Intratympanic Lipopolysaccharide Elevates Systemic Fluorescent Gentamicin Uptake in the Cochlea

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Objectives/Hypothesis: Lipopolysaccharide (LPS), a key component of bacterial endotoxins, activates macrophages and triggers the release of inflammatory cytokines in mammalian tissues. Recent studies have shown that intratympanic injection of LPS simulates acute otitis media (AOM) and results in morphological and functional changes in the inner ear. Here we established an AOM mouse model with LPS to investigate the uptake of ototoxic gentamicin in the inner ear, and elucidated the underlying mechanism by focusing on cochlear inflammation as a result of AOM.

Study Design: Preclinical rodent animal model.

Methods: Fluorescently tagged gentamicin (GTTR) was systemically administered to mice with AOM. Iba1-positive macrophage morphology and inner ear cytokine profile were evaluated by immunofluorescence technique and a mouse cytokine array kit, respectively.

Results: We observed characteristic symptoms of AOM in the LPS-treated ears with elevated hearing thresholds indicating a conductive hearing loss. More importantly, the LPS-induced AOM activated cochlear inflammatory responses, manifested by macrophage infiltration, particularly in the organ of Corti and the spiral ligament, in addition to the up-regulation of proinflammatory cytokines. Meanwhile, GTTR uptake in the stria vascularis and sensory hair cells from all the LPS-treated ears was significantly enhanced at 24, 48, and 72-hour post-treatment, as the most prominent enhancement was observed in the 48-hour group.

Conclusion: In summary, this study suggests that the pathological cochlea is more susceptible to ototoxic drugs, including aminoglycosides, and justified the clinical concern of aminoglycoside ototoxicity in the AOM treatment.

Key Words: Acute otitis media, lipopolysaccharide, aminoglycosides, gentamicin, inflammation, macrophage, cytokine, drug uptake, hearing loss.

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INTRODUCTION

Otitis media (OM) is arguably the most prominent pathology that is associated with acquired hearing deficits, after excluding factors such as genetic predisposition, aging, and history of exposure to noise and ototoxic agents.[1] Regardless of treatment selection, significant short- and long-term adverse effects are expected, and treatment plans for acute otitis media (AOM) typically include the application of antibiotics.

Aminoglycoside is a group of antibiotics that is critical for treating life-threatening Gram-negative bacterial infections.[2] However, aminoglycosides also induce cytotoxicity in the cochlea, the vestibular system, and the kidney. All cells appear to take up aminoglycosides after systemic administration, and while most cells clear the drug rather quickly, inner ear sensory hair cells (HCs) selectively retain the drug.[3] Previous studies have used Texas Red-conjugated gentamicin (GTTR) as a powerful tool to study drug trafficking kinetics in the cochlea. Serum levels of GTTR rapidly increase following intraperitoneal injection and GTTR preferentially accumulates in the stria vascularis (SV).[4] GTTR has a broad fluorescence (dynamic) range with increasing dose, providing a reliable sensitivity to determine the differential uptake of the marker as experimental condition varies.[5]

Inflammation caused by OM is thought to alter the permeability of cochlear membranes that face the middle ear cavity, allowing bacterial toxins and inflammatory

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substances to more readily enter into the inner ear.[6,7] In this study, we explored the effect of Lipopolysaccharide (LPS)-induced AOM on aminoglycoside uptake, as well as the other aspects of the inflammatory response in the cochlea. Following stimulation in the middle ear, fibrocytes in the spiral ligament are responsive to immunogenic signals and subsequently release cytokines, as a relay to initiate immune responses in the inner ear.[8] In previous studies, systemic LPS-induced endotoxemia caused elevation of ototoxic aminoglycoside uptake by cochlear tissues including sensory HCs.[9] This observation is suggestive that similar effects are likely to occur after intratympanic LPS injection, an iconic animal model of OM without live bacterial infection.

Clinically, topical treatment of OM with aminoglycosides, such as neomycin, is effective, but also controversial and many otolaryngologists are concerned that aminoglycoside eardrops may be ototoxic if they enter the middle ear through a ruptured tympanic membrane (TM).[10] Scientific evidence for this specific concern is scarce and clinicians often take precaution and best practice, avoiding the ototopical use of aminoglycosides. The present study also provides the needed justification to support the ototoxic concern upon drug selection in OM treatment in the presence of TM perforation or tympanostomy tube allowing ototoxic ear drops to land in the middle ear.

MATERIALS AND METHODS

Mice

C57BL/6 mice (aged 6–8 weeks, JAX stock #0664) were housed in a Specific Pathogen Free-modified room, without any sound treatment besides the ambient noise. All animal work was carried out using protocols approved by the Institutional Animal Care and Use Committee of the Jerry L. Pettis VA Medical Center, Loma Linda, CA. Animal use procedures conform with federal regulations regarding personnel, supervision, record keeping, and veterinary care.

LPS or PBS Treatment and Tissue Preparation

To perform minimally invasive intratympanic (*i.t.*) injection, a small puncture was initially made by an insulin syringe needle at the anteroinferior quadrant of the TM. Then, another syringe needle, which was connected to a fine flexible polyurethane tube (Fig. 1A), was used to perform the *i.t.* injection. In each animal, 10 μl LPS (Invitrogen, Cat# 00-4976, 1 mg/ml, dissolved in 0.01 M PBS) or phosphate-buffered saline (PBS) was injected into the right ear. At different times after LPS/PBS treatment, mice were cardiac perfused with PBS, then 4% formaldehyde. Cochleae were excised and post-fixed for 2 hours, then proceeded to immunolabeling. For cytokine detection, cochleae from mice not-cardiac perfused were flash-frozen in liquid nitrogen and stored.

Auditory Testing

An auditory brain-stem response (ABR) test was selected to evaluate the hearing function. Briefly, each ear was tested with a closed tube sound delivery system sealed into the ear canal. The ABRs to tone burst stimuli (5-ms duration, 1-ms rise/fall) at 4, 8, 12, 16, 24, and 32 kHz, with 5-dB steps, were recorded using a TDT System 3 (Tucker-Davis Technologies, Alachua, FL). The threshold was determined by the lowest stimulus level that produces a minimally visible response.

Systemic GTTR Application and Cochlear Uptake

After LPS/PBS treatment, fluorescently (Texas Red) tagged gentamicin (GTTR, m.w. = ~1,100 g/mol) was systemically (2 μg/g body weight, *i.p.*) administered to mice 1 hour before cardiac perfusion. Purified GTTR conjugate was produced as previously described.[5] In the present study, GTTR was solely used as a molecular tracer to study its intracochlear uptake, and at a dose greatly lower than that induces ototoxicity when gentamicin (C2, m.w. = 463.6 g/mol) is administered.

