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Endotoxemia-mediated inflammation potentiates aminoglycoside-induced ototoxicity

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Abstract

The ototoxic aminoglycoside antibiotics are essential to treat severe bacterial infections, particularly in neonatal intensive care units. Using a bacterial lipopolysaccharide (LPS) experimental model of sepsis, we tested whether LPS-mediated inflammation potentiates cochlear uptake of aminoglycosides and permanent hearing loss in mice. Using confocal microscopy and enzyme-linked immunosorbent assays, we found that low-dose LPS (endotoxemia) greatly increased cochlear concentrations of aminoglycosides and resulted in vasodilation of cochlear capillaries without inducing paracellular flux across the blood-labyrinth barrier (BLB), or elevating serum concentrations of the drug. Additionally, endotoxemia increased expression of both serum and cochlear inflammatory markers. These LPS-induced changes, classically mediated by Toll-like Receptor 4 (TLR4), were attenuated in TLR4-hyporesponsive mice. Multiday dosing with aminoglycosides during chronic endotoxemia induced greater hearing threshold shifts and sensory cell loss compared to mice without endotoxemia. Thus, endotoxemia-mediated inflammation enhanced aminoglycoside trafficking across the BLB, and potentiated aminoglycoside-induced ototoxicity. These data indicate that patients with severe infections are at greater risk of aminoglycoside-induced hearing loss than previously recognized.

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Author contributions: P.S.S., J.W.K., and Z.D.U. designed the experimental plan. J.W.K. performed all GTTR imaging and vasoactive peptide experiment in C57BL/6 mice; J.W.K. and M.J. performed aminoglycoside serum analyses. J.P.L. and J.A. performed hTR and vasodilation studies. L.Q.D. performed inflammatory marker analyses at 6 hours. Z.D.U. performed all analyses using C3H/HeOuJ and C3H/HeJ mice. J.P.L., M.J., J.J.A., C.P.C., H.L. and P.S.S. performed the ototoxicity analyses. P.S.S. and J.W.K. wrote the manuscript and reviewed all analyses. All authors reviewed and approved the manuscript.

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Introduction

Severe Gram-negative bacterial infections, including meningitis, bacteremia and respiratory infections in cystic fibrosis, are treated with aminoglycoside antibiotics like gentamicin (1–3). These drugs can induce permanent and debilitating hearing loss, particularly in neonates. Approximately 80% of 600,000 admissions into neonatal intensive care units (NICU) in the United States receive aminoglycosides each year (4). The rate of hearing loss in NICU graduates (from all etiologies) is 2–4% compared to 0.1–0.3% of full-term births from congenital etiologies (5). Aminoglycoside-induced ototoxicity could contribute substantially to this increased rate of hearing loss in the NICU population. The irreversibility of hearing loss, particularly in pediatric cases prior to language development, has extensive quality-of-life implications (6–9).

The mechanisms by which circulating aminoglycosides cross the blood-labyrinth barrier (BLB) into the cochlea remain unconfirmed. We previously reported that, *in vivo*, these drugs predominantly cross the BLB into the stria vascularis and are trafficked via marginal cells into endolymph (10). Once in endolymph, these drugs rapidly enter cochlear hair cells via mechanoelectrical transduction (MET) channels located on their apical membranes and induce hair cell death (10–13).

Serious infections induce Systemic Inflammatory Response Syndrome (SIRS), elevating serum concentrations of nitric oxide, vasoactive peptides and inflammatory proteins that can modulate the vascular permeability of the blood-brain barrier (BBB) (14–16). Vasoactive peptides also modulate cochlear uptake of gentamicin across the BLB (17). However, most studies of ototoxicity involve healthy preclinical models, and the effect of induced systemic inflammation on ototoxicity has only recently been reported (18). Here, we used bacterial lipopolysaccharide (LPS) to induce endotoxemia in a classic experimental model of sepsis and inflammation (19) in mice to test the hypothesis that systemic inflammation modulates cochlear concentrations of aminoglycosides and inflammatory markers, and exacerbates aminoglycoside-induced ototoxicity.

Results

Fluorescently-tagged gentamicin (GTTR) is an excellent tracer of gentamicin *in vivo* (10, 13, 17, 20–23). By conjugating Texas Red to gentamicin (450–477 daltons, 3 isoforms), the hydrophobicity of the resulting conjugate (1151–1179 daltons) is increased while serum pharmacokinetics are slowed, providing greater spatiotemporal imaging and a higher signal-to-noise ratio in heterogeneous cellular structures like the cochlea compared to radiolabeled aminoglycosides or immunohistochemistry (10, 13, 24–26). The cytoplasmic intensity of GTTR fluorescence is dose-dependent, unlike gentamicin immunofluorescence where the greater abundance of epitope binding sites can overwhelm the number of available antibodies (13, 20). Thus, changes in cochlear uptake of aminoglycosides can be quantitatively assayed using GTTR and confocal microscopy (13, 17, 20, 23).

Strial uptake of GTTR is enhanced by LPS-induced endotoxemia

In Dulbecco's phosphate buffered saline-treated (DPBS-treated) mice that received an intraperitoneal (*i.p.*) injection of GTTR 1 hour before fixation, intense fluorescence delineated the strial capillaries of the cochlear lateral wall, with moderate diffuse fluorescence distributed throughout the stria vascularis (marginal cells, intra-strial tissues, and basal cells; Fig. 1A, B), as previously described (13). The spiral ligament fibrocytes exhibited reduced intensities of GTTR fluorescence compared to strial cells (Fig. 1A, B and fig. S1A), also as seen previously (13).

Mice intravenously (*i.v.*) injected with 1 mg/kg LPS 24 hours prior to an *i.p.* injection of GTTR 1 hour before fixation had more intense GTTR fluorescence in all regions of the cochlear lateral wall compared to DPBS-treated mice (Fig. 1A, B). DPBS-treated or LPS-treated mice injected with hydrolyzed Texas Red (hTR) 1 hour prior to fixation displayed negligible fluorescence within the lateral wall, as previously described (13, 22).

The mean pixel intensity of GTTR fluorescence was obtained from *xy* optical sections and plotted for specific regions of interest [ROIs; marginal cells, intra-strial tissues (minus capillary structures), basal cells and spiral ligament fibrocytes] at 1 and 3 hours after GTTR injection (Fig. 1C, and fig. S1). In DPBS-treated mice, absolute fluorescence was greatest in marginal cells, with stepwise decreasing fluorescence in intra-strial and basal cells, with a significant drop in spiral ligament fibrocytes (fig. S1A, $P < 0.05$). Strial and spiral ligament fluorescence was significantly greater in basal segments compared to apical segments for each ROI (fig. S1A, $P < 0.01$).

GTTR fluorescence was dose-dependently elevated in selected cell types with increasing LPS dose (Fig. 1C, fig. S1B). At 1 hour, significant increases in GTTR fluorescence were observed in every ROI of basal lateral wall segments dosed with 1 mg/kg or higher LPS (Fig. 1C and fig. S1B, $P < 0.01$ or $P < 0.005$). A similar pattern of increased uptake was observed in the apical coil, but with more variable significance (fig. S1B, $P > 0$, $P < 0.05$, $P < 0.01$, or $P < 0.005$). At 3 hours, GTTR fluorescence in lateral wall cells continued to show dose-dependent elevations with increasing LPS dose, reaching significance at 2.5 and 10 mg/kg LPS (Fig. 1C, and fig. S1B; $P < 0.05$, or $P < 0.01$). The rank order of GTTR fluorescence intensity in individual cell types (marginal cells>intra-strial layer>basal cells>spiral ligament) was generally maintained with increasing LPS dose, with more variable degrees of significance with increasing LPS dose and time (fig. S1A). Strial GTTR fluorescence was significantly greater for each ROI in basal segments compared to apical segments in DPBS-treated or LPS-treated mice (fig. S1A, $P < 0.01$). These data indicate that LPS dose-dependently increased GTTR trafficking across the BLB, particularly in the basal coil of the cochlea, the region most associated with the onset of drug-induced hearing loss and sensory cell death (27, 28).

