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# Transcutaneous auricular vagus nerve stimulation promotes recovery from otitis media by activating the $\alpha 7$ nAChR-mediated anti-inflammatory pathway

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## Abstract

**Background** Otitis media (OM) is an inflammatory disease of the middle ear characterized by mucosal remodeling, effusion, and conductive hearing loss. Although antibiotics and surgical procedures remain standard treatments, their efficacy is often limited by recurrence, antibiotic resistance, and chronic progression. Transcutaneous auricular vagus nerve stimulation (taVNS) is a non-invasive neuromodulatory technique that activates the cholinergic anti-inflammatory pathway through  $\alpha 7$  nicotinic acetylcholine receptor ( $\alpha 7$ nAChR) signaling. This study aimed to evaluate the anti-inflammatory effects of taVNS in a lipopolysaccharide (LPS)-induced mouse model of acute otitis media (AOM) and to assess whether these effects may involve  $\alpha 7$ nAChR-dependent mechanisms.

**Methods** AOM was induced by transtympanic injection of LPS in BALB/c mice, followed by taVNS applied to the auricular concha using biphasic square pulses (0.3 mA, 20 Hz, 200  $\mu$ s). Methyllycaconitine, a selective  $\alpha 7$ nAChR antagonist, was administered to assess receptor involvement. Auditory function, tympanic membrane morphology, and mucosal changes were assessed through auditory brainstem response testing, otoscopic imaging, and histological analysis. Cytokine levels in middle ear effusion and serum were quantified by ELISA, while inflammatory gene and protein expression were analyzed using qPCR and Western blotting. Statistical analyses were performed using one-way ANOVA or Kruskal–Wallis tests with post hoc comparisons.

**Results** taVNS significantly improved hearing thresholds and reduced mucosal thickening and goblet cell hyperplasia in AOM mice. It markedly decreased tumor necrosis factor- $\alpha$ , interleukin (IL)-1 $\beta$ , and IL-6 levels in middle ear effusion, and mechanistically, this anti-inflammatory effect was associated with suppression of NF- $\kappa$ B activation without altering TLR4 or MYD88 expression. These effects were abolished by methyllycaconitine pretreatment. taVNS also reduced spleen enlargement and systemic cytokine concentrations, indicating modulation of both local and systemic inflammation.

**Conclusions** taVNS was associated with attenuation of LPS-induced acute otitis media and reduced NF- $\kappa$ B activation and downstream cytokine expression in a manner consistent with the involvement of  $\alpha 7$ nAChR-related signaling. By attenuating excessive inflammatory responses, taVNS was associated with improved auditory function and reduced middle ear injury, suggesting its potential as a non-invasive therapeutic strategy for LPS-induced acute otitis media.

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## Introduction

Otitis media (OM) is an inflammatory disease of the middle ear that predominantly affects children and remains a leading cause of pediatric hearing loss worldwide. The pathogenesis of OM is closely associated with Eustachian tube dysfunction following upper respiratory tract infection, which facilitates bacterial invasion and triggers mucosal inflammation characterized by epithelial thickening, goblet cell hyperplasia, and effusion accumulation [1–3]. Among bacterial pathogens, *Haemophilus influenzae* is particularly significant, as its lipooligosaccharide shares structural and biological similarities with enterobacterial endotoxins and strongly activates innate immune responses in host tissues [4]. The LPS-induced OM model effectively replicates the pathological features of bacterial OM by activating the Toll-like receptor 4 (TLR4)/MYD88/nuclear factor- $\kappa$ B (NF- $\kappa$ B) signaling pathway [5, 6]. This pathway drives the secretion of pro-inflammatory cytokines, including tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin (IL)-1 $\beta$ , and IL-6, along with secondary mediators such as IL-17 and vascular endothelial growth factor A (VEGF-A), which collectively promote mucosal remodeling, inflammatory cell infiltration, and tissue injury [7]. Therefore, controlling excessive inflammation in the middle ear is a key therapeutic strategy to prevent structural damage, promote epithelial recovery, and restore auditory function in OM.

Traditional therapeutic interventions for OM, including antibiotics and surgical procedures, are effective in managing acute symptoms but are limited by high recurrence rates, antibiotic resistance, chronic progression, and surgical complications [8]. Thus, there is an increasing need for alternative therapeutic approaches that modulate immune responses and facilitate tissue repair without the limitations and adverse effects of conventional pharmacologic interventions [9, 10]. Recent studies have shown that transcutaneous auricular vagus nerve stimulation (taVNS) is a safe and non-invasive neuromodulatory technique that stimulates the auricular branch of the vagus nerve [11, 12]. By activating the cholinergic anti-inflammatory pathway, in which acetylcholine binds to  $\alpha 7$  nicotinic acetylcholine receptors ( $\alpha 7$ nAChRs) on immune cells, taVNS effectively suppresses pro-inflammatory cytokine production and restores immune homeostasis in various experimental models [13–15]. While most previous studies have focused on systemic and organ-level effects of taVNS in conditions such as sepsis, colitis, and neuroinflammation, emerging evidence indicates that it can also modulate localized inflammation in peripheral tissues, including models of gouty arthritis and osteoarthritis [16–20]. These findings suggest that taVNS may provide therapeutic benefits even in anatomically restricted inflammatory conditions such as OM,

where excessive local cytokine production drives tissue damage [21].

However, the potential therapeutic role of taVNS in middle ear inflammation has not yet been clarified, and the contribution of  $\alpha 7$ nAChR-mediated signaling to its anti-inflammatory and tissue-repairing effects remains to be established. Therefore, this study aimed to determine whether taVNS can attenuate middle ear inflammation and promote mucosal recovery and auditory improvement in an LPS-induced acute otitis media (AOM) mouse model. To assess the involvement of  $\alpha 7$ nAChR signaling, methyllycaconitine (MLA), a selective  $\alpha 7$ nAChR antagonist, was administered prior to stimulation [22]. This study provides experimental evidence supporting taVNS as a novel, non-pharmacological approach with potential therapeutic applicability in AOM by modulating inflammatory responses and improving auditory functions.

