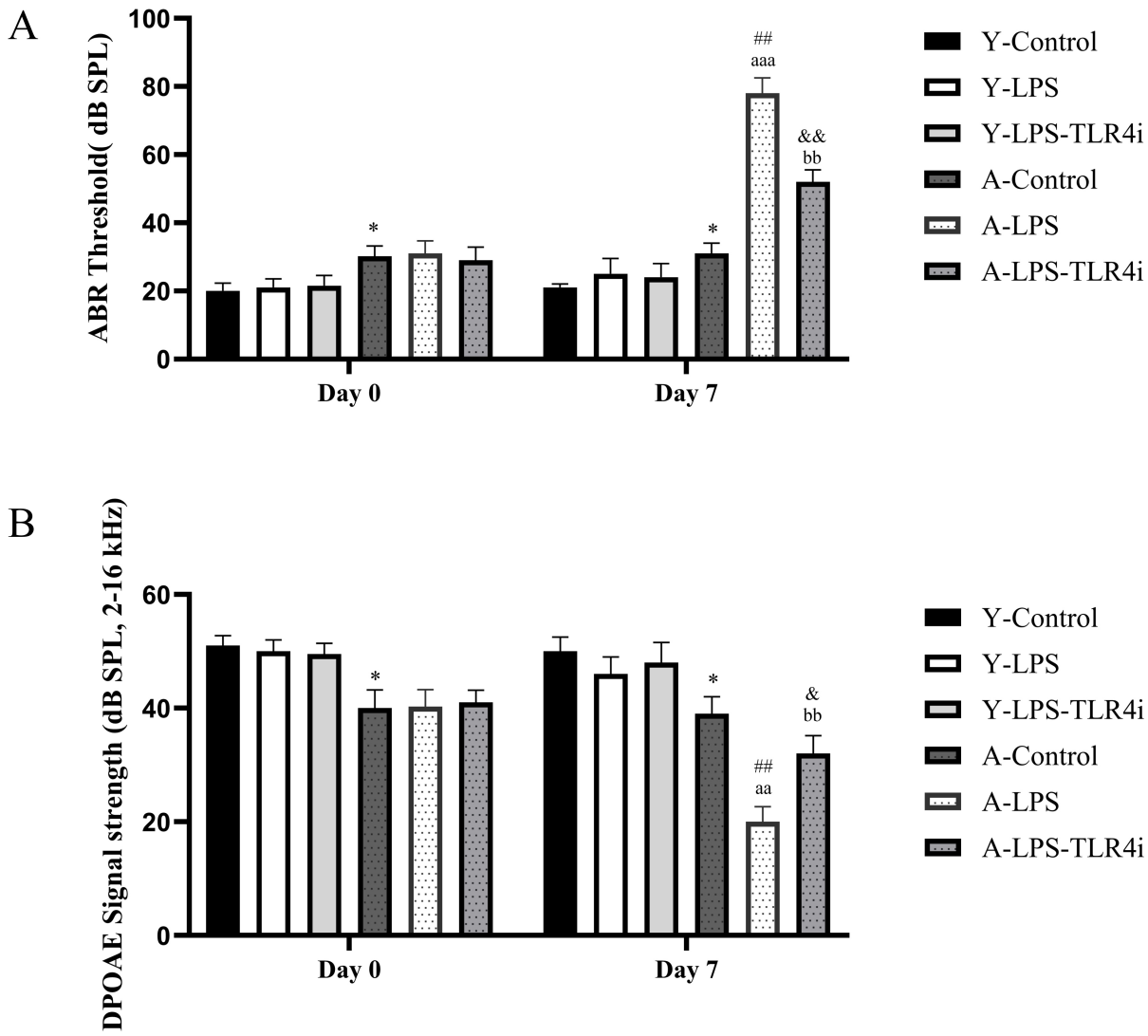

Fig. 3. Inhibition of TLR-4/NF-κB signaling pathway improves hearing function in LPS-treated aged mice. (A) ABR thresholds on Day 0 and Day 7 in the young control group (Y-Control), Y-LPS, Y-LPS+TLR4i, aged control (A-Control), A-LPS, and A-LPS + TLR-4 inhibitor groups. (B) DPOAE amplitudes (2–16 kHz) on Day 0 and Day 7. Bars: mean $\pm$ SD (n = 10 mice/group). *$p < 0.05$ vs the Y-Control group; $^{\#\#}p < 0.01$ vs the A-Control group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$ vs A-LPS; ^{aa}, $p < 0.01$, ^{aaa}, $p < 0.001$ vs the Y-LPS group; ^{bb}, $p < 0.01$ vs the Y-LPS+TLR4i group. Y-Control, young control group; A-Control, aged control.

Expression of TLR-4/NF-κB Pathway and Inflammatory Cytokines in Cochlear Cells

LPS stimulation significantly activated the TLR-4/NF-κB pathway in HEI-OC1 cells and increased the expression of inflammatory factors, including IL-1β, TNF-α, and IL-6. Western blot analysis (Fig. 2A,B) showed that the protein levels of TLR-4, p-NF-κB p65, and total NF-κB p65 were significantly increased in the LPS-treated group ($p < 0.05$). Consistently, qPCR and ELISA results (Fig. 2C–J) confirmed a substantial elevation in both mRNA and protein levels of these inflammatory factors ($p < 0.05$). Furthermore, treatment with TAK-242, a TLR-4 inhibitor, significantly inhibited TLR-4 expression and reduced the associated mRNA and protein levels (Fig. 2). Moreover, ELISA measurements revealed a decrease in the secretion of IL-1β, TNF-α, and IL-6 ($p < 0.05$, Fig. 2H–J). Similarly, BAY 11-7082, an NF-κB inhibitor, effectively suppressed pathway activation and reduced the expression of inflammatory cytokines.

Inhibition of the TLR-4/NF-κB Signaling Pathway Improves Hearing Loss in Mice

At baseline (Day 0), aged mice showed significantly elevated ABR thresholds and reduced DPOAE amplitudes