The same confocal settings were used to compare bilateral images from individual mice, with two acquisitions per location to guarantee data consistency. GTTR fluorescent pixel intensities were obtained by the histogram function of the ImageJ software (Fiji, National Institutes of Health, Bethesda, MD) after the removal of nucleic pixels using Adobe Photoshop. Pixel intensities were statistically compared within each set of images per experiment and not directly compared between different experiments. To normalize data among multiple experimental sets, the mean intensity was ratioed against the control standard and graphed.[11]

Immunolabeling of Iba1-Positive Macrophages

Immunofluorescence labeling was performed using anti-Iba1 rabbit IgG to identify the macrophage. After perfusion and fixation, cochlear tissues were isolated and dissected without decalcification, then processed for immunohistochemical labeling. Tissue was rinsed with 0.5% Triton X-100 in PBS for 30 min and incubated at room temperature for 2 hours in blocking solution (5% normal horse serum; 1% Triton X-100 in PBS). Tissue was then incubated overnight at 37°C with anti-Iba1 rabbit IgG (Cat# ab178846, Abcam; 1:200) in antibody incubation solution (1% normal horse serum; 1% Triton X-100 in PBS). Then specimens were incubated for 2 hours with secondary antibody conjugated to AlexaFluor-647 (Cat# A21245, Invitrogen; 1:500) and phalloidin-488 (Cat# A12379, Invitrogen; 1:1,000) at 37°C. All specimens were rinsed before mounting and microscopic imaging (Olympus FV3000).

Cytokine Array Kit Analysis

A mouse cytokine array kit (R&D systems, Cat #ARY006) was used to simultaneously detect 40 mouse cytokines in LPS-treated cochlear samples, according to the manufacturer's instructions. The chemiluminescent signal on each membrane was collected using an Amersham Imager 600 (GE Healthcare Life Sciences, Pittsburgh, PA). The intensity (pixel density) of each spot on the membrane was quantified using ImageJ software, and corrected for background intensity and normalized to the positive control on the same membrane. The experimental group and the control group each contained five cochlear samples.

Statistical Analyses

Specific statistical methods were chosen based on the data being analyzed. The Student's paired *t*-test was used for GTTR fluorescence intensity analyses. The Mann–Whitney test was used for the assessment of the thickness of TM. For ABR

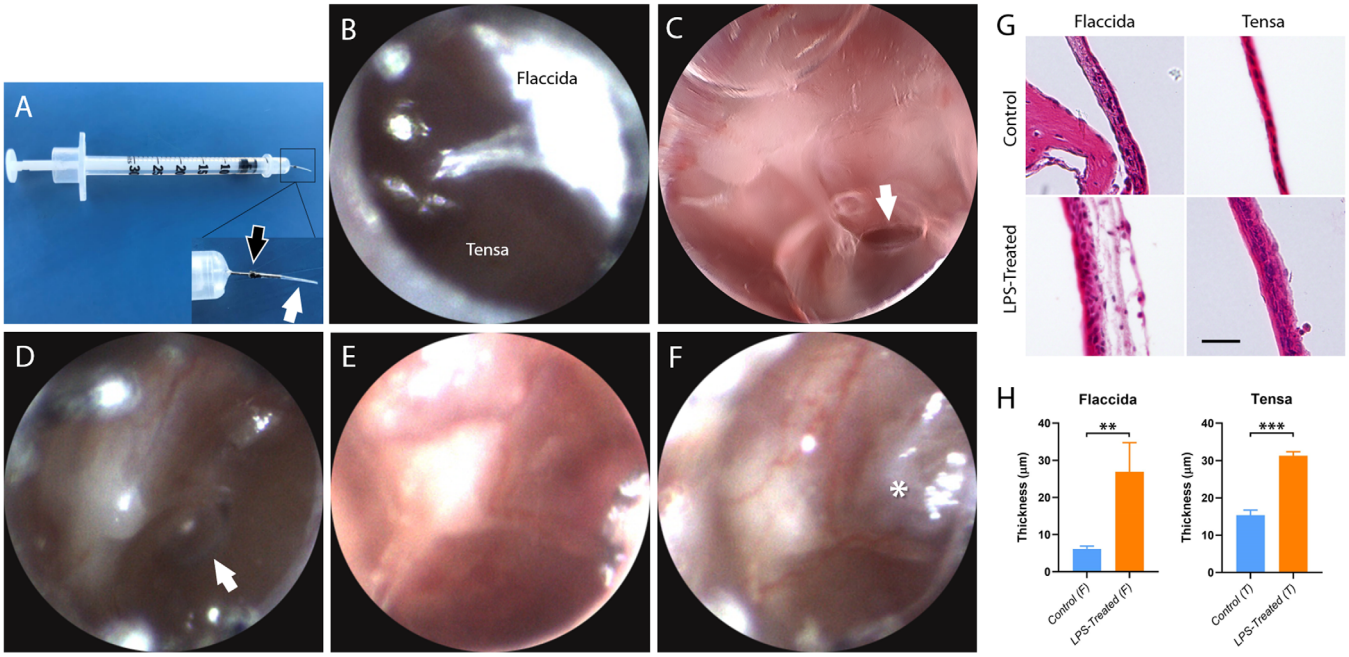

Fig. 1. Mouse model of acute otitis media by intratympanic injection of lipopolysaccharide (LPS). A, To achieve minimally invasive *i.t.* injection, a syringe needle of 31-gauge (black arrow) was connected to a fine polyurethane tube (white arrow, *o.d.* = 0.15 mm). B, Endoscopic findings of a normal tympanic membrane (TM) with Pars tensa and Pars flaccida identified. C, A puncture (arrow) was made at the anteroinferior quadrant of the mouse TM and the middle ear filled with LPS solution immediately after the *i.t.* injection. D–F, Endoscopic findings 48 hours after *i.t.* LPS injection comprised membrane opacity and middle ear effusion (D–F), air pocket (arrow in D), redness and thickening of the membrane with visible vasodilation (E, F), and pseudo-membrane (asterisk in F) at the puncture site. G, H&E staining revealed leukocyte infiltration and epithelial thickening of the TM. Middle ear cavity was located toward the right side of each panel. Error bar = 100 μm. H, Thickness comparison between LPS-treated TMs (n = 9, 10) and non-treated membranes (n = 6, 7). Mann–Whitney tests indicated significant LPS-induced thickening. $^{**}P < .01$, $^{***}P < .001$.

analyses, we used two-way analysis of variance (ANOVA) with Sidak's multiple comparisons. $P < .05$ was considered significant. Sensitivity tests were performed for statistical evaluation of the datasets based on GTTR fluorescence so as to confirm the reproducibility (Table S1).