In cochlear outer hair cells (OHCs), LPS-induced increases in GTTR fluorescence were consistently observed at 1 hour after GTTR injection compared to DPBS-treated mice, particularly at higher LPS doses (>1 mg/kg LPS), and trended towards significance at higher doses of LPS (2.5 mg/kg) at 3 hours post-GTTR injection (fig. S2A,B). In apical coils, LPS did not consistently modulate GTTR fluorescence in OHCs 1 hour after GTTR injection,

and trended towards significance at 3 hours with higher doses of LPS (1 mg/kg). These data suggest that LPS accelerated GTTR entry into basal OHCs, and that dynamic increases in GTTR uptake kinetics at later time points, or by apical OHCs, were dissipated by the low doses of GTTR used here.

Renal function is impaired at higher doses of LPS

Endotoxemia and sepsis affect vascular function in multiple organ systems, and can induce decreased glomerular filtration rates and renal dysfunction (29). Since GTTR is readily taken up by renal proximal tubules (21, 23), decreased glomerular filtration rates and renal dysfunction should reduce proximal tubule uptake of GTTR. As an internal control, we assessed renal GTTR fluorescence intensities during endotoxemia. In DPBS-treated mice, 1 or 3 hours after GTTR injection, GTTR fluorescence was localized as intense puncta close to the lumen, and as diffuse fluorescence in the cytoplasm of proximal tubule cells, while distal tubule cells had visibly less diffuse fluorescence and no puncta (fig. S3A), as seen previously (21). In LPS-treated mice, diffuse GTTR fluorescence within proximal tubule cells at 1 hour was significantly reduced only at 10 mg/kg LPS compared to DPBS-treated control tissues (fig. S3C; $P<0.001$). At 3 hours, cytoplasmic fluorescence in proximal tubule cells of DPBS-treated mice was markedly increased compared to that at 1 hour (fig. S3C) due to longer exposure. However, at 3 hours, only mice treated with 10 mg/kg LPS had significantly less cytoplasmic GTTR fluorescence compared to DPBS-treated mice (fig. S3; $P<0.05$), as expected during endotoxemic shock (29).

Low dose LPS increases cochlear but not serum concentrations of aminoglycosides

Given that 10 mg/kg LPS can decrease both glomerular filtration rates (29) and renal uptake of GTTR (fig. S3), we assessed serum concentrations of GTTR 24 hours after LPS administration using immunoturbidimetry. Serum concentrations of GTTR were significantly higher in mice dosed with 2.5 and 10 mg/kg LPS than in DPBS-treated mice at 1 or 3 hours after GTTR injection (Fig. 2A; Tables S1 and S2, $P<0.01$ and $P<0.05$, respectively). Serum concentrations of GTTR in mice dosed with 0.1 and 1 mg/kg LPS were not statistically different from controls at 1 or 3 hours of GTTR exposure (Fig. 2A; Table S2). Serum concentrations of GTTR in mice dosed with 2.5 mg/kg LPS were significantly higher than in 0.1 and 1 mg/kg LPS-treated mice at both 1 and 3 hour time points (Fig. 2A; Table S2, $P<0.05$ and $P<0.01$, respectively). To verify that serum concentrations for unconjugated gentamicin were not altered by exposure to 1 mg/kg LPS for 24 hours, we used enzyme-linked immunosorbent assays (ELISA). Both GTTR and gentamicin serum concentrations were unchanged in LPS-treated mice compared to DPBS-treated mice at 1 and 3 hour time points after drug injection (Fig. 2B, D). Serum gentamicin was lower at 3 hours after injection, while GTTR has a longer serum half-life, as described previously (13). In contrast, cochlear concentrations of both GTTR and gentamicin were significantly increased in LPS-treated mice compared to DPBS-treated mice at both 1 and 3 hours after injection (Fig. 2C, E, $P<0.05$).

Six mice at higher LPS doses died within 24 hours after LPS injection (Table S1), as predicted (30). However, 1 mg/kg LPS was not fatal, as expected (30), yet induced acute weight loss (fig. S4A) associated with endotoxemia and sepsis (31). Given that 1 mg/kg LPS

did not increase serum concentrations, yet elevated cochlear concentrations, of GTTR and gentamicin, we used 1 mg/kg LPS for subsequent experiments.

Low dose LPS does not increase paracellular flux across the BLB

Endotoxemia can change the volume of distribution for drugs, including gentamicin (32). Hydrolyzed Texas Red (hTR; a.k.a. sulforhodamine 101; molecular mass: 679) is a membrane-impermeant fluorophore (33, 34). We used hTR to test whether exposure to LPS for 24 hours enhanced paracellular flux across the BLB into the interstitial spaces of the stria vascularis and spiral ligament (35), using neonatal (P6) mice with an immature BLB as a positive control (21, 36). In P6 mice treated with the hTR for 1 hour, fluorescence was distributed throughout stria tissues, with weaker intensities in stria marginal and basal cells (Fig. 3A, fig S5A). The spiral ligament of P6 mice exhibited less fluorescence compared to stria tissues (fig. S5A, $P<0.01$). In adult DPBS-treated and LPS-treated mice exposed to hTR for 1 hour, significantly less fluorescence was observed in stria cells and spiral ligament fibrocytes compared to P6 mice (Fig. 3A, fig. S5A, $P<0.05$ or $P<0.005$). No statistical differences in hTR fluorescence could be observed between DPBS-treated and LPS-treated adult mice for any ROI (Fig. 3A; fig. S5A).

Morphometry of cochlear lateral wall tissues revealed that the diameters of stria capillaries in P6 pups were visibly and significantly larger than in DPBS-treated adult mice (Fig. 3B, C, E; Table 1, $P<0.0001$). LPS treatment significantly vasodilated a subset of both apical and basal stria capillaries in adult mice compared to DPBS-treated mice (Fig. 3D, E, fig. S5C; Table 1, $P<0.0001$), resulting in a bimodal distribution of capillary diameters indicative of acute inflammation. Intriguingly, fixed stria capillary diameters may underestimate actual physiological diameters (37).

These data indicate that the BLB was relatively impermeable to hTR in adult mice compared to the immature and more permeable BLB of neonatal pups, as has been found for another fluorophore, FM1-43 (38, 39). Furthermore, the paucity of hTR flux across the BLB of adult mice was not altered by LPS treatment, suggesting that the physical integrity of the adult BLB was retained despite LPS-induced vasodilation of stria capillaries.

Low dose LPS induces robust pro-inflammatory marker expression

We previously reported that two inflammatory-mediated vasoactive peptides – histamine and serotonin – can modulate cochlear uptake of GTTR (17). Twenty-four hours after LPS injection, serum histamine concentrations were not modulated, while serum serotonin concentrations were significantly decreased in a dose-dependent manner in LPS-treated mice (Table 2, fig. S6, $P<0.05$ or $P<0.001$). This suggested that the loss of vasoconstrictive serotonin is associated with stria vasodilation and potentially increased stria vascular permeability to aminoglycosides.