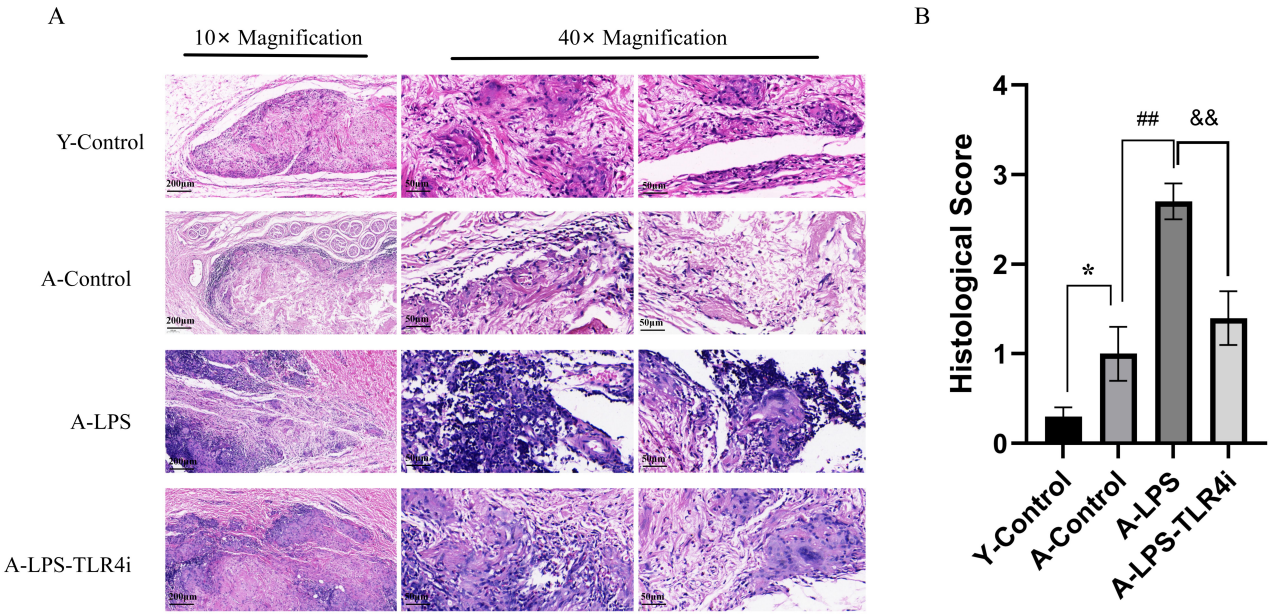

Fig. 4. Inhibition of the TLR-4/NF-κB signaling pathway reduces inflammation and cell damage in mouse cochlear tissue (HE staining). (A) Representative HE staining images of cochlear sections from the Y-Control, A-Control, A-LPS, and A-LPS-TLR4i groups. Images were captured at 10× and 40× magnification. (B) Semi-quantitative histological damage scores (0–3 scale), based on criteria including inflammatory cell infiltration, cellular disarray, and tissue integrity. Bars: mean ± SD (n = 10 mice/group). *$p < 0.05$ vs the Y-Control group; $^{\#\#}p < 0.01$ vs the A-Control group; $^{\&\&}p < 0.01$ vs the A-LPS group.

compared with young controls ($p < 0.05$; Fig. 3A,B), confirming age-related auditory decline before treatment. By Day 7, LPS exposure induced only mild changes in young mice ($p > 0.05$), whereas it induced a pronounced deterioration in aged mice, with ABR thresholds rising to ~80 dB SPL and DPOAE amplitudes substantially reduced compared to the A-Control group ($p < 0.05$). Importantly, the magnitude of functional loss was significantly greater in aged mice than in young ($p < 0.05$), indicating an additive, age-dependent vulnerability to LPS. Co-treatment with the TLR-4 inhibitor partially restored auditory function in both age groups, with a more significant absolute improvement observed in aged mice; however, values did not completely return to baseline.

These data demonstrate that LPS induces more severe auditory dysfunction in aged mice, underscoring an age-related aggravation of cochlear susceptibility.

Inhibition of the TLR-4/NF-κB Signaling Pathway Reduces Inflammation and Cellular Damage in Cochlear Tissue