RESULTS

Establishment of the LPS-Induced Mouse AOM Model

Intratympanic injection of LPS has been widely adopted to establish animal AOM models.[12–14] In our AOM mouse model, empirical clinical manifestations were detected by otic endoscopy as well as histological hematoxylin and eosin (H&E) staining (Fig. 1). The normal appearance of the TM was thin and transparent with light reflection (Fig. 1B). Immediately after the *i.t.* LPS injection, an aperture in the membrane could be seen, and the middle ear cavity was filled with translucent liquid (Fig. 1C). After 48 hours, the TM became congested and thickened, light reflection disappeared, the middle ear cavity was filled with turbid liquid (Fig. 1D–F), membrane perforation healed, and a healing pseudomembrane covered on the prior perforation (Fig. 1F). Microscopic images of H&E-stained samples confirmed the thickening of TM (Fig. 1G,H). The membrane thickening appeared more prominent at the inner mucosal epithelial layer facing the middle ear cavity (Fig. 1G).

LPS Treatment Enhanced GTTR Uptake by Cochlear Tissues

Three treatment groups were initially tested for 24, 48, and 72-hour post-injection, to determine the effective time of *i.t.* LPS injection on GTTR uptake, by outer hair cells (OHC) and by the SV. After LPS treatment, the SV GTTR uptake increased by 71%, 32%, and 18% in the 24, 48, and 72-hour groups, respectively (Fig. 2A). In comparison, the increase of OHC GTTR uptake was 32%, 43%, and 27%, respectively (Fig. 2B). A significant correlation existed between the SV and OHC GTTR uptake for each post-treatment time point, manifested by a significant non-zero slope of the linear regression (Figs. 2C, S1 and Table S2). Given the greatest OHC GTTR uptake enhancement, 48-hour LPS post-treatment was selected for the following experiments, and we expanded the sample size in this group to detect treatment effect in detail in terms of region-specific GTTR uptake. In all ears (n = 8) with LPS treatment for 48 hour, more intensive OHC GTTR fluorescence was observed among all three cochlear turns (Fig. 3).

As to the GTTR uptake by cochlear lateral wall tissues, a similar trend was observed. GTTR fluorescence intensity in marginal cells, intermediate cells (interstitial space), basal cells, and fibrocytes (including spiral ligament) was more intense than that of the contralateral control ear, and the increase of immunofluorescence intensity in intermediate cells was the most obvious

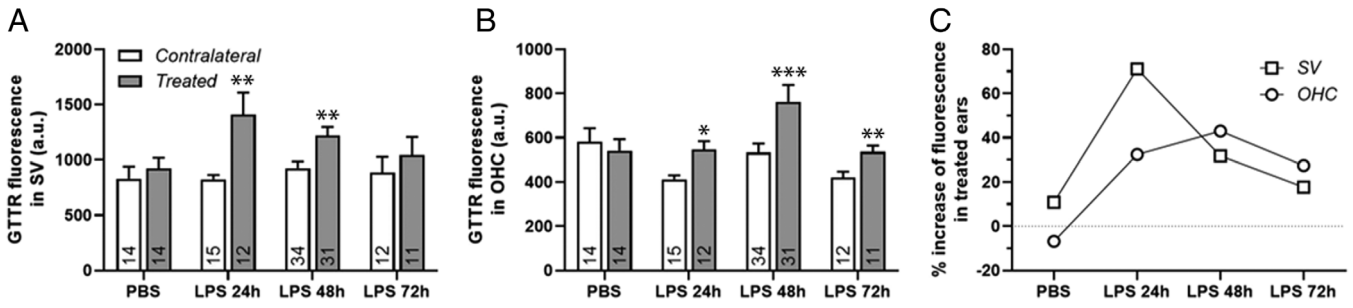

Fig. 2. GTTR uptake in the stria vascularis (SV) and by outer hair cells (OHC) from non-treated contralateral ears, or ears treated with lipopolysaccharide (LPS) or PBS. A, Compared with the contralateral control ears, GTTR uptake in the SV was significantly enhanced at all three LPS post-treatment time points, but not in the control group with *i.t.* PBS treatment. B, Similarly, GTTR uptake by the OHCs increased by 32.5%, 43.1%, and 27.4% in *i.t.* LPS groups at 24, 48, and 72 hours post-treatment, respectively. The number of examined cochlear locations was denoted in each bar, with 3–5 locations from each mouse. (PBS group, 4 mice; LPS-24 hour group, 4 mice; LPS-48 hour, 8 mice; LPS-72 hour group, 4 mice. ***$P < .0001$, **$P < .001$, *$P < .05$, and *t*-tests). C, Bilateral comparison of GTTR uptake was represented in the format of % increase of fluorescence intensity in the treated ear, for the SV and OHCs.

(Fig. 4A,B). The enhancement of GTTR fluorescence intensities in intermediate cells in the middle and basal turns and the whole cochlea including all three turn locations was significant (Fig. 4C). These results indicated the *i.t.* LPS elevated the GTTR uptake in HCs and lateral wall tissues of the cochlea.

LPS Treatment Activated Iba1-Positive Macrophages in the Fibrocyte Layer of the Lateral Wall but not That in the SV

In the untreated contralateral control ear, a certain number of Iba1-positive macrophages with ramified shape were present in the fibrocyte layer of the cochlear lateral wall (Fig. 5A; first column). Forty-eight hour after *i.t.* LPS, these immune cells were activated. The number of macrophages in the fibrocyte layer increased drastically in all three turns, and the morphology of Iba1-positive macrophages greatly altered. In brief, the change could be described by vigorous branching and enlarged cell volume (Fig. 5A; second column). As previously reported,[15] Iba1-positive macrophages were also detectable in the SV but appeared rather inactive after LPS treatment as we observed no change in either number or morphology (Fig. 5B).

LPS Treatment Also Activated Iba1-Positive Macrophages in the Organ of Corti

In the absence of LPS treatment, and distinguishable from the morphology of macrophages in the cochlear lateral wall, Iba1-positive macrophages exhibited various shapes in the organ of Corti depending on their locations. The shape of Iba1-positive macrophages in the apex is typically dendritic while macrophages in the base are predominantly amoeboid. The shape of macrophages in the middle turn is between these two extremities, typically a partially arborized shape (Fig. 6A).[16] At 48-hour post-treatment, the number of macrophages increased throughout the organ of Corti, and the apical macrophages changed from the typical dendritic shape to a more arborized shape (Fig. 6B).