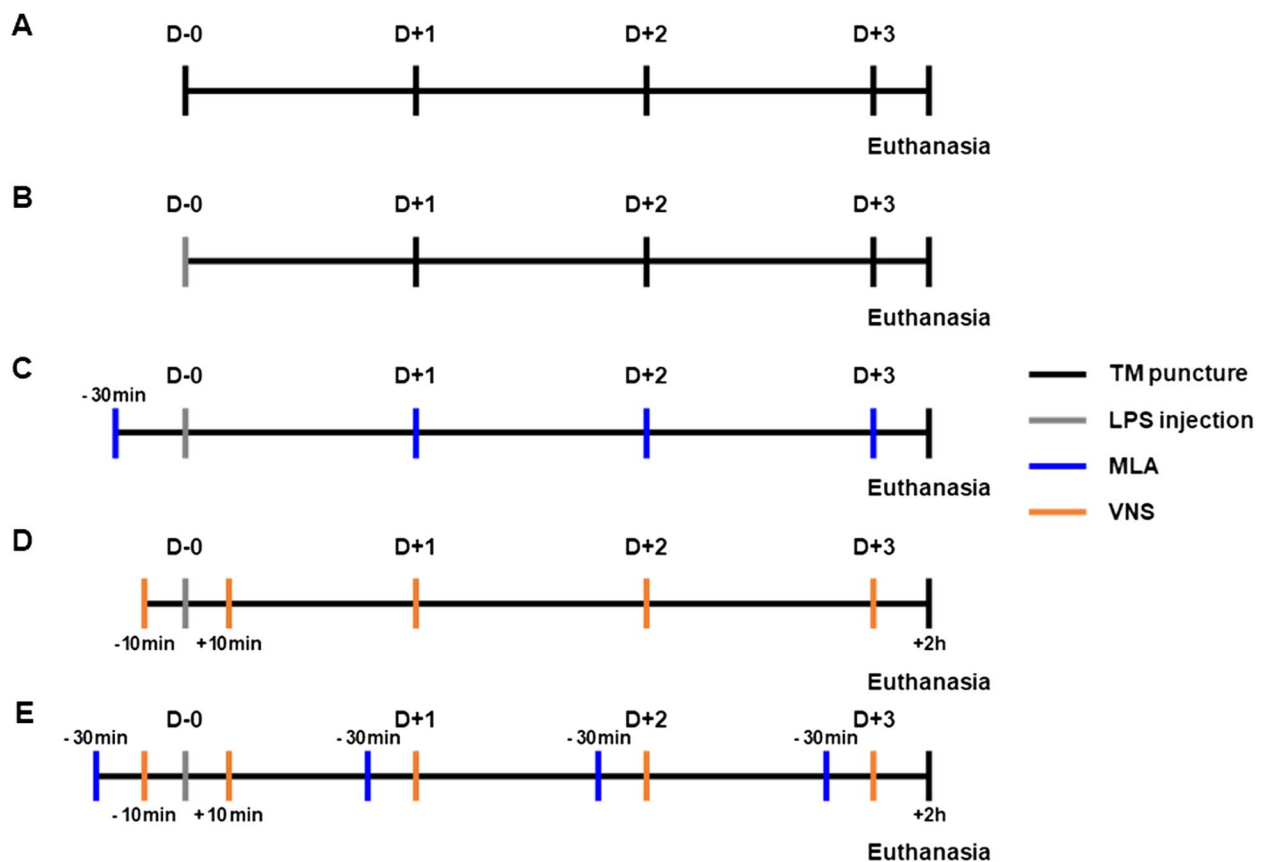
## Materials and methods

### Animal subjects

Male BALB/c mice (4–6 weeks old) were purchased from KOATECH (Pyeongtaek, Republic of Korea) and housed under a 12-h light/dark cycle with free access to food and water. All experimental procedures were approved by the Institutional Animal Care and Use Committee of Seoul National University Hospital (Permit No. 0720232101). The animals were maintained in a facility accredited by AAALAC International (#001169) in accordance with the Guide for the Care and Use of Laboratory Animals, 8th edition [23].

### Study design

This study was designed to investigate the therapeutic effects of non-invasive taVNS on LPS-induced AOM in mice and to assess whether these effects may involve  $\alpha 7$  nicotinic acetylcholine receptor ( $\alpha 7$ nAChR) signaling (Fig. 1). Mice were randomly assigned to five groups: control, OM, OM+MLA, VNS, and VNS+MLA. The control group underwent tympanic membrane puncture only, without LPS injection, taVNS, or MLA administration. The OM group was subjected to LPS-induced acute otitis media without taVNS. In the OM+MLA group, the  $\alpha 7$ nAChR antagonist methyllycaconitine (MLA) was administered daily to mice with LPS-induced AOM. The VNS group consisted of mice with LPS-induced AOM that received taVNS treatment 10 min before and after LPS injection, followed by daily stimulation until euthanasia. The VNS+MLA group received MLA administration 30 min prior to each taVNS session; on the first day, MLA was administered only before the taVNS session performed prior to LPS injection. Baseline assessments were performed under normal conditions prior to any experimental



**Fig. 1** Overview of the experimental protocol. **A** Experimental schedule for the Control **B** OM groups illustrating baseline conditions and inflammation induction. **C** In the OM + MLA group,  $\alpha 7$ nAChR antagonist MLA was applied prior to LPS injection and administered once daily thereafter, including on the day of euthanasia. **D** taVNS administration in the VNS group showing the period of daily stimulation following LPS injection. **E** In the VNS + MLA groups, MLA was applied 30 minutes prior to stimulation. This schematic summarizes the overall grouping and temporal sequence

manipulation. ABR testing was conducted at baseline and on Day 3 following the final taVNS or MLA administration, prior to euthanasia. Otoscopic imaging was performed at baseline and again on Day 3 following the final taVNS or MLA administration. All animals were euthanized on Day 3, 2 h after the final taVNS or MLA administration, for subsequent tissue and sample collection. Histological evaluation, spleen analysis, and inflammatory marker assessment were conducted to characterize both local and systemic immune responses.

#### AOM induction

AOM was induced by transtympanic injection of LPS (20  $\mu$ L, 2 mg/mL; L6511, Sigma-Aldrich, St. Louis, MO, USA) into the middle ear cavity under general anesthesia with Zoletil (30 mg/kg; Virbac, Carros, France) and xylazine (10 mg/kg). Control mice underwent tympanic membrane perforation without LPS injection.

#### Transcutaneous vagus nerve stimulation (taVNS)

Vagus nerve stimulation was applied transcutaneously to the auricular concha using clip-type electrodes under isoflurane anesthesia (induced with 2.0% isoflurane and maintained at 1.5% with an oxygen flow of 2 L/min). Biphasic square pulses (0.3 mA, 20 Hz, 200  $\mu$ s) were delivered using a Model 2100 Isolated Pulse Stimulator (A-M Systems, Sequim, WA, USA). Stimulation was administered for 5 min, followed by a 5-min rest, and repeated once (total of 10 min). On the day of LPS injection, stimulation was applied twice (10 min before and after injection) and subsequently once daily until euthanasia.

#### Methyllycaconitine (MLA) administration

To confirm whether the effects of taVNS were mediated through  $\alpha 7$ nAChR signaling, methyllycaconitine citrate (MLA; Tocris Bioscience, Bristol, UK), a selective  $\alpha 7$ nAChR antagonist, was used. MLA was dissolved in normal saline and administered intraperitoneally at

5 mg/kg, 30 min before taVNS in the VNS+MLA group. In the OM+MLA group, MLA was administered without subsequent stimulation.

### Otoscopic imaging

Otoscopic images were obtained at defined time points using a portable digital otoscope connected to a smartphone camera. Baseline images were captured before any experimental manipulation. In the control group, imaging was performed three days after tympanic membrane puncture, prior to euthanasia. In the OM group, imaging was performed three days after LPS injection, prior to euthanasia, to assess disease progression. In the VNS and MLA-treated groups, images were acquired after the completion of stimulation sessions following the same schedule.