HE staining showed significant differences in cochlear tissue morphology among the groups (Fig. 4A). In the Y-Control group, hair cells and spiral ganglion (SG) neurons were orderly arranged with intact structural integrity. The A-Control group exhibited mild to moderate degeneration. The A-LPS group showed severe cellular degeneration and pronounced inflammatory infiltration. Conversely, the A-LPS-TLR4i group showed a substantial reduction in pathological damage, with a relatively restored cochlear structure and alleviated inflammation. Histological scoring (Fig. 4B) revealed that A-Control mice had higher scores than Y-Control mice ($p < 0.05$), A-LPS mice demonstrated further increases versus A-Control mice ($p < 0.05$); and scores were significantly reduced in the A-LPS-TLR4i group compared with A-LPS ($p < 0.05$).

Collectively, these results suggest that inhibition of the TLR-4/NF-κB pathway can effectively alleviate cochlear tissue inflammation and structural damage.

Suppression of the TLR-4/NF-κB Signaling Pathway Downregulates the Expression of Inflammatory Cytokines in Cochlear Tissues of Hearing-Impaired Mice

Western blot analysis showed that TLR-4, p-NF-κB p65, and NF-κB p65 protein levels significantly increased in the A-Control group compared to the Y-Control group ($p < 0.05$), further elevated after LPS stimulation, and reduced upon TLR-4 inhibition. Moreover, the p-NF-κB p65/NF-κB p65 ratio exhibited a similar trend ($p < 0.05$, Fig. 5A,B). Consistently, qRT-PCR revealed that mRNA levels of *NF-κB p65*, *TNF-α*, *IL-6*, *IL-1β*, and *TLR-4* were upregulated in the A-Control group, further enhanced in A-LPS, and suppressed by inhibitor treatment ($p < 0.05$, Fig. 5C–G). Similarly, ELISA results were consistent with these observations, demonstrating increased protein levels of TNF-α,