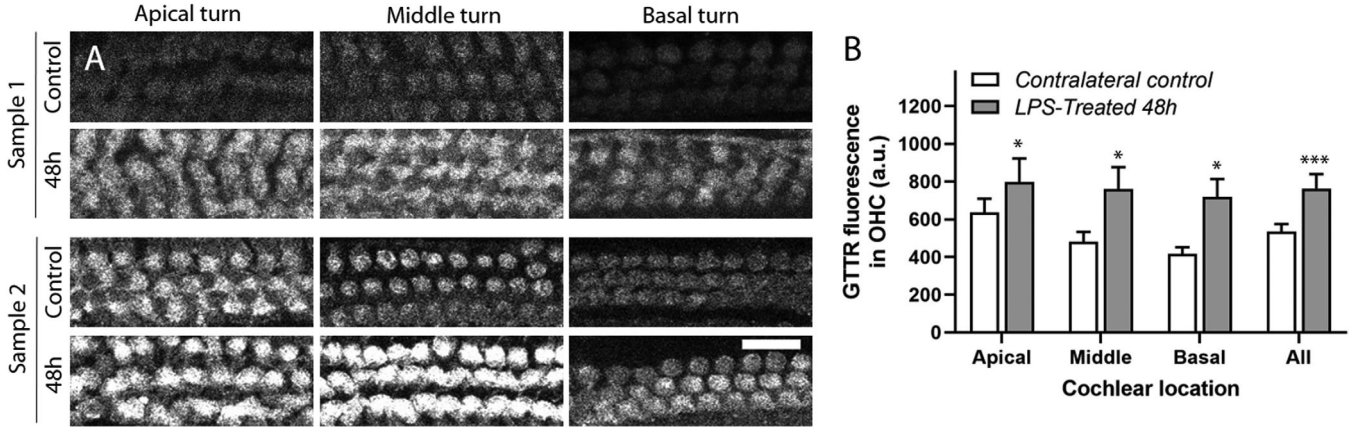

Fig. 3. Lipopolysaccharide (LPS) significantly enhanced GTTR uptake by outer hair cells (OHCs). GTTR (2 mg/kg) was injected *i.p.* 1 hour after *i.t.* LPS treatment for 48 hours. A, Representative images demonstrated increased GTTR fluorescence intensity in OHCs at various longitudinal cochlear locations, compared to the contralateral control ears without any treatment. Scale bar = 20 μm. B, The fluorescence intensity of the sub-cuticular zone of individual OHCs was scored from each confocal stack. Six stacks were imaged from each cochlea, either LPS-treated or control, with two stacks per location from the apical turn, middle turn, or basal turn. The Student's paired *t*-test indicated that *i.t.* LPS treatment exerted a statistically significant effect on GTTR fluorescence in the OHC sub-cuticular zone in all three different turn locations compared to the control ($n = 8$ mice, ***$P < .001$, *$P < .05$). Error bars, SD. a.u., arbitrary unit.