### Auditory brainstem response (ABR) test

Click-evoked ABRs were recorded in a sound-attenuating chamber using the RZ6/BioSigRZ system (Tucker-Davis Technologies, Alachua, FL, USA). Baseline ABR recordings were obtained under normal conditions prior to any experimental manipulation. Follow-up ABR measurements were performed on Day 3, after completion of the final taVNS or MLA administration and prior to euthanasia. Mice were anesthetized with Zoletil (30 mg/kg) and xylazine (10 mg/kg). Subdermal needle electrodes were placed at the vertex (active), ipsilateral mastoid (reference), and contralateral mastoid (ground), maintaining electrode impedance below 1 k $\Omega$ . Click stimuli (100  $\mu$ s, alternating polarity) were presented at 21/s across 90–10 dB SPL in 10 dB decrements using SigGen RP software, with 512 responses averaged per intensity level [24]. Responses were amplified 5,000 $\times$  with a Medusa preamplifier, filtered between 100 and 1,500 Hz, and digitized at a sampling rate of 14 kHz. The ABR threshold was defined as the lowest intensity that produced a reproducible wave II response. Recordings were performed separately for both ears.

### Evaluation of systemic immune response through spleen analysis

Spleens were dissected, photographed with a scale reference, and analyzed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Length and surface area were measured from calibrated images, and mean values were used for analysis. For total splenocyte quantification, spleens were mechanically dissociated in RPMI 1640 medium (Welgene Inc., Gyeongsan, Republic of Korea) and filtered through 70  $\mu$ m strainers. After red blood cell lysis, cells were washed, resuspended in medium, and counted using trypan blue exclusion on an

automated cell counter (TC20, Bio-Rad, Hercules, CA, USA).

### Histological analysis of middle ear tissues

Middle ear tissues were fixed in 4% paraformaldehyde, decalcified in 14% EDTA for 10 days, embedded in paraffin, and sectioned at 4  $\mu$ m. Sections were stained with hematoxylin and eosin (Abcam, Cambridge, UK) to assess mucosal morphology and with periodic acid–Schiff (PAS; Roche, Mannheim, Germany) to visualize goblet cells. Stained sections were examined under a light microscope, and quantitative analyses were performed using ImageJ software. Mucosal thickness and goblet cell counts were measured at comparable anatomical regions across samples.

### Enzyme-linked immunosorbent assay (ELISA)

Middle ear effusion (MEE) was collected by transtympanic injection and aspiration of 30  $\mu$ L saline, followed by centrifugation (3,000 $\times$ g, 15 min, 4  $^{\circ}$ C). The supernatants were used for analysis. Whole blood was obtained by retro-orbital bleeding, and serum was separated by centrifugation at 2,000 $\times$ g for 10 min at 4  $^{\circ}$ C. IL-6 (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA), IL-1 $\beta$ , and TNF- $\alpha$  (BioLegend, San Diego, CA, USA) levels were measured using commercial ELISA kits according to the manufacturers' instructions. Absorbance was read at 450 nm with a 570 nm reference, and standard curves were generated using a four-parameter logistic regression model.

### Quantitative real-time polymerase chain reaction (qPCR)

Total RNA was extracted from middle ear tissues using QIAzol Lysis Reagent (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesized from total RNA using the PrimeScript<sup>TM</sup> RT reagent kit (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. qPCR was performed with TB Green<sup>®</sup> Premix Ex Taq<sup>TM</sup> (Takara Bio Inc.) on a LightCycler<sup>®</sup> 480 System (Roche, Meylan, France). Gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase, and relative quantification was calculated using the  $2^{-\Delta\Delta C_t}$  method. Primer sequences for target genes are provided in Table 1.

### Western blot

Proteins were extracted from middle ear tissues using the EzRIPA Lysis Kit (WSE-7420, Atto, Tokyo, Japan) and quantified using the BCA assay. Equal amounts of protein (20  $\mu$ g) were loaded onto 10% SDS–polyacrylamide gels for electrophoresis, alongside a molecular weight marker (PageRuler<sup>TM</sup> Prestained Protein Ladder, Bio-Rad). Proteins were transferred to PVDF

**Table 1** Primer sequences used for quantitative real-time PCR analysis

| Primer  | Forward (5' → 3')           | Reverse (5' → 3')            |
|---------|-----------------------------|------------------------------|
| TLR4    | ACTCAGCAAAGTCCTGA<br>TGAC   | GAGGTGGTGAAGCCATGCCA         |
| MYD88   | ACCTGTGTCTGGTCCATT<br>GCCA  | GCTGAGTGCAAACCTTGGT<br>CTGG  |
| P65     | AGGCTTCTGGGCCTTATGTG        | TGCTTCTCTGCCAGGAATAC         |
| TNF-α   | GGTGCCTATGTCTCAGCC<br>TCTT  | GCCATAGAAGCTGATGAGA<br>GGGAG |
| IL-1β   | TGGACCTTCCAGGATGAG<br>GACA  | GTTCATCTCGAGCCTGTAGTG        |
| IL-6    | TTCCATCCAGTTGCCTTCTTG       | TTGGGAGTGGTATCTCTGTGA        |
| IL-17   | CAGACTACCTCAACCGTT<br>CCAC  | TCCAGCTTTCCTCCGCATTGA        |
| VEGF-A  | CTGCTGTAACGATGAAGC<br>CCTG  | GCTGTAGGAAGCTCATCTCTCC       |
| α7nAChR | GCCAGCAACATCTGATTC<br>CGTG  | GGCATTTCGCCACCATCAGGGT       |
| GAPDH   | CATCACTGCCACCCAGAA<br>GACTG | ATGCCAGTGAGCTTCCCG<br>TTCAG  |

membranes, blocked with 5% BSA, and incubated overnight at 4 °C with primary antibodies diluted in 5% BSA in TBS-T: TLR4 (1:50, 48–2300; Invitrogen), NF-κB p65 (1:1,000, 8242; Cell Signaling Technology, Danvers, MA, USA), and β-actin (1:10,000, A1978; Sigma-Aldrich) as a loading control. Horseradish peroxidase-conjugated secondary antibodies were applied, and signals were visualized using an enhanced chemiluminescence detection kit (EzWestLumi plus; Atto). Protein band intensities were quantified using ImageJ software.

**Statistical analysis**

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were conducted using GraphPad Prism (version 9.0; GraphPad Software, San Diego, CA, USA). Group comparisons were made using one-way ANOVA followed by Tukey’s post hoc test when normality was satisfied (Shapiro–Wilk test). When normality was not met, the Kruskal–Wallis test with Dunn’s correction was used. All quantitative measurements were performed in at least triplicate, and mean values were analyzed. Statistical significance was defined as  $p < 0.05$ . Each group initially included 10 mice, and the final sample sizes ( $n = 8–10$ ) are indicated in the figure legends, while detailed group-wise sample numbers and corresponding mean ± SEM values are provided in the supplementary file. No animals were excluded from the analyses beyond those explicitly indicated in the Methods or Supplementary Data.

**Results**

**Hearing threshold changes following AOM induction and taVNS treatment**

Click-evoked ABR measurements were conducted to evaluate hearing threshold changes before and after experimental interventions (Fig. 2B). ABR testing revealed significant hearing loss in OM-induced mice compared with the control group ( $p < 0.0001$ ). taVNS treatment markedly attenuated this threshold elevation ( $p = 0.002$ ) (Fig. 2C). The OM, OM+MLA, and VNS+MLA groups exhibited comparable hearing thresholds, indicating that MLA administration alone did not affect hearing loss but abolished the protective effect of taVNS.