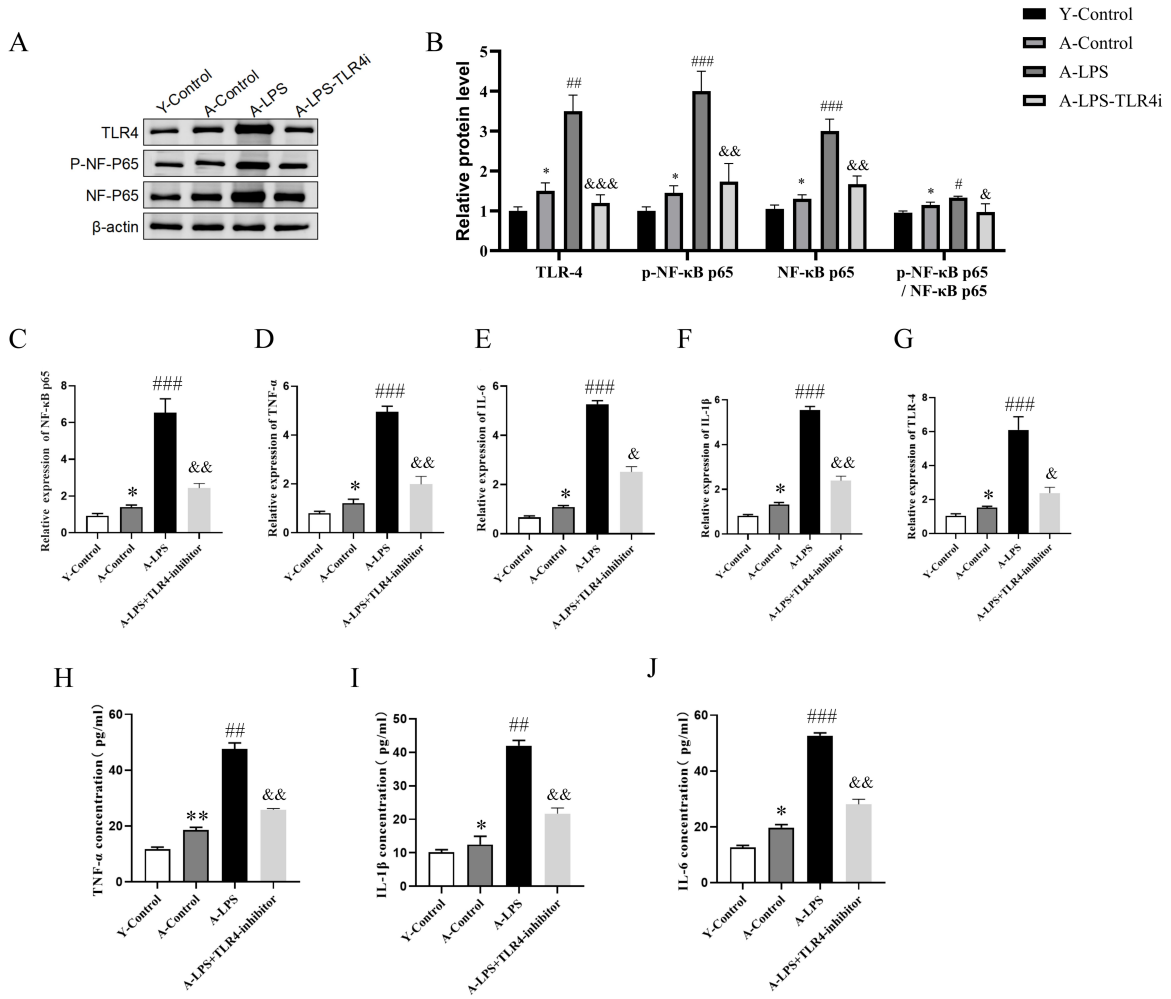

Fig. 5. Inhibition of the TLR-4/NF-κB pathway downregulates the expression of inflammatory factors in the cochlear tissue of deaf mice. (A) Western blot analysis of TLR-4, p-NF-κB p65, and total NF-κB p65 protein levels in cochlear tissues. (B) Densitometric quantification of the p-NF-κB p65/NF-κB p65 ratio (β-actin–normalized; Y-Control = 1.0). (C–G) Relative mRNA expression levels of *NF-κB p65* (C), *TNF-α* (D), *IL-6* (E), *IL-1β* (F), and *TLR-4* (G) in cochlear tissues measured using qRT-PCR. (H–J) Expression levels of TNF-α (H), IL-1β (I), and IL-6 (J) in cochlear tissues determined employing ELISA. Bars: mean $\pm$ SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$ vs. the Y-Control group; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs the A-Control group; $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, $^{\&\&\&}p < 0.001$ vs the A-LPS group.

IL-1β, and IL-6 in the A-Control and A-LPS groups, which were substantially decreased after inhibitor administration ($p < 0.05$, Fig. 5H–J).

These results suggest that aging and LPS synergistically activate the TLR-4/NF-κB pathway in cochlear tissues, whereas pharmacological inhibition of this pathway effectively mitigates the associated inflammatory response.

Discussion

We conducted *in vitro* analyses using HEI-OC1 cochlear cells and employed a C57BL/6 mouse model to elucidate the pivotal role of TLR-4/NF-κB signaling in cochlear inflammation and ARHL development. We observed that, under inflammatory stimulation such as LPS, this signaling pathway is significantly activated, resulting in upregulation of various downstream pro-inflammatory factors, thereby inducing cochlear cell damage and apoptosis. However, intervention with specific inhibitors, TAK-242 or BAY 11-7082, effectively