LPS binds to and activates Toll-like Receptor 4 (TLR4) to induce the secretion and transcription of acute phase inflammatory cytokines and chemokines that orchestrate immune responses (40, 41). Given that LPS enhanced cochlear uptake of aminoglycosides, we assessed whether LPS modulated serum and cochlear expression of acute phase

inflammatory markers. Six hours after injection, LPS significantly elevated serum concentrations of all acute phase pro-inflammatory proteins tested: TNF α , IL-1 α , IL-1 β , IL-6, IL-8 (a.k.a. KC, CXCL1), MIP-1 α and MIP-2 α (Fig. 4A). In cochlear homogenates, LPS significantly elevated protein concentrations of all early phase pro-inflammatory markers, particularly IL-6 and IL-8, but not IL-1 β (Fig. 4B). Interestingly, LPS significantly elevated the anti-inflammatory cytokine IL-10 in serum, but not in vascular-perfused cochlear homogenates (Fig. 4A, $P < 0.05$, and 4B, respectively). Exposure to gentamicin for 3 hours did not modulate serum or cochlear inflammatory protein concentrations (Fig. 4A, B), indicating that gentamicin (at the doses used here) was not a confounding factor. Overall, LPS-induced changes in the serum concentrations of acute phase pro-inflammatory proteins were reflected in cochlear homogenates, except for IL-1 β and the anti-inflammatory cytokine IL-10 (Fig. 4A, B).

Twenty-four hours after LPS injection in C57BL/6 mice, plasma concentrations of most tested inflammatory proteins were not elevated (or were below the limit of detection; fig. S7A), consistent with previous findings (42). Although IL-6 and MIP-1 α concentrations remained significantly elevated compared to DPBS-treated controls, they were substantially lower compared to the 6 hour time point (Fig. 4A, fig. S7A). In cochlear homogenates of C57BL/6 mice 24 hours after LPS injection, concentrations of all tested inflammatory proteins were significantly elevated, particularly IL-6, IL-8 and MIP-1 α (Fig. 5A, fig. S7D). These findings suggested that LPS triggers robust inflammatory responses both systemically and in the cochlea within 6 hours. Furthermore, 24 hours after LPS injection, cochleae continued to exhibit significantly elevated concentrations of tested inflammatory proteins (Fig. 5A), akin to that induced by middle ear administration of LPS or bacteria (43, 44).

To determine if increased cochlear inflammatory protein concentrations were due to local (parenchymal) gene transcription, mRNA levels were assayed 6 or 24 hours after LPS injection (with or without gentamicin). At 6 hours, LPS significantly increased (5-fold) mRNA expression for all 8 inflammatory genes tested, particularly *Il-6*, *Il-8* and *Mip-1 α* (Fig. 4C). Gentamicin alone did not modulate cochlear mRNA expression of any inflammatory genes tested (Fig. 4C). Twenty-four hours after LPS injection, cochlear mRNA levels remained significantly elevated for most inflammatory genes tested, except *Tnfa* and *Il-1 β* (fig. S7G), with greater increases for later-expressing acute phase cytokines: *Il-6*, *Il-8* and *Mip-2 α* (Fig. 5B, fig. S7G). These data show that LPS challenge upregulated cochlear mRNA transcription for inflammatory proteins within 6 hours, with sustained mRNA transcription for later-expressing acute phase inflammatory proteins at 24 hours, as in other organs during endotoxemia including the liver, lung (41), brain (45) and eye (46).

Reduced LPS-mediated inflammation in TLR4-hyporesponsive C3H/HeJ mice

The C3H/HeJ mouse strain is homozygous for an inactivating short-nucleotide polymorphism in TLR4, resulting in greatly attenuated inflammatory responses to LPS exposure (47). Twenty-four hours after LPS injection, plasma concentrations of all tested inflammatory proteins in C3H/HeJ mice, and the control C3H/HeOuJ mouse strain, were not different from those in DPBS-treated mice of the same strain (fig. S7A–C). Unlike control

C3H/HeOuJ mice, C3H/HeJ mice did not experience significant weight loss (fig. S4B, $P<0.001$).

LPS significantly elevated cochlear homogenate concentrations of all acute phase pro-inflammatory proteins 24 hours after injection in control C3H/HeOuJ and C57BL/6 mice compared to DPBS-treated mice of the same strains (Fig. 5A and fig. S7D–F; $P<0.05$). Several inflammatory proteins (TNF α , IL-6, IL-8, MIP-1 α and MIP-2 α) were more elevated in C57BL/6 mice compared to C3H/HeOuJ mice after LPS treatment (Fig. 5A). Crucially, several, mostly later-expressing acute phase inflammatory proteins (IL-1 α , IL-6, IL-8, and MIP-1 α) were significantly attenuated in cochlear homogenates of LPS-treated C3H/HeJ mice compared to LPS-treated C3H/HeOuJ and C57BL/6 mice (Fig. 5A; $P<0.05$).

Similar trends were seen with cochlear mRNA levels for tested inflammatory genes for both C3H/HeOuJ and C3H/HeJ mice after LPS treatment. LPS significantly increased cochlear mRNA expression of most inflammatory markers tested (not *Il-1 β*) in C3H/HeOuJ mice, particularly later-expressing acute phase pro-inflammatory cytokines: *Il-6*, *Il-8*, *Mip-1 α* and *Mip-2 α* (fig. S7H). In contrast, LPS did not modulate cochlear mRNA expression for tested pro-inflammatory markers in C3H/HeJ mice (fig. S7I). When mRNA expression was compared between strains, there was attenuated mRNA expression for later-expressing acute phase pro-inflammatory cytokines (*Il-8*, *Mip-1 α* and *Mip-2 α*) in LPS-treated C3H/HeJ cochleae compared to LPS-treated C57BL/6 and C3H/HeOuJ mice, and also for *Il-6* compared to LPS-treated C57BL/6 mice (Fig. 5B). mRNA expression of early phase pro-inflammatory genes (*Tnf α* , *Il-1 α* and *Il-1 β*) was weakly or not significantly elevated in all three mouse strains. Interestingly, mRNA for the anti-inflammatory cytokine *Il-10* was transcribed at significantly higher levels in C3H/HeJ mice compared to control C3H/HeOuJ or C57BL/6 mice (Fig. 5, fig. S7G–I, $P<0.05$). These data demonstrate that cochlear expression of acute phase inflammatory markers was upregulated in LPS-treated control C3H/HeOuJ and C57BL/6 mice, and this upregulation was attenuated for later-expressing inflammatory markers in TLR4-hyporesponsive C3H/HeJ mice 24 hours after LPS injection (Fig. 5).