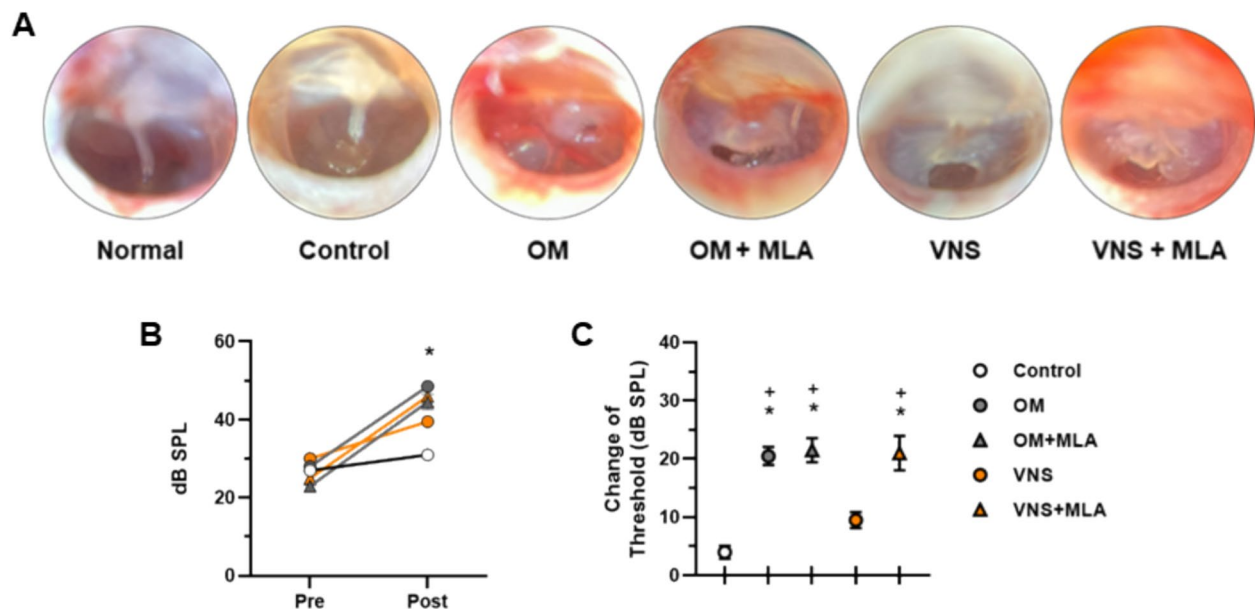
**Morphological changes in the tympanic membrane**

Otoscopic imaging was performed to assess morphological changes in the tympanic membrane (Fig. 2A). At baseline, all groups exhibited intact, smooth, and translucent membranes without signs of inflammation. Three days after intervention, the control group maintained a translucent tympanic membrane with only a small perforation at the injection site. The OM group displayed typical inflammatory features, including marked redness, opacity, and effusion. Both the OM+MLA and VNS+MLA groups showed persistent redness and opacity comparable to OM. The VNS group exhibited an opaque tympanic membrane with a visible perforation but reduced redness and no effusion, suggesting partial resolution of inflammation.

Histological analysis confirmed significant mucosal thickening in the OM group ( $p < 0.0001$ ), which was markedly reduced by taVNS ( $p = 0.043$ ). MLA administration negated this improvement, with OM+MLA and VNS+MLA groups showing persistent mucosal hypertrophy comparable to OM (Fig. 3A, B). PAS staining revealed a similar trend in goblet cell hyperplasia (Fig. 3A, C). The OM group demonstrated a pronounced increase in goblet cell counts compared with the Control group ( $p < 0.0001$ ). taVNS significantly reduced goblet cell counts relative to OM ( $p < 0.0001$ ), and this effect was abolished by MLA injection.

**Effects of taVNS on systemic immune responses**

Spleen morphometric analysis demonstrated significant enlargement in the OM group compared with controls (length,  $p = 0.012$ ; area,  $p = 0.002$ ), consistent with systemic immune activation. The VNS group exhibited a significant reduction in spleen length ( $p = 0.028$ ), whereas MLA pretreatment abolished this effect (Fig. 4B, C). Total splenocyte counts showed a similar



**Fig. 2** Otoloscopic and auditory functional assessment following taVNS treatment in LPS-induced AOM. **A** Representative otoscopic images of the tympanic membrane under normal conditions and from each experimental group (Control, OM, OM + MLA, VNS, and VNS + MLA). **B** Auditory brainstem response (ABR) thresholds measured before and after experimental manipulation. The OM group exhibits marked elevation of thresholds, which is significantly reduced by taVNS, while MLA administration negates this effect. **C** Quantification of the change in ABR threshold ( $\Delta$ threshold = post – pre) confirming that taVNS mitigates LPS-induced hearing loss, whereas  $\alpha$ 7nAChR blockade reverses this protection. Data are presented as mean  $\pm$  SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test (\* $p < 0.05$  vs. Control, + $p < 0.05$  vs. VNS;  $n = 10$  mice per group)

trend, with a partial reduction in the VNS group but no significant difference in MLA-treated groups (Fig. 4D).

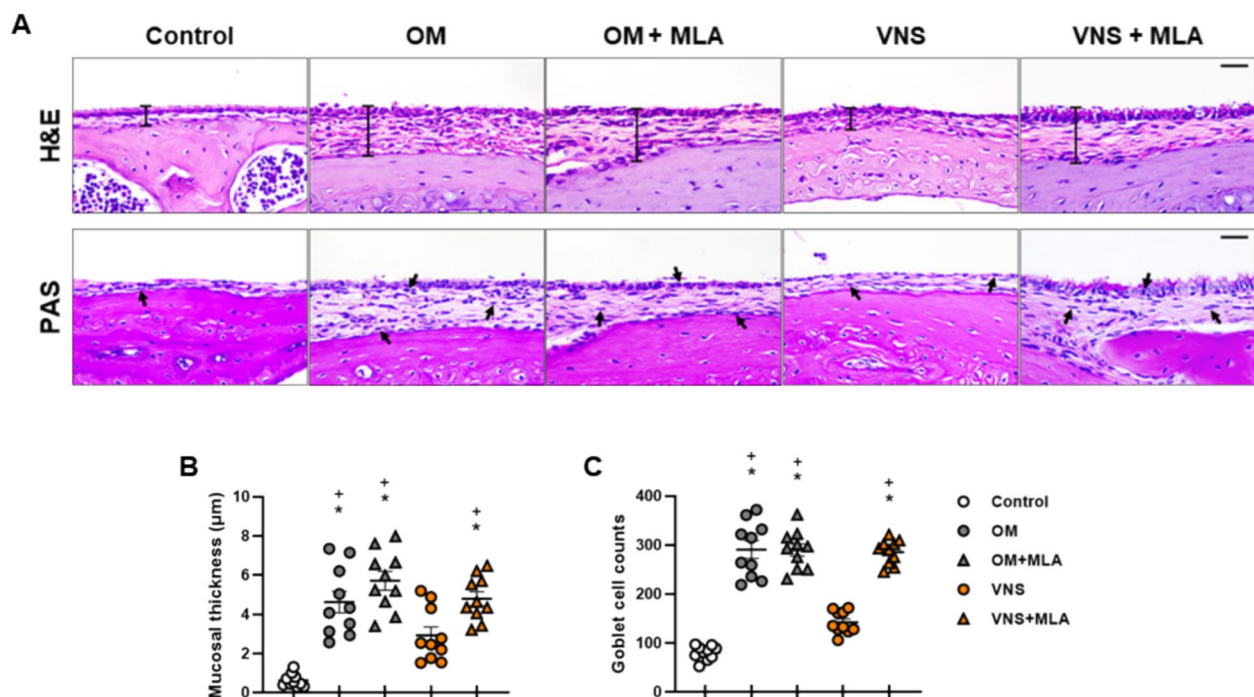
#### taVNS attenuates local and systemic cytokine production