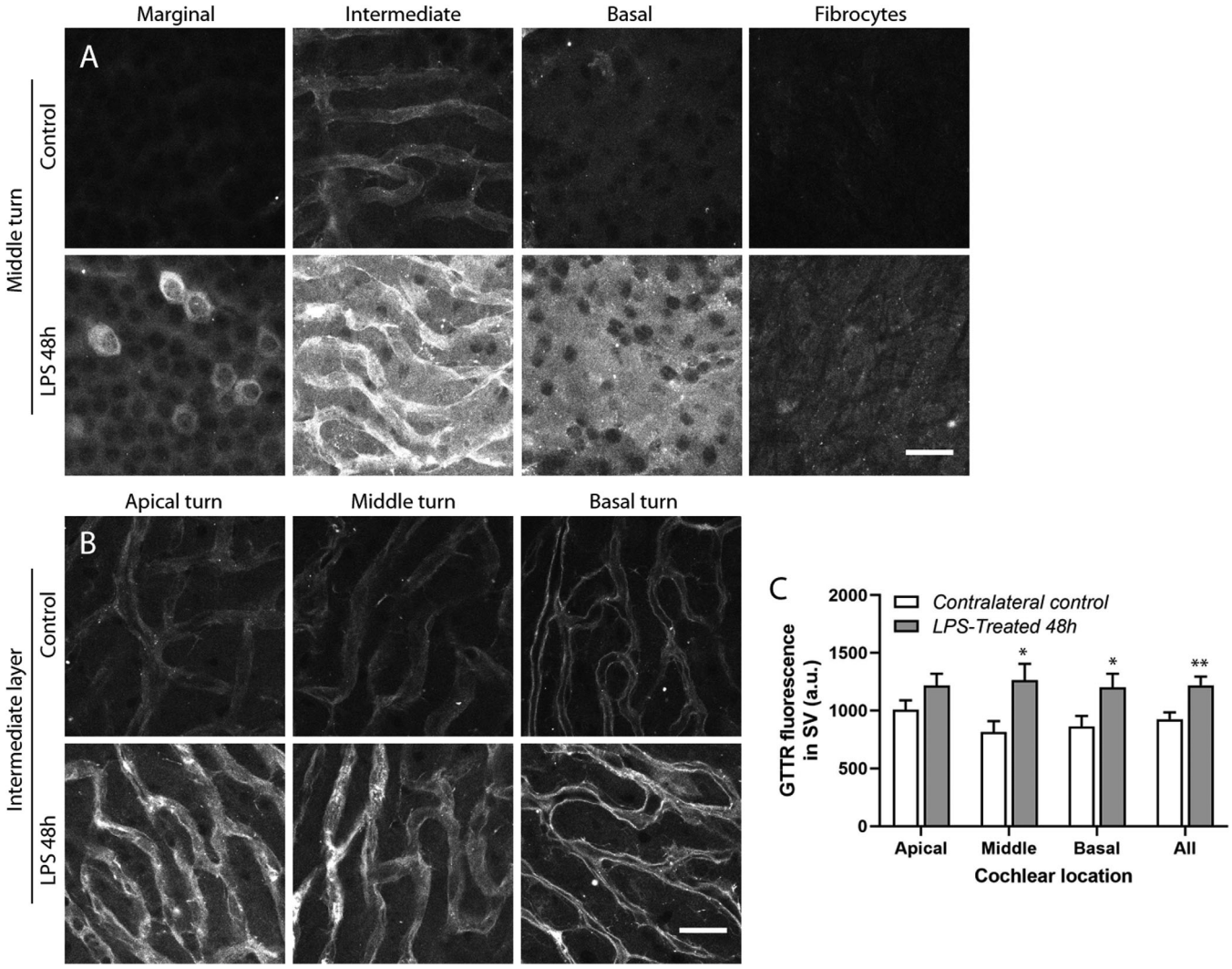

Fig. 4. Lipopolysaccharide (LPS) significantly enhanced GTTR uptake in the cochlear lateral wall. GTTR (2 mg/kg) was injected *i.p*. for 1 hour after *i.t*. LPS treatment for 48 hours. A, In wholemounts of the *i.t*. LPS-treated and control cochlear lateral wall, disparate levels of GTTR fluorescence were observed in the marginal cell layer, the intermediate cell layer, the basal cell layer, and the fibrocyte layer (spiral ligament) in the contralateral control ear (four images in the first row), among which the fluorescence intensity in the intermediate cell layer and the basal cell layer was relatively high. Compared to the control ears, GTTR fluorescence intensity was more robust in the respective cell layers in the LPS-treated ears (four images in the second row), and the increase of fluorescence intensity in the intermediate cell layer was the most noticeable. Scale bar = 20 μm. B, Increased GTTR fluorescence intensities were observed in the intermediate cell layer along the longitudinal axis from the basal turn to the apical turn in the LPS-treated ear compared to the control ear. Scale bar = 20 μm. C, GTTR fluorescence intensity was quantified from the intermediate cell layer, and significantly higher intensity was found in middle and basal turns, and in the entire cochlear combining the three turn locations in the LPS-treated ear ($n = 8$ mice, $^{**}P < .01$, $^{*}P < .05$). Error bars, SD. a.u., arbitrary unit.

LPS Treatment Induced the Change of Multiple Cytokines in Cochleae

To test if the cochlear cytokine levels also altered by *i.t*. LPS, paired whole cochlear lysates from pooled samples ($N = 7$) were processed to evaluate the cochlear cytokine profile at 48-hour post-treatment, using mouse cytokine arrays (Fig. 7A). Images of array membrane incubated with the LPS samples and the untreated controls (Fig. 7B) showing six references (A1-2, A23-24, and F1-2) exhibited the most intense immunoblotting signals, whereas the negative control (F23-24) and all the blanks (on rows E&F) exhibited undetectable signals. Seventeen cytokines were present in both LPS-treated and control samples (Fig. 7B,C). Of these, the expression of 10 cytokines, including both pro-inflammatory and anti-inflammatory, was significantly up-regulated in the LPS-treated cochlear tissues (Fig. 7C).

LPS Treatment Induced Conductive Hearing Loss

Intratympanic LPS resulted in elevated ABR thresholds at all tested frequencies. The hearing threshold increased by 11.7 to 20.8 dB at 48-hour post-treatment (Fig. 8A). The parallel threshold elevation indicated a dominant conductive hearing loss, rather than sensorineural hearing loss, in accordance with previous findings.[14,17] HC survival was evaluated according to

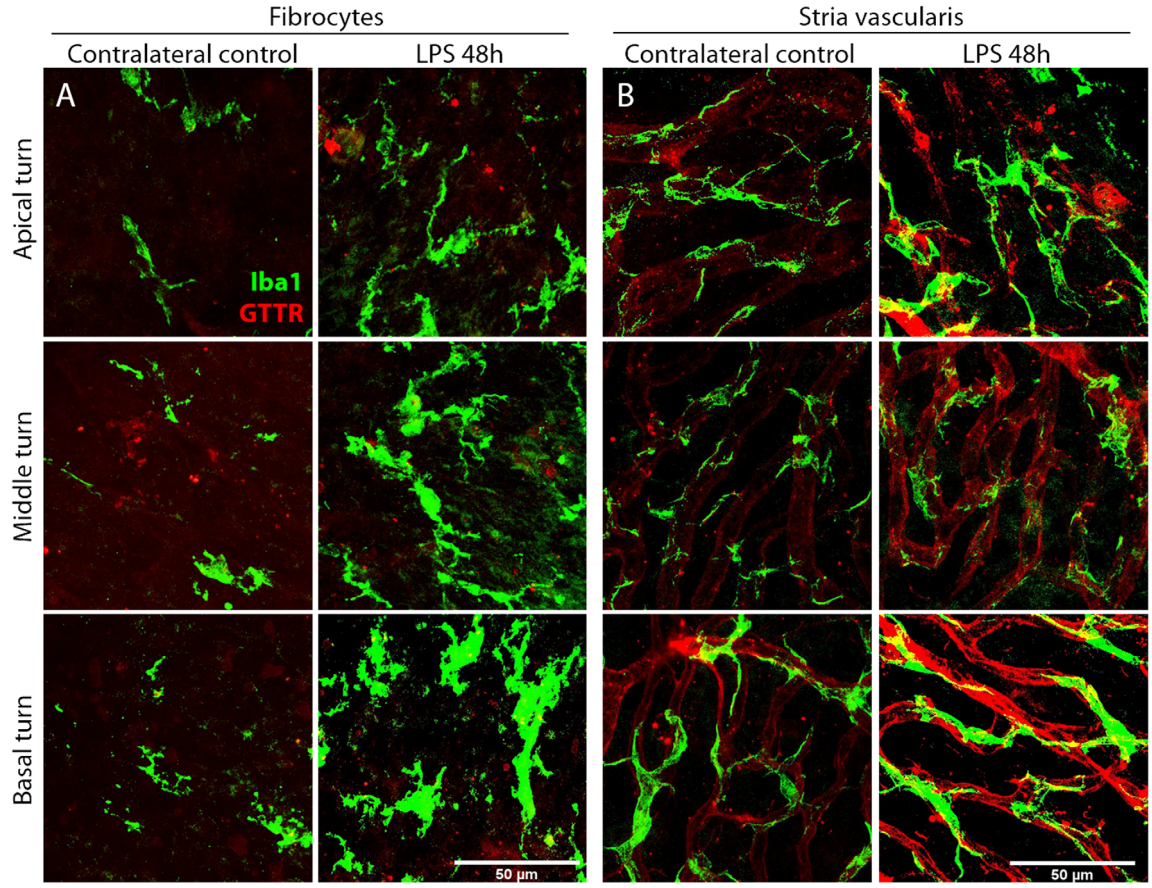

Fig. 5. Lipopolysaccharide (LPS) treatment modified Iba1-positive macrophages morphologically and numerically in the spiral ligament but not in the stria vascularis. After *i.t.* LPS treatment for 48 hours, GTTR (2 mg/kg) was administered (*i.p.*) for 1 hour prior to cochlear tissue collection including the LPS-treated ear and the contralateral control ear. GTTR fluorescence signals (red) in the cochlear lateral wall facilitated the identification of the layered anatomy. Each panel represented a z-projection confocal image through a segmented depth of the lateral wall, at various cochlear locations from the apex to the base. A, Compared to the contralateral control ear, a significant increase of Iba1-positive macrophages (green) in the spiral ligament was observed in all three turns with LPS treatment, meanwhile, the morphology of Iba1-positive macrophages also changed greatly, including enlargement and additional branching. B, In contrast, this distribution and morphology change of Iba1-positive macrophages was negligible in the SV. Scale bar = 50 μm fits all panels.

phalloidin labeling at 24, 48, and 72-hour post-injection. Consistent with the ABR result, both OHCs and inner hair cells (IHCs) largely survived and the degree of survival was comparable between LPS-treated and control ears (Fig. 8B), with the exception of 24-hour time point, at which *i.t.* LPS resulted in an OHC loss in the middle turn.