Cochlear lateral wall uptake of GTTR is attenuated in endotoxemic C3H/HeJ mice

Given that LPS-mediated inflammatory responses are attenuated in TLR4-hyporesponsive C3H/HeJ mice, we hypothesized that LPS-enhanced GTTR uptake would also be attenuated in the cochlear lateral wall of TLR4-hyporesponsive C3H/HeJ mice. We injected C3H/HeJ and control C3H/HeOuJ mice with LPS, and 24 hours later injected GTTR. We found significantly enhanced GTTR fluorescence in strial cells and fibrocytes of LPS-treated C3H/HeOuJ mice compared to that in DPBS-treated C3H/HeOuJ mice (Fig. 6, fig. S8A, $P<0.05$). LPS also significantly enhanced GTTR fluorescence in strial cells (but not fibrocytes) in LPS-treated C3H/HeJ mice compared to that in DPBS-treated C3H/HeJ mice (Fig. 6, fig. S8B, $P<0.05$). Crucially, however, LPS-enhanced GTTR uptake was significantly attenuated ($P<0.05$) in marginal cells, intermediate cells and fibrocytes, with a downward trend in basal cells, in TLR4-hyporesponsive C3H/HeJ mice compared to control C3H/HeOuJ mice (Fig. 6). The residual expression of inflammatory markers and cochlear uptake of GTTR in C3H/HeJ mice was likely due to activation of other innate immune system receptors, such as

TLR2 (48). These data indicate that TLR4-mediated inflammation mediated (at least in part) LPS-enhanced cochlear uptake of GTTR.

LPS significantly dilated a subset of stria capillaries in C3H/HeOuJ mice compared to DPBS-treated C3H/HeOuJ mice, as we observed for C57BL/6 mice (Fig. 3E,F; Table 1, $P<0.0001$). Endotoxemia also significantly dilated a smaller subset of stria capillaries in TLR4-hyporesponsive C3H/HeJ mice compared to DPBS-treated C3H/HeJ mice (Fig. 3G; Table 1, $P<0.0001$). However, the degree of capillary dilation in LPS-treated C3H/HeJ mice was significantly attenuated compared to LPS-treated C3H/HeOuJ mice (Fig. 3G; Table 1, $P<0.0001$).

Repeated low dose LPS exposure exacerbates kanamycin ototoxicity

Since LPS-induced endotoxemia increased the cochlear uptake of aminoglycosides, we hypothesized that chronic exposure to LPS would exacerbate aminoglycoside-induced ototoxicity, as determined by auditory brainstem response (ABR) threshold shifts. Given that chronic dosing with gentamicin is systemically lethal to mice, we used a related aminoglycoside – kanamycin – with a well-established protocol (27). One group of C57BL/6 mice received 1 mg/kg LPS the day before kanamycin treatment, and on the 5th and 10th day during a 14-day course of kanamycin treatment (700 mg/kg, twice daily). ABR thresholds were obtained from age-matched mice 1, 10 and 21 days after kanamycin treatment, and shifts from pre-treatment thresholds were determined (Fig. 7A,B; figs. S9–S11; tables S3–S5). At all timepoints, DPBS-treated and LPS-treated mice had negligible threshold shifts. One day after chronic dosing, kanamycin induced significant threshold shifts at 16, 24, and 32 kHz compared to DPBS-treated and LPS-treated mice (fig. S11A; table S3), as expected (27). Endotoxemia exacerbated the frequency range of kanamycin-induced threshold shifts (12–32 kHz) compared to age-matched control mice without kanamycin treatment (fig. S11A, table S3).

The 3-week recovery timepoint is a well-established primary benchmark for many preclinical ototoxicity studies (27, 49, 50). At 21 days post-treatment, kanamycin-treated mice exhibited a significant permanent threshold shift (PTS) only at 32 kHz compared to DPBS-treated and LPS-treated mice. Mice that received both LPS and kanamycin had greater and more significant PTS at 16, 24 and 32 kHz compared to kanamycin-treated mice or mice treated with DPBS or LPS alone (Fig. 7A; table S5). Mice receiving both LPS and kanamycin also had significant PTS at 12 kHz compared to mice treated with DPBS or LPS alone and at 8 kHz compared to mice treated with LPS alone mice (Fig. 7A; table S5). During the 3-week recovery period, kanamycin-induced threshold shifts did not change significantly at individual frequencies between timepoints (figs. S11A–C). However, there was a loss of significant threshold shifts at 16 and 24 kHz in kanamycin-treated mice at 10 and 21 days post-treatment compared to DPBS-treated and LPS-treated mice that were present 1 day post-treatment (figs. S11A–C; tables S3–5). This likely reflected the onset of slight, non-significant threshold shifts in DPBS-treated and LPS-treated mice at 10 and 21 days post-treatment (figs. S11A–C). Endotoxemia, however, increased the degree of kanamycin-induced threshold shifts at 21 days, and over a wider frequency range (8–32 kHz) compared to 1 day post-treatment (figs. S11A–C; tables S3 and S5). These increased

threshold shifts trended towards significance at higher frequencies, and reached statistical significance at 32 kHz ($P<0.05$). Thus, endotoxemia significantly exacerbated kanamycin-induced PTS.

Drug-induced PTS and sensory hair cell death occur in a dose-dependent fashion (27). To verify whether endotoxemia exacerbated kanamycin-induced hair cell death, we obtained cytochleograms of cochlear hair cell survival at 21 days post-treatment (Fig. 7B, table S7, S9). DPBS-treated and LPS-treated mice had minimal hair cell loss, mostly at the extreme basal (very high frequency) region of the cochlea. Kanamycin induced OHC loss over a wider frequency range in basal cochlear locations, but this was not significant compared to age-matched control mice. LPS-induced endotoxemia significantly enhanced kanamycin-induced OHC loss, and over a wider frequency range (8–64 kHz), compared to non-endotoxemic mice. These data indicate that endotoxemia exacerbated kanamycin-induced OHC death.

We then used hTR to test whether chronic LPS and kanamycin exposure altered the flux of membrane-impermeant hTR across the BLB. Significant threshold shifts were present immediately after chronic treatment with kanamycin, with or without LPS, at 16, 24, and 32 kHz compared to DPBS-treated and LPS-treated mice (figs. S11D, S12; table S6). Cytochleogram data revealed significantly greater OHC loss in the basal (32–64 kHz) regions of LPS+kanamycin-treated mice compared to all other groups (fig. S11E; table S8). Nonetheless, no differences in hTR fluorescence intensity were detected in stria cells or spiral ligament fibrocytes between LPS+kanamycin-treated mice and control DPBS-treated or chronic LPS-treated adult mice (fig. S11F). However, all cochlear ROI in adult mice had significantly less fluorescence compared to P6 mice (figs. S5B, S11F). Thus, neither chronic endotoxemia nor chronic kanamycin treatment that induced auditory threshold shifts and OHC loss increased the flux of hTR across the adult BLB.

In endotoxemic mice, there was more kanamycin-induced OHC loss, over a wider frequency range, at 3 weeks compared to that immediately post-treatment (Fig. 7B, fig. S11E; table S9). There was also more OHC loss in mice treated with kanamycin only in the 16–32 kHz frequency region, corresponding to the PTS in this region 3 weeks post-treatment compared to that immediately post-treatment (Fig. 7, fig. S11D, E; table S9). These increasing losses of OHCs during the recovery period after ototoxic insult have been described previously (51). LPS-treated and DPBS-treated mice also had significant OHC loss in the 32–64 kHz region compared to 3 weeks earlier (table S9) that was attributed to the onset of strain-specific age-related hearing loss in these 11-week old mice (52). Nonetheless, kanamycin-induced OHC loss in endotoxemic mice greatly exceeded these age-related losses (Fig. 7B, tables S7, S9).