preserved cochlear cell function and significantly reduced apoptosis, underscoring the crucial role of TLR-4/NF-κB activation in mediating hearing loss. Furthermore, the substantial downregulation of pro-inflammatory markers after pathway inhibition suggests that targeted modulation of this pathway can significantly alleviate the local inflammatory microenvironment, reduce tissue damage, and delay the progression of hearing impairment.

Mechanistically, TLR-4 is a key pattern recognition receptor (PRR) initially identified for its ability to recognize LPS and activate downstream immune responses. Previous studies have reported that TLR-4 activation triggers a signaling cascade involving adaptor molecules such as MyD88, which promotes the nuclear translocation of transcription factors like NF-κB and increases the expression of pro-inflammatory factors [13,16]. The TLR-4/NF-κB pathway has been associated with numerous conditions, including metabolic disorders, neurological diseases, and both acute and chronic inflammation. In the context of otology, abnormal activation of this pathway is closely linked to cochlear damage induced by environmental stressors, such as noise exposure. Indeed, previous research has demonstrated that noise stimulation significantly activates this pathway in the cochlea, resulting in hair cell damage, apoptosis of spiral ganglion neurons, and disruption of cochlear structures [17].

Therefore, we suggest that TLR-4/NF-κB pathway plays a key role in mediating both acute inflammatory damage and the progression of chronic inflammation in ARHL. Targeted intervention of this signaling axis may represent a promising approach for preventing and managing ARHL, with its potential to alleviate cochlear inflammation, preserve auditory function, and delay progression of hearing degradation.