DISCUSSION

Previous studies have shown that *i.t.* LPS can cause inflammatory cell infiltration and the release of inflammatory factors in tympanum and middle ear mucosa.[12–14] Results from the present study indicate that similar inflammatory responses can also be induced in the cochlea.

OM and Sensorineural Hearing Loss

OM is arguably the most prevalent disease in humans and a major public health concern. Here, we studied the pathological responses of LPS treatment and their effect on hearing in an AOM mouse model. Our results indicated that there is no loss of OHC, up to 72 hour after LPS treatment. In contrast, the observed ABR threshold shift was an indicator of conductive hearing loss, presumably due to the middle ear effusion upon AOM. However, this does not strictly mean that AOM does not result in impairment of sensorineural function in the cochlea. As our initial focus was on the uptake of GTTR by OHCs, the experimental design inevitably included a bias toward selecting cochlear locations with intact OHC morphology. HC loss is typically considered to proceed with the damage of spiral ganglion neurons and their dendrite fibers that innervate corresponding inner HCs.[18,19] Recent work on cochlear synaptopathy suggested that this is not always true.[20–22] For instance, moderate noise exposure can result in suprathreshold hearing deficit without obvious HC loss.[21,23] Furthermore, the cochlear immune activity likely has a unique role in cochlear synaptopathy.[24,25] Thus, it is a legitimate

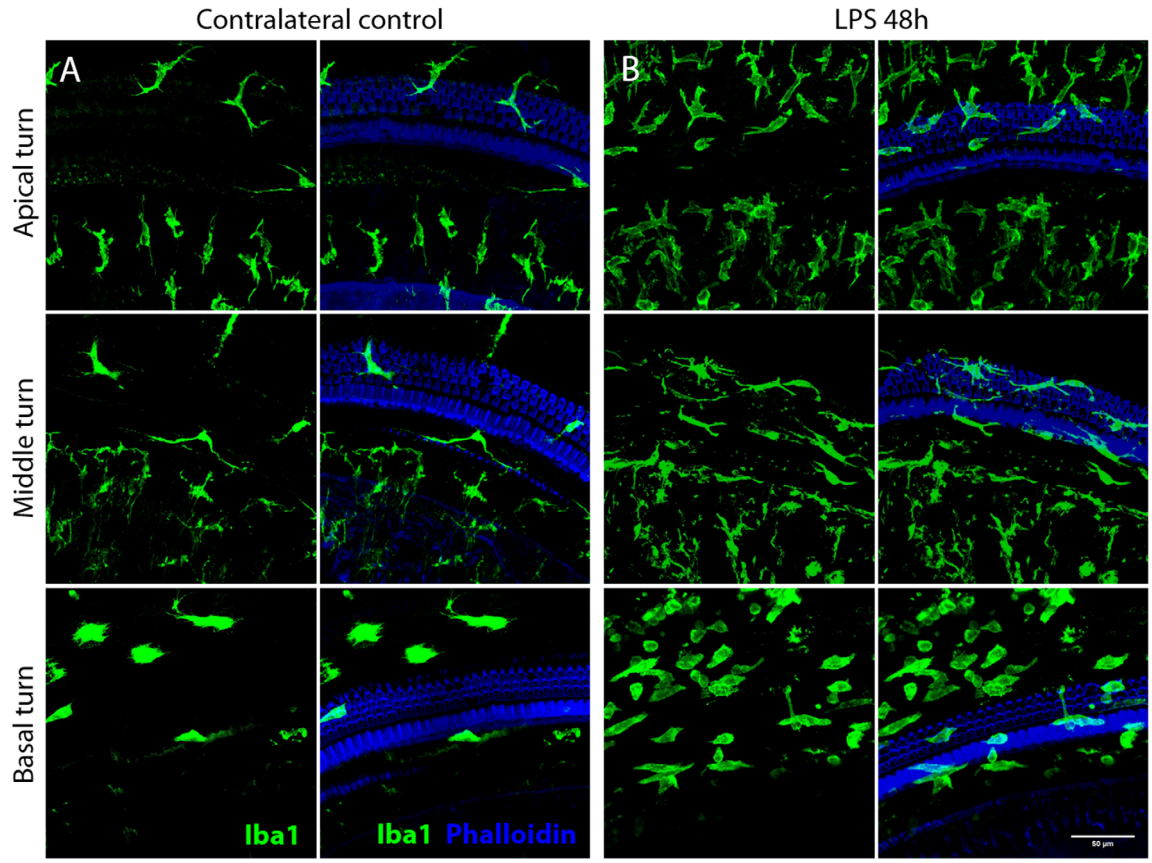

Fig. 6. Lipopolysaccharide (LPS) treatment stimulated morphological change of Iba1-positive macrophages in the organ of Corti. Cochlear tissues were collected after LPS treatment for 48 hours, then immunolabeled with anti-Iba1 antibody (green) and phalloidin (blue) to identify Iba1-positive macrophages and the structure of organ of Corti, respectively. Each panel represented a z-projection confocal image through the depth of sensory epithelium. A, In the organ of Corti of the contralateral control ear, a small number of macrophages were identified in the apical turn, the middle turn, and the basal turn. The characteristic morphology of apical macrophages was dendritic, in comparison, amoeboid shapes in the basal turn. The macrophage morphology in the middle turn was between the two extremities, exhibiting an arborized. B, After LPS treatment, the number of macrophages increased significantly in all three turns, and the apical macrophages changed from the typical dendritic shape to a more arborized form. Scale bar = 50 μm fits all panels.

question whether *i.t.* LPS could lead to synaptic damage to some extent, though subsequent work is warranted.