Acute LPS-induced endotoxemia does not alter ABR thresholds

Transtympanic injection of LPS can induce ABR threshold shifts in a dose-dependent manner (53, 54). Systemic LPS (0.5 mg/kg/day for 2 days) did not change ABR thresholds or endolymphatic potentials (18). Since chronic LPS alone did not induce ABR threshold shifts at 6, 16 or 27 days after the last LPS injection (Fig. 7; figs. S9–S11), we tested whether acute systemic LPS exposure modulated auditory thresholds. Twenty-four hours

after injection with 1 mg/kg LPS, no significant threshold shifts were observed within, or between, DPBS-treated or LPS-treated groups (fig. S13). The persistence of sensitive auditory function during acute LPS challenge suggests that the physical integrity of the BLB remained intact, since physical disruption of the BLB is thought to impair sensitive cochlear performance (35).

Discussion

The easy availability and low cost of aminoglycosides contributes to their frequent use worldwide (28). Clinical use of aminoglycosides is limited due to the risk of acute nephrotoxicity, and more critically, permanent hearing loss. The risk of ototoxicity is proportional to dose and duration of aminoglycoside therapy (28). Additional factors predisposing patients to aminoglycoside-induced ototoxicity include age, renal dysfunction, mitochondrial mutations, and concurrent exposure to other ototoxic drugs (like loop diuretics) or noise (55–60).

We used LPS-induced inflammation as a model for aminoglycoside pharmacotherapy of severe Gram-negative bacterial infections. LPS binds to ubiquitous TLR4 receptors to initiate immune response signaling cascades (40, 41). Lysis of bacteria by aminoglycosides and immune cells releases LPS and other bacterial ligands into the interstitial and vascular fluids, potentiating the inflammatory response (the Jarisch-Herxheimer reaction) (61, 62).

We found that low doses of LPS (1 mg/kg) significantly increased the expression of acute phase inflammatory markers in serum, plasma and cochlear tissues, mimicking low-grade sepsis. Furthermore, acute or chronic endotoxemia did not modulate the paracellular flux of membrane-impermeant hTR across the BLB, nor attenuate cochlear function. Nonetheless, LPS-induced endotoxemia increased cochlear concentrations of GTTR and gentamicin, without modulating the serum concentrations of these compounds. Simultaneous exposure to chronic endotoxemia and kanamycin significantly increased PTS and OHC death compared to age-matched mice treated with kanamycin, LPS or DPBS alone.

Endotoxemia and cochlear inflammation

Until recently, the cochlea had been considered an immunologically-privileged compartment (63). Here, we show that endotoxemia can elevate cochlear expression of inflammatory markers. Although vascular inflammatory proteins could potentially be trafficked across the BLB, significantly elevated serum concentrations of IL-1 β and IL-10 were not reflected in cochlear tissues, implying that the BLB is not passively permeable to serum inflammatory proteins. Endotoxemia also significantly increased cochlear mRNA expression of inflammatory proteins. A similar parenchymal response to endotoxemia has been observed in ocular, pulmonary and cerebral tissues (42, 45, 46). The cochlear expression of inflammatory markers was greatly attenuated in LPS-treated mice with hyporesponsive TLR4, as was stria vasodilation and GTTR uptake. Thus, endotoxemia-induced inflammation appears to be associated with changes in BLB physiology that enhanced cochlear loading with gentamicin.

Since cochleae are pooled from several mice to determine cochlear inflammatory marker protein concentrations and mRNA levels, it was not possible to correlate any potential tonotopic gradient in inflammatory protein expression with GTTR uptake, auditory threshold shifts and OHC loss in the basal regions of the cochlea. To accomplish this correlative analysis will require development of more sensitive biochemical or quantitative immunofluorescence techniques.

Endotoxemia alone did not induce ABR threshold shifts, corroborating a recent study showing that low dose LPS exposure has little effect on endolymphatic potentials and auditory thresholds (18). Disruption of the physical integrity of the striaal BLB is thought to elevate ABR thresholds (35). This implies that the BLB remained physically intact, even though endotoxemia enhanced aminoglycoside trafficking across the BLB.

Trafficking across the BLB

Several mechanisms have been proposed for the trafficking of small compounds like aminoglycosides across tight junction-coupled endothelial barrier layers, like the blood-brain barrier and the BLB (see fig. S14). Paracellular flux across the BLB is not thought to occur under normal physiological conditions (10, 64), but may arise during inflammation, as has been reported for the blood-brain barrier (15). Pathophysiological opening of paracellular routes can occur by immune cell-dependent and immune cell-independent mechanisms (65). In our panel, IL-8 and MIP-2 α , strong chemotactic signals for immune cell recruitment and diapedesis into the parenchyma beyond the blood vessels (66, 67), were highly elevated. Immune cells are capable of secreting cytotoxic molecules that disrupt tight junctions between adjacent endothelial cells, opening paracellular trafficking routes (68). Alternatively, the tight junction-coupling between adjacent endothelial cells could break down independently of immune cell activity allowing paracellular flux through disrupted endothelial tight junctions (69). Although we did not use markers for immune cell-mediated injury or electron-dense tracers, the lack of hTR flux into the intra-striaal tissues of adult mice contraindicated a major contribution by paracellular trafficking during acute endotoxemia. Furthermore, chronic endotoxemia with or without chronic kanamycin treatment did not increase hTR flux across the adult striaal BLB, corroborating analogous experiments with mannitol following chronic ototoxic drug treatment (64).

Unlike membrane-impermeant hTR, GTTR rapidly traversed the adult BLB into striaal tissues, and entered cochlear hair cells within 30 minutes (10, 13, 21), suggestive of transcellular trafficking across the BLB. This could occur via several mechanisms characterized in other cell systems. Aminoglycosides and GTTR can permeate through non-selective cation channels, including the MET channel expressed by hair cells (11, 12), and TRPV4 channels expressed by endothelial cells (70, 71). The sodium-glucose transporter-2 (SGLT2) traffics aminoglycosides into cells, and facilitates aminoglycoside-induced cytotoxicity (72). LPS treatment can upregulate endothelial cation channel expression (73). If endotoxemia increases the expression of aminoglycoside-permeant ion channels or transporters, it will be crucial to determine if these channels enable LPS-enhanced trafficking of aminoglycosides across the BLB.

Endocytotic and transcytotic trafficking across the BLB has been described previously (74) and is increased during endotoxemia in non-cochlear capillary beds (75). While aminoglycosides strongly interact with negatively-charged phospholipid membranes (76), hydrophobic passage (or diffusion) through the membrane is slow (77). How endotoxemia potentiates aminoglycoside trafficking routes across the BLB remains to be determined.

Endotoxemia exacerbates ototoxicity

Chronic kanamycin dosing induced PTS at only 32 kHz. Endotoxemia significantly exacerbated the degree of kanamycin-induced PTS at 32 kHz, and at additional lower frequencies. Furthermore, endotoxemia significantly potentiated the degree of kanamycin-induced OHC death, predominantly in the basal region of the cochlea. These data support prior observations that bacteremia and hyperthermia (an experimental model for sepsis-induced fever) enhanced aminoglycoside-induced ototoxicity in humans and mice, respectively (78, 79). Endotoxemia also potentiated aminoglycoside-induced nephrotoxicity (80), and heightened the degree of cisplatin-induced PTS (81).