Cytokine analysis of MEE revealed elevated TNF- $\alpha$  ( $p = 0.0002$ ), IL-1 $\beta$  ( $p = 0.0004$ ), and IL-6 ( $p < 0.0001$ ) concentrations in the OM group compared with the Control group. In the VNS group, taVNS significantly suppressed these cytokine levels (TNF- $\alpha$ ,  $p = 0.0006$ ; IL-1 $\beta$ ,  $p = 0.016$ ; IL-6,  $p = 0.012$ ), whereas MLA treatment prevented this suppression, resulting in cytokine profiles comparable to OM (Fig. 5A–C). Similarly, in serum samples, TNF- $\alpha$  ( $p < 0.0001$ ), IL-1 $\beta$  ( $p = 0.0008$ ), and IL-6 ( $p = 0.0003$ ) levels were significantly elevated in the OM group relative to controls. In the VNS group, taVNS markedly reduced IL-1 $\beta$  ( $p = 0.034$ ) and IL-6 ( $p = 0.021$ ) levels, but MLA pretreatment abolished these effects (Fig. 5D–F).

#### Modulation of inflammatory and immune-related gene expression by taVNS

qPCR analysis was conducted to examine transcriptional changes in inflammatory and immune-related genes in middle ear tissues (Fig. 6). The expression levels of upstream regulators TLR4 and MYD88 showed no statistically significant differences among the groups, indicating that taVNS did not influence the initiation of TLR4/

MYD88 signaling. taVNS significantly downregulated several downstream inflammatory mediators. NF- $\kappa$ B p65 expression was markedly reduced in the VNS group compared with OM ( $p = 0.006$ ), whereas MLA treatment abolished this effect. TNF- $\alpha$  mRNA levels were significantly decreased by taVNS ( $p = 0.0081$ ), but this reduction was also negated by MLA. IL-1 $\beta$  expression followed a similar pattern, showing a significant decrease in the VNS group compared with OM ( $p = 0.0396$ ) and loss of this effect after MLA administration. IL-6 expression was likewise decreased following taVNS ( $p = 0.0374$ ) but not in MLA-treated mice. Pro-inflammatory cytokines associated with adaptive immune activation were similarly affected. IL-17 mRNA levels were markedly reduced by taVNS ( $p = 0.0005$ ), and VEGF-A expression—a marker of inflammation-induced vascular permeability—was also significantly decreased ( $p = 0.004$ ). In both cases, MLA pretreatment eliminated these effects.  $\alpha$ 7nAChR mRNA expression itself remained unchanged among all groups, indicating that taVNS did not alter receptor expression at the transcriptional level. Collectively, these findings indicate that taVNS is associated with reduced expression of downstream pro-inflammatory cytokines and NF- $\kappa$ B signaling without affecting TLR4, MYD88, or  $\alpha$ 7nAChR expression, and that these effects are attenuated by  $\alpha$ 7nAChR antagonism with MLA.



**Fig. 3** Histological evaluation of middle ear mucosa following taVNS in LPS-induced AOM. **A** Representative hematoxylin and eosin (H&E) stained (upper panels) and periodic acid–Schiff (PAS) stained (lower panels) sections of the middle ear mucosa from each group (Control, OM, OM + MLA, VNS, and VNS + MLA). The OM group shows pronounced mucosal thickening and inflammatory cell infiltration, which were attenuated by taVNS, whereas MLA pretreatment abolishes this effect. Black arrows indicate goblet cells stained positively by PAS. Scale bars: 30  $\mu$ m; magnification: 400 $\times$ . **B** Quantitative analysis of mucosal thickness showing a significant increase in the OM group compared with Control, which was markedly reduced by taVNS. MLA pretreatment negated this reduction, resulting in mucosal hypertrophy comparable to OM. **C** Quantification of goblet cell counts demonstrating a significant increase in the OM group which was alleviated by taVNS and MLA administration abolished this effect. All experiments were independently repeated at least three times. Data are presented as mean  $\pm$  SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test (\* $p < 0.05$  vs. Control, + $p < 0.05$  vs. VNS;  $n = 10$  mice per group)