Although AOM did not directly cause structural damage in the inner ear, it did elevate the cochlear susceptibility to an ototoxic drug if administered during the morbidity period. The results of the present study also validated the concerns of many clinicians with respect to the potential ototoxicity when aminoglycoside drops are used to treat AOM.[10]

Activated Immune Activity in the Cochlea Under AOM

A key finding in the present study is that cochlear macrophages and inflammatory factors respond to LPS treatment. Previous findings on inner ear gene expression were reported for acute and chronic OM models using inflammatory gene arrays.[26,27] These studies together with our results suggest similar inflammatory pathways are involved in the escalated aminoglycoside ototoxicity.

In our AOM model, the boundary of the inflammatory microenvironment expanded to the cochlea from the middle ear. In the cochlea, LPS treatment altered the number and distribution of Iba1-positive macrophages, as well as their morphology in the spiral ligament and in the organ of Corti. Some macrophages changed from the typical dendritic shape to a more arborized form with an enlarged cell body. These morphological changes indicate the activation of macrophages and potential participation in nonspecific immune responses. The number and morphology of macrophages in the SV showed little modification in response to AOM, consistent with previous report.[6] Upon inflammatory stimulation, macrophages migrated from the vasculature to the spiral ligament and the spiral limbus, through capillaries embedded in these perilymph-filled regions of the mammalian cochlea.[28–30] However, the capillary environment of the SV[31–34] does not typically allow chemotactic migration of leukocytes into the intrastrial space. The number of macrophages in the SV demonstrates little variation even in conditions of severe inflammation.[14,15]

Cytokines and chemokines are extracellular signaling molecules that mediate cell–cell communication and have critical roles in many biological processes. Previous

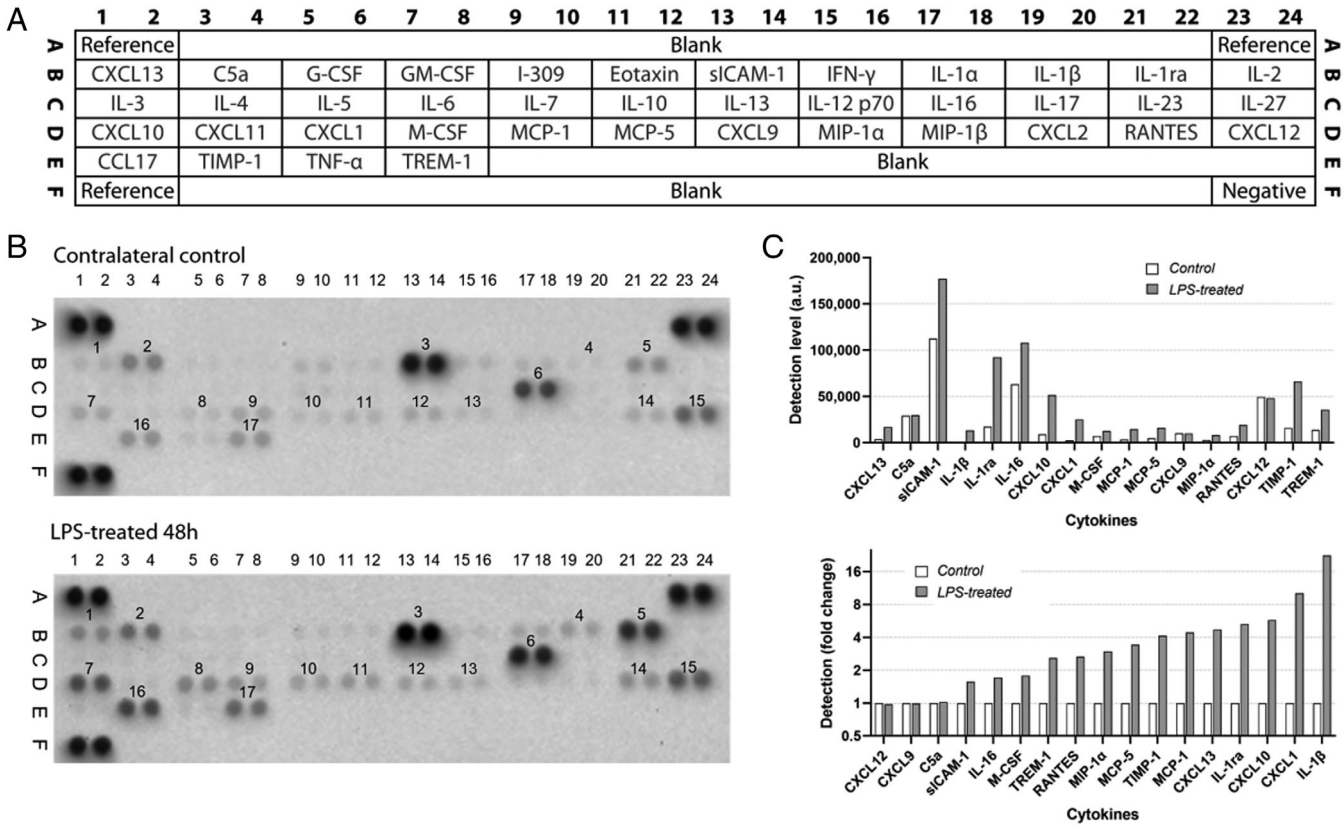

Fig. 7. Cytokine Array detection of multiple analytes in mouse cochlear tissues with *i.t.* Lipopolysaccharide (LPS) treatment. A, The alignment of 40 cytokines in duplicates on the mouse cytokine array. Reference, reference spots; Negative, negative control. B, Cytokine array blots probed with pooled, untreated contralateral control samples and *i.t.* LPS samples at 48 hours post-treatment ($N = 7$ each). Each blot represents immunoreactive labeling against the respective antibodies. The blots marked with digits are the cytokines that were clearly expressed in cochlear tissues or significantly regulated between the paired samples. 1:CXCL13; 2:C5a; 3:sICAM-1; 4:IL-1β; 5:IL-1ra; 6:IL-16; 7:CXCL10; 8:CXCL1; 9:M-CSF; 10:MCP-1; 11:MCP-5; 12:CXCL9; 13:MIP-1α; 14:RANTES; 15:CXCL12; 16:TIMP-1; 17:TREM-1. Note the absence of labeling of the negative control and blank slots. C, The detection level of marked cytokines was presented in the format of paired comparison (top) and in ranked fold change due to LPS treatment (bottom). The relative expression levels of each cytokine were determined by the average pixel intensity between the corresponding duplicates on the array, and the respective signals on the two arrays were compared after signal correction using the pixel intensity of the reference spots. Compared to the untreated control ears, the expression of various cytokines including IL-1β, CXCL1, CXCL10, IL-1ra, CXCL13, MCP-1, and TIMP-1 was significantly increased in the cochlear tissues after *i.t.* LPS treatment. a.u., arbitrary unit.

studies demonstrated that inflammatory cytokines following bacterial stimulation in the middle ear were released by resident fibrocytes,[35,36] while cochlear macrophages also release many cytokines to participate in immunity. A variety of chemokines, adhesion molecules, and other inflammation-related factors were detected previously,[37] in accordance with the increased interleukins and chemokines found in our study. It is expected that some of these cytokines activate immune cell proliferation and extend the inflammatory response, while others could be involved in processes related to inner ear tissue remodeling.