Kanamycin-induced OHC loss occurred in a narrower tonotopic range than for drug-induced PTS, as reported previously (27, 82). This mismatch between the broader frequency ranges of PTS and narrower tonotopic regions of OHC loss has been attributed to functional dysregulation of the stria vascularis, hair cell mechanotransduction and/or synaptic activity in surviving hair cells (12, 82, 83). Although partial recovery of auditory function after kanamycin treatment has been described previously, it only occurred in regions with lower threshold shifts and greater OHC survival (82). The basis for this partial recovery is thought to be drug clearance from cochlear tissues, facilitating the repair of hair cell and stria physiology incurred during sub-lethal toxicity to resume optimal auditory function (25, 82, 84). However, once threshold shifts exceed >40 dB (akin to the LPS+kanamycin group), no functional or anatomical recovery of hair cells occurred, resulting in a PTS (82, 85).

Endogenous inflammatory responses to Gram-negative bacterial infections are crucial to controlling infection and host survival. However, the clinical use of the life-saving bactericidal aminoglycosides can inadvertently heighten the existing inflammatory response via bacteriolytic release of endotoxins, i.e., the Jarisch-Herxheimer reaction (61, 62), to potentiate both cochlear uptake of aminoglycosides and ototoxicity. Thus, efforts to ameliorate aminoglycoside-induced ototoxicity (and nephrotoxicity) using pharmacotherapeutic agents, for example D-methionine (86), should also aim to demonstrate otoprotection in preclinical models with induced inflammation.

The progression of acute phase inflammation is characterized by changing expression patterns of specific inflammatory proteins over time (40, 41), while the onset of aminoglycoside-induced ototoxicity in mice requires several days of administration (27). We tested inflammation-enhanced cochlear uptake of aminoglycosides only at 24 hours, and this potentiated uptake could be greater at other time points after induction of endotoxemia. We induced endotoxemia using LPS from *E. coli*, yet, LPS from other bacteria can also activate TLR4. It will be important to determine if other TLR signaling cascades (particularly TLR3 for viremia) also potentiate aminoglycoside-induced ototoxicity. If inner ear inflammation increases the penetration of non-ototoxic antibiotics (e.g., cephalosporins) into the cochlea,

then this phenomenon could better preserve auditory and vestibular function during bacterial labyrinthitis (87).

We conclude that endotoxemia-induced inflammation potentiated ototoxicity by increasing aminoglycoside trafficking across the BLB into the cochlea. *In toto*, these data suggest that patients receiving aminoglycoside pharmacotherapy for life-threatening bacterial infections are at greater risk of ototoxicity than previously recognized.

Materials and Methods

Study design

The objective was to test the hypothesis that LPS-induced inflammation increased cochlear concentrations of aminoglycosides without renal dysfunction or increased serum aminoglycoside concentrations in C57BL/6 mice. We then verified LPS-induced systemic and cochlear inflammation using multiplex ELISAs and quantitative RT-PCR (44) in C57BL/6 mice, and in mice with hypofunctional TLR4 activity (C3H/HeJ) and their strain control (C3H/HeOuJ). We then tested whether LPS-induced inflammation exacerbated aminoglycoside-induced ototoxicity using a well-established protocol for C57BL/6 mice (27). All experiments were approved by the Institutional Animal Care and Use Committee of Oregon Health & Science University, and followed the ARRIVE reporting guidelines (88).

Serum and cochlear concentrations of GTTR, gentamicin, serotonin and histamine

GTTR was prepared as before (20). Mice, chosen at random, received a tail vein injection of DPBS or LPS, followed by an *i.p.* injection of GTTR (2 μ g/g), gentamicin (20 μ g/g) or hTR (2 μ g/g molar equivalent to GTTR) 24 hours later (fig. S15). LPS-treated mice received one dose (0.1, 1, 2.5, or 10 mg/kg) of LPS (*E. coli* serotype 0111:B4). One or 3 hours after GTTR, gentamicin or hTR injection, cardiac blood samples were obtained prior to cardiac perfusion with DPBS followed by 4% paraformaldehyde, and cochleae and kidneys were immersion fixed. Fixed tissues were counter-labeled with Alexa-488-conjugated phalloidin and examined by confocal microscopy for fluorophore intensity or capillary diameter analyses by operators blinded to treatment groups (20). Serum concentrations of the gentamicin epitope of GTTR were obtained by OHSU Diagnostic Services (13). Serum and cochlear concentrations of gentamicin or GTTR were also assayed by ELISA according to the manufacturer's instructions (EuroProxima) to determine concentrations. Serum serotonin and histamine concentrations were obtained using ELISA kits (Rocky Mountain Diagnostics).

Inflammatory protein and mRNA analyses at 6 hours

C57BL/6 mice were randomly grouped and treated as described in fig. S16. For multiplex ELISAs, cochleae were homogenized prior to performing multiplex ELISAs in duplicate (44). For quantitative RT-PCR, excised cochleae were placed in RNeasy lysis buffer (Qiagen) and stored at 80° C. Tissue RNA was extracted, reverse-transcribed using an RT² first-strand kit, prepared for RT-PCR using custom PCR arrays optimized for reaction conditions, primers,

and probes (SABiosciences), and analyzed using the SABiosciences PCR Array Data Analysis Web Portal (44).

Inflammatory protein and mRNA analyses at 24 hours

Twenty-four mice for each strain (C57BL/6, C3H/HeOuJ, C3H/HeJ) were randomly grouped and treated as shown in fig. S16. For ELISAs, 8 cochleae from 4 mice per sample were pooled in protein extraction buffer prior to multiplex ELISA, in triplicate. Absolute protein concentrations from DPBS-treated mice were subtracted from LPS-treated mice, and the 95% Student's confidence intervals propagated. For mRNA levels, RNA from each pair of cochleae were obtained, and cDNA samples analyzed in triplicate using RT² qPCR Primer Assays read on an Applied Biosystems Step One Plus qRT-PCR. Relative expression levels were calculated using the Ct method normalized to GAPDH.

Ototoxicity studies

ABRs to pure tones were used to obtain measures of cochlear function prior to and after treatments to determine threshold shifts (20, 72). For toxicity studies, mice were randomly divided into 4 groups (i) DPBS only; (ii) LPS only; (iii) kanamycin only and (iv) LPS plus kanamycin (fig. S17). Mice received 700 mg/kg kanamycin (or DPBS) twice daily for 14 consecutive days (27). Mice received a tail vein injection of 1 mg/kg LPS (or DPBS) the day before kanamycin treatment, and on the 5th and 10th day during kanamycin treatment. ABRs were obtained before kanamycin treatment, and 1, 10 and 21 days after kanamycin treatment prior to fixation and cytochleogram analyses by operators blinded to treatment groups (27). Additional mice in each group received hTR for 1 hour after ABR testing on day 14 of chronic LPS and/or kanamycin treatment, prior to fixation and processing for hTR fluorescence intensity and cytochleogram analyses as described above. Neonatal pups (P6) were used as positive controls.

Statistical analyses

Statistical analyses were chosen based on data under analysis. In brief, Wilcoxon signed-rank test was used for GTTR intensity analyses; the Mann-Whitney U test for ordinal variables (e.g., Fig. 2A); and Student's unpaired t-test for single variable analyses (e.g., Fig. 2B–E). For vasodilation analyses, we used 1-way ANOVA with Tukey post-hoc tests. For ELISA studies in C57BL/6 mice, we used a 1-way ANOVA with Bonferroni multiple comparison correction with family-wise 95% confidence levels, while comparisons of mRNA levels in C57BL/6 mice were determined as significant if the 95% confidence interval (CI) does not overlap with 1 (control-treated baseline). To compare ELISA and mRNA data between strains, a 1-way ANOVA with either Bonferroni or Dunnett's post-hoc tests and a family-wise 95% confidence level was used. For ABR and cytochleogram analyses, we used 2-way ANOVA with Bonferroni or Tukey post-hoc tests. The statistical analyses used, number of replicate measurements or mice are stated in each figure legend or in the Supplementary Materials; and *P* values <0.05 were considered significant.