#### Protein expression of TLR4 and NF- $\kappa$ B in the middle ear tissue

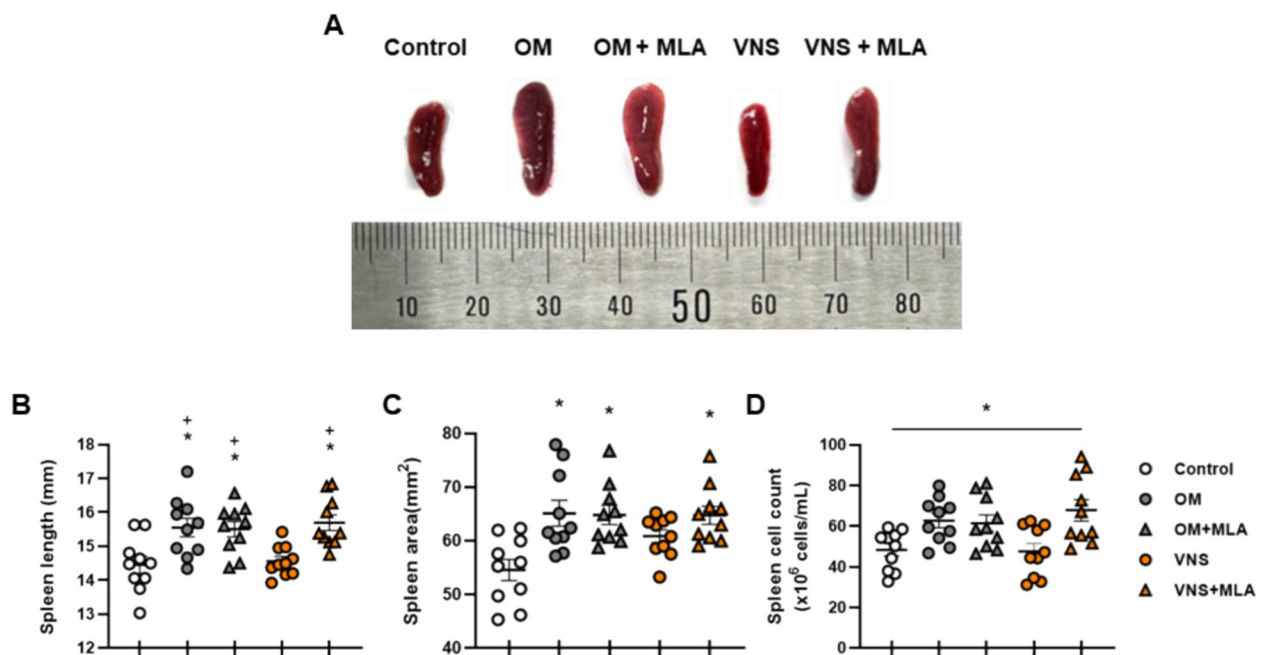
Western blot analysis was performed to assess the protein expression of TLR4 and NF- $\kappa$ B p65 in middle ear tissues (Fig. 7). TLR4 expression was significantly elevated in all LPS-treated groups compared with the control group (OM,  $p = 0.0029$ ; OM + MLA,  $p = 0.0021$ ; VNS,  $p = 0.0008$ ; VNS + MLA,  $p = 0.0005$ ). No significant differences were observed among the inflamed groups, indicating that neither taVNS nor MLA altered TLR4 protein levels. In contrast, NF- $\kappa$ B p65 expression displayed a distinct pattern. The OM group showed a marked increase compared with control ( $p < 0.0001$ ), consistent with inflammation-induced NF- $\kappa$ B activation. taVNS significantly reduced NF- $\kappa$ B p65 expression relative to OM ( $p = 0.0079$ ), restoring levels comparable to those of the control group ( $p = 0.1838$ ). However, MLA treatment abolished this effect, with both OM + MLA and VNS + MLA groups remaining significantly elevated compared with control (both  $p < 0.0001$ ). These findings indicate that taVNS is associated with reduced NF- $\kappa$ B

p65 expression without altering upstream TLR4 protein levels, and that this effect is attenuated in the presence of MLA.

#### Discussion

This study demonstrates that taVNS alleviates LPS-induced AOM by improving auditory function and reducing middle ear inflammation. taVNS significantly enhanced auditory performance and attenuated mucosal thickening and goblet cell hyperplasia, accompanied by decreased cytokine levels in both middle ear effusion and serum. At the molecular level, taVNS suppressed NF- $\kappa$ B activation and downregulated pro-inflammatory mediators, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-17, and VEGF-A, without altering upstream TLR4/MYD88 signaling. These anti-inflammatory effects were abolished by MLA administration, confirming that  $\alpha 7$  nicotinic acetylcholine receptor ( $\alpha 7$ nAChR) signaling mediates the regulatory actions of taVNS [25–27].

AOM is characterized by rapid activation of innate immune signaling, primarily via the TLR4/MYD88/



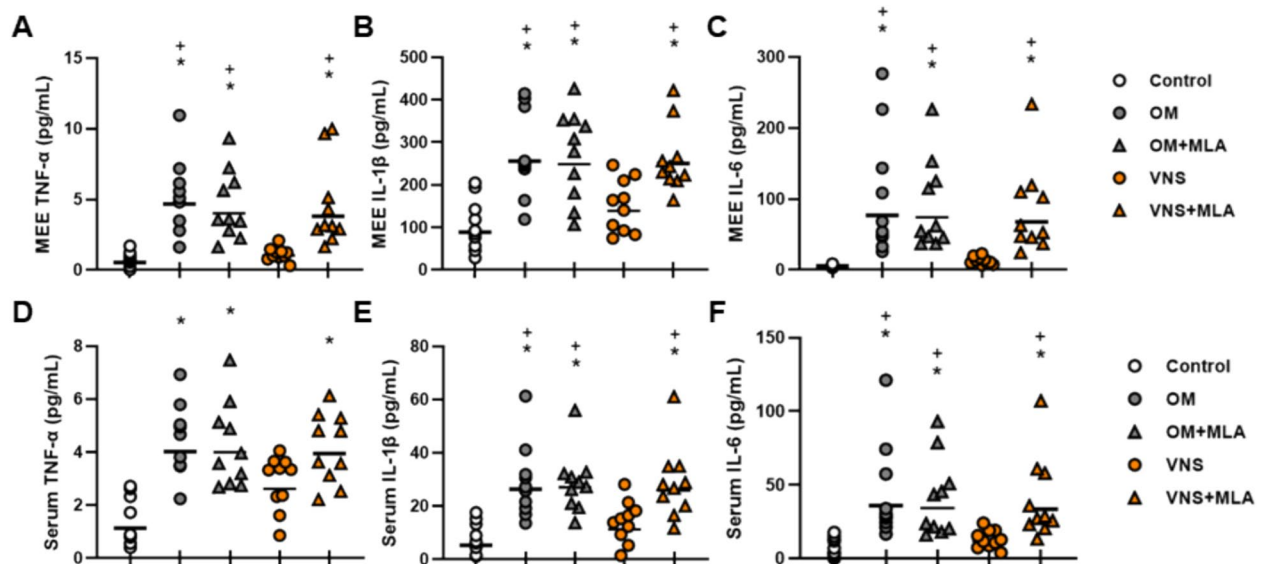
**Fig. 4** Effects of taVNS on systemic immune responses in LPS-induced AOM. **A** Representative images of spleens from each experimental group (Control, OM, OM + MLA, VNS, and VNS + MLA), showing splenic enlargement in inflamed mice. **B** Quantitative analysis of spleen length and **C** surface area demonstrating significant splenomegaly in the OM group compared with Control, which was attenuated by taVNS. MLA pretreatment abolished the effect of taVNS, resulting in spleen dimensions comparable to those of the OM group. **D** Total splenocyte counts showing a similar trend, with partial reduction in the taVNS group and no significant difference following MLA administration. All experiments were independently repeated at least three times. Data are presented as mean  $\pm$  SEM. Statistical significance was determined by one-way ANOVA followed by Tukey's post hoc test (\* $p < 0.05$  vs. Control, + $p < 0.05$  vs. VNS;  $n = 10$  mice per group)

NF- $\kappa$ B axis, which drives mucosal remodeling, effusion formation, and conductive hearing loss [2, 28]. By selectively suppressing NF- $\kappa$ B activation, taVNS appears to attenuate excessive production of inflammatory mediators while preserving basal immune function. This selective modulation aligns with the physiological role of the vagus nerve as a homeostatic regulator of immune tone [29, 30]. Moreover, the reduced expression of IL-17 and VEGF-A observed in this study suggests that taVNS influences both innate and adaptive immune pathways involved in middle ear inflammation [31, 32]. The anti-inflammatory effects observed here are consistent with activation of the cholinergic anti-inflammatory pathway, a vagus nerve-dependent neuroimmune circuit that modulates peripheral inflammation [33, 34]. In this pathway, acetylcholine binding to  $\alpha 7$ nAChRs on immune cells inhibits NF- $\kappa$ B nuclear translocation and subsequent cytokine production [35]. Previous studies in models of sepsis, colitis, and arthritis have demonstrated that vagus nerve stimulation suppresses systemic inflammation through this mechanism [16–20]. The present findings extend these results to the middle ear, showing that taVNS suppresses NF- $\kappa$ B and related cytokines while maintaining TLR4/MYD88 signaling, thereby