Altered Aminoglycoside Ototoxicity Under AOM

Aminoglycoside ototoxicity is a complex process occurring in a dose-dependent manner,[2] primarily affecting sensory HCs in the cochlea and vestibular labyrinth. Additional factors further predispose patients to enhanced ototoxicity, including noise exposure and individual genetic susceptibility.[38–41] Here we also observed AOM-altered aminoglycoside uptake. Modified OHC uptake of aminoglycoside in vivo or in vitro has been documented by many investigators,[5,42–45] the systemic aminoglycosides are trafficked from the strial capillaries across the SV into endolymph,[4,46,47] then cochlear HCs primarily take up aminoglycosides in endolymph across their apical membranes by mechanoelectrical transduction (MET) channels.[3,4,47–49] Aminoglycosides could also enter cells via an endocytosis pathway,[48,50–52] or via nonselective cation channels, including transient receptor potential (TRP) channels.[4,49,53–56]

Previous studies reported that systemic LPS increased the expression of acute-phase inflammatory markers in cochlear tissues, and it increased cochlear concentrations of GTTR and gentamicin.[9] This endotoxemia-induced inflammation was deemed to associate with the physiological modification of the SV and subsequently enhanced cochlear loading with gentamicin. Our results indicated that LPS-induced local acute inflammation also increased GTTR uptake in the inner ear. In the AOM model, systemic GTTR could enter the

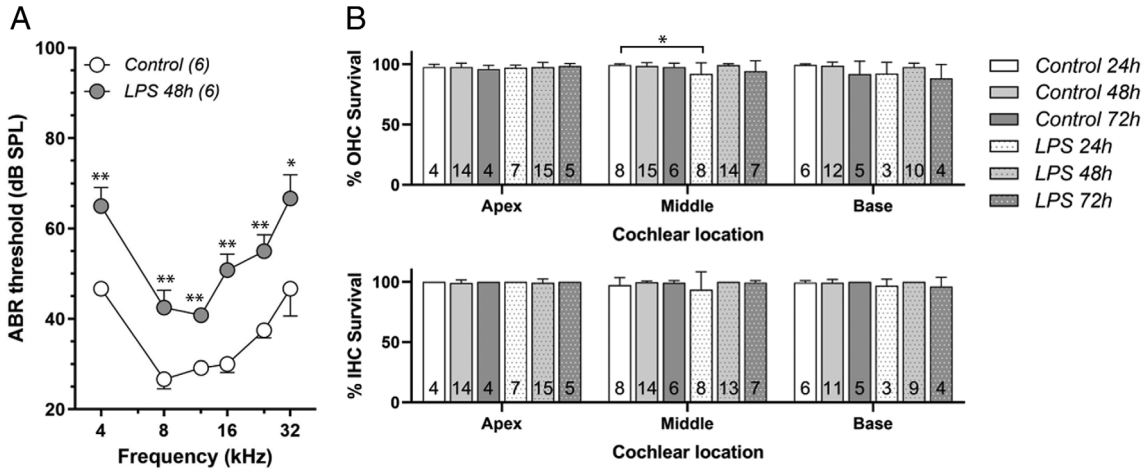

Fig. 8. Intratympanic lipopolysaccharide (LPS) injection caused conductive hearing loss. A, LPS-treated ears exhibited parallel ABR threshold elevation 48 hours later, in the degree of 15–20 dB at all tested frequencies, a characteristic indicator of conductive hearing loss. Control group, $n = 6$; LPS group, $n = 6$; $^{*}P < .05$, $^{**}P < .01$. Error bars, SD. B, Hair cell survival was not altered by *i.t.* LPS treatment examined 24, 48, and 72 hours post-treatment, suggesting the ears were free of sensorineural hearing loss. Hair cells were quantified based on phalloidin labeling along the length of the cochlea, including apex, middle, and base. LPS did not cause hair cell loss in either OHC population (top) or IHC population (bottom), except the 24 hours time point at the middle cochlear turn for OHCs (F [1,30] = 7.325, $P = .011$; Sidak's multiple comparisons test, $P = .025$). $N = 4$–8 mice per group, and 1–2 sites at each cochlear location per mouse were examined. $^{*}P < .05$. Error bars, SD.

inner ear via the inflamed, and often fluid-filled middle ear. However, the observation of the uniform increase of GTTR uptake throughout the cochlea is more consistent with a local enhancement mechanism pertaining to the blood-labyrinth barrier.

Although AOM raises the cochlear susceptibility to aminoglycosides, the direct relationship between the middle ear inflammation and the inner ear sensitivity of aminoglycosides remains unclear. We speculated that increased chemokines could directly modulate the activity of endocytosis, and sensitize the expression of selected aminoglycoside-permeant channels, such as MET and TRP channels within the cochlea.[49,54,55,57] For instance, TRPV1 expression could be upregulated in the scenario of inflammation[58–60] and translocated from the vesicular reservoir to the plasma membrane via exocytosis.[61] Besides, aminoglycoside antibiotics are commonly used by a topical approach in the context of AOM with or without tympanic perforation in the clinic setting, while the aminoglycoside analog, GTTR, was systemically administered in the present study. Given the rapid middle-ear tissue remodeling and membrane thickening upon the LPS-induced inflammation, it is not straightforward to predict how the efficacy of transtympanic aminoglycosides is modulated. Thus, further studies with local aminoglycoside treatment are warranted, possibly using a variety of OM animal models. Regardless, the results of this study suggest that the pathological cochlea is more susceptible to ototoxic aminoglycosides, providing a novel aspect in searching for ototoxicity countermeasures without compromising their beneficial anti-bacterial properties.

CONCLUSION

LPS-induced AOM rendered the cochlea more susceptible to ototoxicity from aminoglycosides. Cochlear inflammation altered the permeability of the blood-labyrinth barrier, allowing substance in the vasculature to readily enter the inner ear. Thus, the ototoxic risk is elevated in the pathological cochlea, and the clinical concern of aminoglycoside ototoxicity in the AOM treatment is justified.

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