Materials and Methods

Preparation of GTTR

Gentamicin sulfate (200 mg/ml in 0.1 M potassium bicarbonate, pH 10) and succinimidyl esters of Texas Red (Invitrogen, CA; 10 mg/ml in dimethyl formamide) were agitated together for 1 week at room temperature to produce an approximately 100:1 molar ratio of gentamicin:Texas Red. A high ratio of free gentamicin to Texas Red esters ensures that only one Texas Red molecule is conjugated to any individual gentamicin molecule. We used reversed phase chromatography using C-18 columns (Grace Discovery Sciences, IL) to purify the conjugates from unconjugated gentamicin, and contamination by unreacted Texas Red (20).

Serum and cochlear levels of GTTR, gentamicin, serotonin and histamine

The care and use of all animals reported in this study were approved by the Animal Care and Use Committee of Oregon Health & Science University in keeping with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*. C57BL/6 mice (15–25 g; 4–7 weeks old) with normal Preyer's reflex were divided into control and LPS-treated groups. The control group mice received a tail vein injection of DPBS (50 μ l/10g) at 0 hours, then an intraperitoneal (*i.p.*) injection of gentamicin (20 mg/kg) or GTTR (2 mg/kg) 24 hours later (see fig. S11). The LPS group received an intravenous (*i.v.*) injection of LPS followed by an *i.p.* injection of GTTR or gentamicin 24 hours later. The LPS group received one of several dosages of LPS (*E. coli* serotype 0111:B4, Sigma-Aldrich Co., St Louis, MO, USA; 0.1, 1, 2.5, or 10 mg/kg in DPBS, 50 μ l/10 g). Six mice (5 out of 19 in 10 mg/kg group, 1 out of 13 in 2.5 mg/kg group) died within 24 hours after LPS injection, as expected (30).

One or 3 hours after gentamicin or GTTR injections, mice were sacrificed, blood samples obtained prior to cardiac perfusion with DPBS followed by 4% paraformaldehyde in DPBS. Cochleae were then excised and immersion fixed in 4% paraformaldehyde. The level of the gentamicin epitope of GTTR in serum samples was obtained by particle-enhanced turbidimetric inhibition immunoassay by OHSU Diagnostic Services (13). The Mann-Whitney U test was used to test for differences between groups. Serum serotonin and histamine levels were obtained from samples collected 3 hours after GTTR (27 hours after LPS or DPBS) injection using ELISA fast track kits for serotonin or histamine (Rocky Mountain Diagnostics, Colorado Springs, CO, USA) by the Oregon Clinical and Translational Research Institute. The Mann-Whitney U test was used to test for differences between groups.

Cochlear imaging

At 1 or 3 hours after GTTR injection, anesthetized mice were transcardially perfused with DPBS, then 4% paraformaldehyde; the cochleae and kidneys excised and post-fixed. The lateral wall and cochlear coils containing the organ of Corti were isolated, and kidneys vibrotome-sectioned ~70 μ m thick. Prepared tissues were permeabilized with 0.5 % Triton X-100 for 45 min, rinsed, labeled with Alexa-488-conjugated phalloidin, post-fixed for 15 minutes and rinsed. Tissues from the quarter turn of the basal coil adjacent to the hook

region were mounted on slides and examined using a Bio-Rad MRC 1024 ES laser scanning confocal system attached to a Nikon Eclipse TE300 inverted microscope. All GTTR fluorescence images were acquired at the same laser intensity and gain settings, including control tissues. Images of each ROI from the red channel were manually segmented for pixel intensity determination (ImageJ). To normalize data between experimental sets, the mean intensity was ratioed against the control specimen (intensity at 1 hour time point of control animal, at each ROI area) and plotted. The Mann-Whitney U test was used to test for the difference in the relative fluorescence intensity of GTTR between control and experimental groups at each time point and each ROI. Absolute GTTR fluorescence intensity between cell layers of the cochlear lateral wall was compared using Wilcoxon signed rank test after background intensity was subtracted.

In addition, mice in each group received 2 $\mu\text{g/g}$ hTR (equimolar to 2 $\mu\text{g/g}$ GTTR) for 1 hour, 24 hours after LPS injection, then fixed and processed for intensity analysis as described above by blinded operators. Neonatal pups (P6) were used as a positive control. Differences between groups were determined using 1-way Analysis of Variance (ANOVA) with Tukey post-hoc tests. In addition, stria capillary calibers in apical and basal cochlear lateral wall segments were determined as described previously (20).

Gentamicin ELISAs

Paraformaldehyde-perfused cochleae from 4 mice in each DPBS or LPS group at each time point (1 or 3 hours) were excised and stored at -80°C . Thawed cochleae were weighed and then homogenized using BioMasher III kits (DiagnoCine, NJ), with 100 μl homogenizing buffer (SDB, 1.15g Na_2HPO_4 ; 0.2g KH_2PO_4 ; 0.2g KCl ; 30g NaCl ; 0.5 ml Tween 80 in 1 liter distilled water, pH 7.4; EuroProxima, Arnhem, The Netherlands). After removal of bone fragments, the protein extract was centrifuged at 4°C , and the supernatant used for gentamicin ELISA according to the manufacturer's instructions (EuroProxima, Arnhem, The Netherlands) to determine gentamicin or GTTR levels in serum and cochleae (normalized to cochlear mass). Student's unpaired t-test was used to compare gentamicin levels between groups.

Inflammatory protein and mRNA analyses: 6 hours

As shown in fig. S12, the vehicle control group was anesthetized prior to a tail vein injection of DPBS *i.v.* at 0 hours, followed by an *i.p.* injection of DPBS at 3 hours. Using the same time points, a second group received DPBS *i.v.* followed by gentamicin *i.p.*; the third group received 1 mg/kg LPS *i.v.*, followed by DPBS *i.p.*; and the last group received 1 mg/kg LPS, followed by gentamicin *i.p.* At the 6 hour time point, all mice were anesthetized and cardiac blood collected, the vasculature perfused with ice-chilled DPBS prior to excision of both cochleae and stored at -80°C . Each group contained 16 mice. Gentamicin was administered at a dose of 300 mg/kg, a non-lethal dose to determine any effect of gentamicin on inflammatory protein serum levels (27).

Recent advances allow multiplex ELISAs to measure multiple cochlear inflammatory proteins simultaneously within a single sample (44). Thawed cochleae from 10 mice were homogenized as above with 100 μl T-PER (extraction buffer, source). Bone fragments were

removed by centrifugation at 4°C, and protein concentration of the supernatant determined using the Bradford assay and normalized prior to performing multiplex ELISA using Q-Plex Array (Quansys Biosciences, USA) in duplicate, as described previously (44).