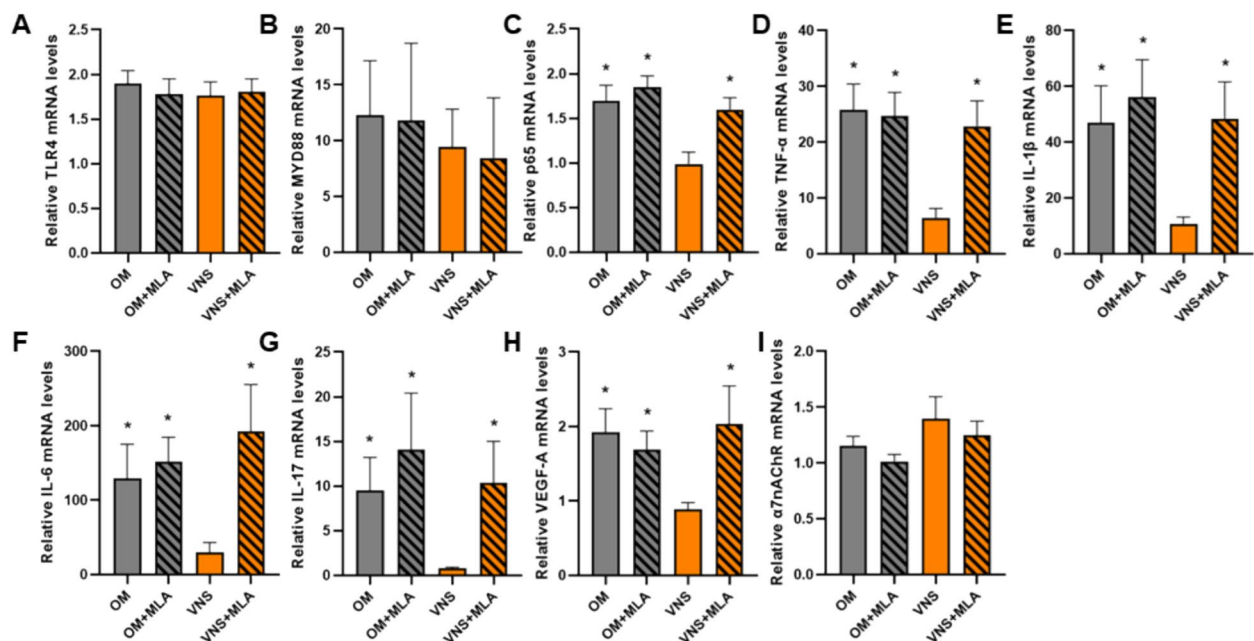
modulating inflammation without impairing innate immune recognition.

Previous studies have also shown that vagus nerve activation modulates immune responses and suppresses cytokine release through  $\alpha 7$ nAChR-dependent signaling [33, 36]. Recent evidence further indicates that vagus nerve stimulation can influence localized inflammatory processes through the same mechanism [19, 37]. Building on this background, the present study investigated whether taVNS could attenuate inflammation in an AOM model. Our findings demonstrate that the effects of taVNS extend beyond systemic circulation to local tissue inflammation. taVNS reduced splenic enlargement and systemic cytokine levels while concurrently suppressing inflammatory changes in the middle ear. These results suggest that taVNS may modulate inflammatory responses within the middle ear microenvironment, providing experimental evidence supporting its therapeutic potential in AOM. Future studies are warranted to determine whether similar effects occur in other localized inflammatory conditions.

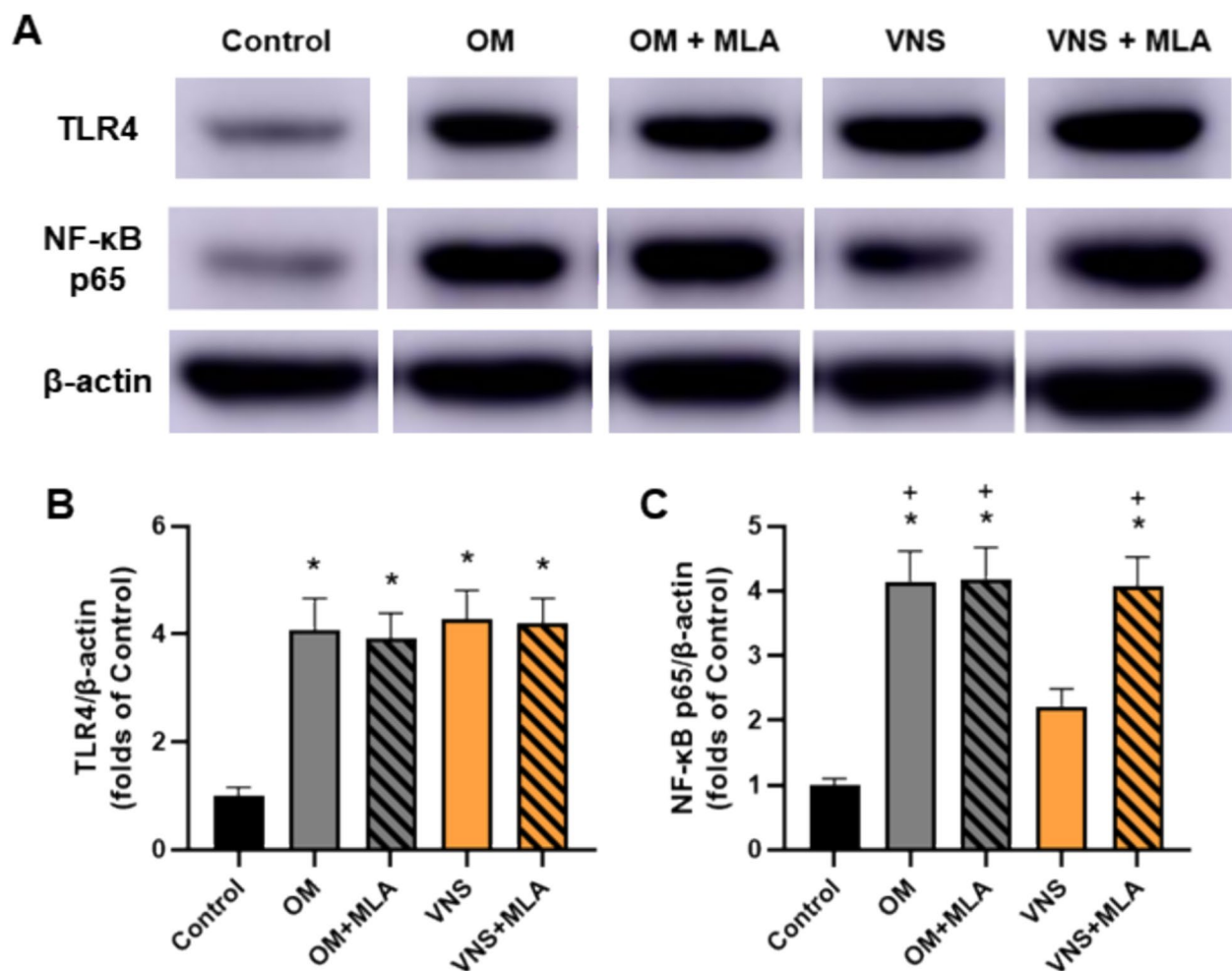
Although this study did not employ live bacterial inoculation, it utilized an LPS preparation whose structural composition closely resembles the lipooligosaccharide