Cells respond to stress or inflammatory stimuli by activating the crucial NF-κB signaling pathway. Previous studies have reported that dysregulated activation of this pathway is strongly linked to neuroinflammation, neuronal death, and neurodegeneration—key features of neurodegenerative diseases [18,19]. For instance, inhibiting the NF-κB pathway has been reported to enhance neuronal function, reduce inflammation, and impede disease progression [20,21]. Our study shows that cochlear inflammation can be significantly reduced by inhibiting the TLR-4/NF-κB pathway, thereby mitigating hearing loss. Furthermore, evidence indicates that NFκB1 subunits are upregulated in the aging cochlea, with IL-1β and interleukin-18 receptor accessory protein (IL-18RAP) increasing as early as 6 months of age, followed by the significant upregulation of multiple immune-related genes, such as caspase-1 (*CASP1*), interleukin-18 receptor 1 (*IL18R1*), interleukin-1 beta (*IL1B*), and toll-like receptor 9 (*TLR9*), between 9 and 12 months [22]. These results indicate that this pathway accelerates the age-related hearing loss by modulating the expression of pro-inflammatory factors. Furthermore, these studies demonstrated that IL-1β and IL-18R1 are localized in the spiral ligament, spiral lamina, and organ of Corti. In contrast, TNFα and PTGS2 are widely distributed across the stria vascularis and spiral ligament of the lateral cochlear wall. This suggests that the inflammatory response is not restricted to a single cochlear cell type but rather involves multiple structural regions.

These observations are consistent with our finding that LPS-induced inflammatory signaling broadly impacts cochlear cells, further highlighting the complexity of inflammation in cochlear damage and hearing degradation. Notably, pro-inflammatory molecules such as TNF-α and IL-6 exhibit neurotoxic effects, and increased levels of these cytokines are strongly associated with hearing loss and neuronal damage [23,24]. The long-term expression of these inflammatory factors can exacerbate cochlear structural damage, accelerating hearing loss. Therefore, blocking excessive activation of the TLR-4/NF-κB pathway, along with other related inflammatory pathways, may reduce cochlear inflammation and slow or prevent the progression of age-related hearing loss.

This study demonstrates the role of TLR-4/NF-κB signaling to cochlear inflammation and the progression of age-related hearing loss; however, several limitations should be acknowledged. First, as this study was conducted using a mouse model, the results may not accurately reflect the physiological characteristics of the human cochlea, warranting further validation using human samples. Second, other signaling pathways involved in cochlear inflammation, such as the JAK/STAT and MAPK pathways, were not examined in this study. Third, *in vitro* experiments were performed using only the HEI-OC1 cell line, which, although widely used as a cochlear model, may not fully represent the diversity of cochlear cell types *in vivo*. Future studies should incorporate additional cochlear-derived or primary cells to enhance the generalizability of the findings.

Additionally, histological analyses focused exclusively on aged cohorts, where structural pathology is most pronounced, and did not include Y-LPS (young LPS-induced mice) samples, indicating a limitation which should be addressed in future studies. Finally, the study was of short duration and could not determine whether blocking the TLR-4/NF-κB pathway produces persistent effects on hearing recovery. Future studies should explore therapeutic approaches targeting multiple pathways concurrently and extend observation periods to assess long-term outcomes.

Conclusion

This study reveals that inhibition of the TLR-4/NF-κB signaling pathway alleviates age-related hearing loss by decreasing cochlear inflammation and protecting cochlear cells from apoptosis. Evidence from both *in vitro* cellular and *in vivo* mouse model experiments suggests that inhibition of this pathway not only preserves cell viability and reduces apoptosis induced by inflammatory stimuli such as LPS but also decreases the secretion of key pro-inflammatory cytokines. Our findings indicate that targeted modulation of this pathway represents a promising therapeutic approach for managing cochlear inflammation, inhibiting cellular damage, and ultimately reducing the progression of age-related hearing loss.

Availability of Data and Materials

All data generated or analyzed during this study are available within the article. Further inquiries can be directed to the corresponding author.

Author Contributions

YBL is responsible for the guarantor of integrity of the entire study, study design, literature research, clinical studies, data analysis, statistical analysis, manuscript preparation & editing, and drafted the first version of the manuscript; HZ is responsible for the literature research, experimental studies, statistical analysis; HMZ is responsible for the literature research, data acquisition; YJH is responsible for the study concepts & design, definition of intellectual content, data analysis, manuscript review. All authors contributed to critical revision of the manuscript for important intellectual content. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All experimental procedures were conducted in strict accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the Jiangsu Hanjiang Biotechnology Co., Ltd. (Approval No. HJSW24070801, 8 July 2024).

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Conflict of Interest

The authors declare no conflict of interest.

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