**Fig. 5** taVNS attenuates local and systemic cytokine production in LPS-induced AOM. **A–C** Cytokine concentrations in middle ear effusion (MEE) showing significant increases in **(A)** TNF- $\alpha$ , **(B)** IL-1 $\beta$ , and **(C)** IL-6 levels in the OM group compared with Control. In the VNS group, taVNS treatment markedly suppressed all three cytokines, whereas MLA pretreatment abolished this effect, resulting in cytokine profiles comparable to OM. **D–F** Serum cytokine levels showing a similar pattern, with elevated **(D)** TNF- $\alpha$ , **(E)** IL-1 $\beta$ , and **(F)** IL-6 in OM mice compared with Control and significant reductions following taVNS treatment. MLA administration prevented these reductions, restoring cytokine levels to those observed in OM. All experiments were independently repeated at least three times. Data are expressed as mean  $\pm$  SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test or, when normality was not satisfied, the Kruskal–Wallis test with Dunn's correction (\* $p < 0.05$  vs. Control, + $p < 0.05$  vs. VNS;  $n = 10$  mice per group)



**Fig. 6** taVNS modulates the expression of inflammatory and immune-related genes in middle ear tissues. **A,B** mRNA expression levels of upstream regulators **(A)** TLR4 and **(B)** MyD88 showed no significant differences among groups, indicating that taVNS did not alter initiation of the TLR4/MyD88 signaling pathway. **C–I** Expression of downstream inflammatory mediators and cholinergic pathway markers. **C** NF- $\kappa$ B p65, **(D)** TNF- $\alpha$ , **(E)** IL-1 $\beta$ , **(F)** IL-6, **(G)** IL-17, and **(H)** VEGF-A were significantly reduced following taVNS treatment. MLA pretreatment abolished these reductions, restoring expression to levels comparable to OM. **I**  $\alpha 7$ nAChR expression remained unchanged across all groups. All experiments were independently repeated at least three times. Data are expressed as mean  $\pm$  SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test or, when normality was not satisfied, the Kruskal–Wallis test with Dunn's correction (\* $p < 0.05$ ;  $n = 8$ –10 mice per group)



**Fig. 7** taVNS suppresses NF- $\kappa$ B activation without altering TLR4 expression in middle ear tissues. **A** Representative Western blot images showing the protein expression of TLR4 and NF- $\kappa$ B p65 in middle ear tissues across experimental groups (Control, OM, OM + MLA, VNS, and VNS + MLA). Each lane represents a cropped band obtained from the original blot, and  $\beta$ -actin was used as the loading control. **B,C** Densitometric quantification of TLR4 and NF- $\kappa$ B p65 protein expression. **B** TLR4 expression was significantly increased in all LPS-treated groups compared with Control and was not affected by taVNS or MLA treatment. In contrast, **(C)** NF- $\kappa$ B p65 expression was markedly elevated in the OM group, significantly reduced by taVNS, and restored by MLA pretreatment. All experiments were independently repeated at least three times. Data are expressed as mean  $\pm$  SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey's post hoc test or, when normality was not satisfied, the Kruskal–Wallis test with Dunn's correction (\* $p < 0.05$  vs. Control, + $p < 0.05$  vs. VNS;  $n = 10$  mice per group)

of *H. influenzae*, a major pathogen responsible for otitis media [2]. *H. influenzae* lipooligosaccharide exhibits both structural and functional similarity to *Salmonella* LPS, resulting in comparable endotoxin activity and innate immune activation [4]. Previous infection studies have shown that *H. influenzae* activates inflammatory responses via TLR2- and TLR4-mediated signaling, leading to MYD88- and NF- $\kappa$ B-dependent cytokine expression and mucosal inflammation in the middle ear [38]. Therefore, the LPS-induced AOM model used in this study provides a biologically relevant representation that captures key innate immune features of infection-driven inflammation. Future investigations employing

live *H. influenzae* inoculation will be essential to confirm whether taVNS produces similar protective effects under infectious conditions.

In addition to the limitations associated with the LPS-induced model, an additional mechanistic consideration should be acknowledged. The present study demonstrates that taVNS exerts anti-inflammatory and functional benefits in acute otitis media; however, the precise neural and cellular mechanisms underlying these effects were not fully delineated. In particular, while pharmacological blockade with the  $\alpha 7$ nAChR antagonist MLA supports the involvement of cholinergic anti-inflammatory signaling, we did not directly assess vagus nerve activity

or cell-type-specific NF- $\kappa$ B activation within the middle ear tissue. In this context, the primary aim of this study was to evaluate the therapeutic potential of taVNS in a disease-relevant inflammatory context. The tissue-level analyses performed in this study, including cytokine profiling, histological assessment, and evaluation of NF- $\kappa$ B signaling, provide consistent evidence supporting modulation of inflammatory responses following taVNS treatment. Future studies incorporating cell-specific imaging approaches and neural pathway-targeted interventions, such as immunofluorescence-based assessment of immune cell activation or vagotomy-based validation, will be valuable for further refining the mechanistic understanding of taVNS-mediated immunomodulation in otitis media.

## Conclusion

In conclusion, the present study investigated the effects of transcutaneous auricular vagus nerve stimulation (taVNS) in an LPS-induced acute otitis media (AOM) mouse model and demonstrated that taVNS is associated with reduced inflammatory responses and improved auditory outcomes. The observed effects were accompanied by decreased NF- $\kappa$ B activation and downstream pro-inflammatory cytokine expression, with pharmacological findings suggesting a potential involvement of  $\alpha 7$  nicotinic acetylcholine receptor ( $\alpha 7$ nAChR)-related cholinergic signaling. Despite inherent limitations including the absence of sham or VNS-only control groups, the present study provides experimental evidence indicating that taVNS may modulate inflammatory signaling pathways under pathological inflammatory conditions. Taken together, these results suggest a possible association between taVNS and vagus nerve-related anti-inflammatory mechanisms in LPS-induced AOM, supporting the relevance of taVNS as a non-invasive neuromodulatory approach that warrants further investigation.

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s41232-026-00408-6>.

Additional File 1.

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## Authors' contributions

N.S. conducted the experiments, analyzed the data, prepared the figures, and drafted the manuscript. S.P. assisted with data acquisition and figure preparation. M.-W.S., S.Y.L., and J.-H.L. provided conceptual advice and reviewed the manuscript. M.K.P. supervised the study, provided overall direction, and

approved the final version of the manuscript. All authors reviewed the final manuscript.

## Data availability

No datasets were generated or analysed during the current study.

## Declarations

### Competing interests

The authors declare no competing interests.

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