Recent advances enable quantitative RT-PCR analyses on primer arrays of multiple genes for inflammatory protein genes to quantify cochlear gene expression (44). Six pairs of excised cochleae from each group were placed in *RNAlater* (Ambion, Inc, Austin, TX, USA) and stored at -80° C. Tissue RNA was extracted with the Qiagen RNeasy Mini Kit, and sample concentrations of RNA equalized. RNA was reverse-transcribed using an RT² first-strand kit (SABiosciences Corp, Frederick, MD, USA), and prepared for real-time polymerase chain reaction (RT-PCR), using custom PCR arrays optimized for reaction conditions, primers, and probes (from SABiosciences Corp). The data was analyzed using the SABiosciences PCR Array Data Analysis Web Portal. The housekeeping gene used was glyceraldehyde-3-phosphate dehydrogenase (GAPDH) has been verified to standardize samples routinely (43). One-way ANOVA with Bonferroni multiple comparison correction was used to compare gene expression and protein levels.

Inflammatory protein and mRNA analyses: 24 hours

Twenty-four mice of each strain (C57BL/6, C3H/HeOuJ, C3H/HeJ; 5–6 weeks old) were anesthetized prior to a tail vein injection of LPS (1mg/kg), or DPBS (5μl/g; see fig. S12). Twenty-four hours later, mice were anesthetized, cardiac blood collected in Na₂EDTA in pyrogen-free sterile epitubes, the vasculature perfused with ice-cold DPBS, cochleae excised and stored at -80 C. For ELISAs of cochlear inflammatory proteins, 8 cochleae from 4 mice were pooled in protein extraction buffer prior to multiplex ELISA using a Quansys® platform, as described above, and measured in triplicate (2.5, 1.5, and 1mg/ml protein concentration) for each sample. Absolute protein concentrations from DPBS-treated mice were subtracted from LPS-treated mice, and the 95% Student's confidence intervals propagated. One-way ANOVA using Dunnett's post-hoc multiple comparison test was used to compare protein levels.

For mRNA levels of cochlear inflammatory proteins, cochleae from 8 mice of each strain (N=4 LPS, N=4 DPBS) were excised and processed as described above. RNA from each pair of cochleae was extracted using an RNeasy kit, and 400ng of RNA from each sample reverse-transcribed using an RT² first strand kit. cDNA samples were analyzed in triplicate using RT² qPCR Primer Assays read on an Applied Biosystems Step One Plus qRT-PCR. Relative expression levels were calculated using the Ct method normalized to GAPDH. One-way ANOVA using Dunnett's post-hoc multiple comparison tests was used to compare mRNA levels.

ABRs and drug-induced ototoxicity studies

ABRs to pure tones were used to ensure normal cochlear function prior to toxicity studies and determine threshold shifts following kanamycin treatment (20, 72). Briefly, needle electrodes were placed subcutaneously below the test ear, at the vertex, with a ground electrode near the paw. Each ear of each anesthetized mouse was stimulated individually with a closed tube sound delivery system sealed into the ear canal. The ABR to 1 ms rise-

time tone burst stimuli at 4, 8, 12, 16, 24 and 32 kHz was recorded and thresholds obtained for each ear. Only mice with normal bilateral, baseline ABR thresholds were used. For toxicity studies, mice were divided into 4 groups, (i) DPBS only; (ii) LPS only; (iii) kanamycin only and (iv) LPS plus kanamycin (fig. S13). Mice received 700 mg/kg kanamycin (or DPBS) twice daily for consecutive 14 days (27). Mice received a tail vein injection of 1 mg/kg LPS (or vehicle) the day before the first day on kanamycin treatment, and every 5th day thereafter during kanamycin treatment (2 additional injections). ABRs were obtained before kanamycin treatment, and 1, 10 and 21 days after kanamycin treatment. Mice were subsequently euthanized, fixed, labeled with Alexa 488-conjugated phalloidin and Hoechst nuclear dyes, prior to imaging with an Olympus FV1000 confocal microscope for hair cell apices and nuclei. Blinded operators constructed cytochromeograms by counting the presence or absence of hair cells along the length of the cochlea (27) in ImageJ software. The frequency map and the length of each cochlea were obtained using the Measure Line and MosaicJ plugins in the ImageJ software. A 2-way ANOVA using Bonferroni post-hoc multiple comparison test correction was used to test for differences between groups.

For BLB permeability studies following chronic LPS and/or kanamycin treatment (as described above), except, on day 14 mice in each of the 4 groups underwent ABR testing, then received 2 $\mu\text{g/g}$ hTR (equimolar to 2 $\mu\text{g/g}$ GTTR) for 1 hour prior to fixation and processing for confocal imaging, pixel intensity and cytochrome analyses by blinded operators as described above. Differences between groups were determined using 1-way ANOVA with Tukey post-hoc tests.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Accessible Summary: Inflammation potentiates antibiotic-induced hearing loss

Aminoglycoside antibiotics are essential for treating life-threatening infections, despite the known risk of permanent hearing loss by damaging cochlear sensory cells in the inner ear in healthy subjects. Because severe infections also trigger widespread inflammation, Koo and colleagues tested the effect of inflammation on aminoglycoside-induced hearing loss as a model of clinical dosing with these drugs. Systemic inflammation increased cochlear uptake of aminoglycosides and, surprisingly, cochlear expression of inflammatory markers. Inflammation also greatly exacerbated aminoglycoside-induced hearing loss and cochlear sensory cell death compared to healthy subjects. It is hoped this information will assist clinicians to choose the appropriate pharmacotherapy for patients with severe infections to better protect life-long hearing health, and encourage the development of otoprotective drugs to also demonstrate efficacy against aminoglycoside-induced ototoxicity during infection-induced inflammation.

Fig. 1. Cochlear lateral wall uptake of GTTR is enhanced by LPS

(A) In xz planes of the cochlear lateral wall 1 hour after GTTR injection, F-actin labeling (green,) revealed tight junctions (arrowheads) between marginal cells (MC), with amorphous labeling in basal cells (BC, arrows). In DPBS-treated mice, intense GTTR fluorescence (red) distinguished strial capillaries (c), with less intense fluorescence in marginal cells, intra-strial layer (IS), and basal cells of the stria vascularis. The spiral ligament (SL) fibrocytes presented substantially less intense GTTR fluorescence compared to strial cells. LPS-treated mice displayed more intense GTTR fluorescence in the lateral wall (right panel) compared to DPBS-treated mice (left panel). (B) A focal series of xy planes through marginal cells, intra-strial tissues, basal cells, and fibrocytes at successively lower xy planes in the z -axis, 1 hour after GTTR injection. LPS-treated mice exhibited more intense GTTR fluorescence in grayscale (right panels) compared to DPBS-treated mice (left panels). Scale bar, 50 μm . (C) Mean pixel intensities of GTTR fluorescence in lateral wall ROIs (excluding capillary structures) are dose-dependently increased with increasing doses of LPS at 1 and 3 hours after GTTR injection (relative to DPBS-treated mice at 1 hour), with statistical significance in every cell type at 1 hour at 1 mg/kg or higher dose of LPS (Wilcoxon signed-rank test; $*P<0.05$; $**P<0.01$; $***P<0.001$; error bars=s.e.m.; N as in Table S1).

Fig. 2. Low dose LPS does not alter serum concentrations but does alter cochlear concentrations of aminoglycosides

(A) Using immunoturbidimetry, GTTR serum concentrations were significantly higher in 2.5 and 10 mg/kg LPS-treated mice than in controls at 1 or 3 hours. There was no difference between DPBS-treated mice and those dosed with 0.1 and 1 mg/kg LPS, nor between mice dosed with 2.5 and 10 mg/kg LPS. Serum concentrations of GTTR in 2.5 mg/kg LPS-treated mice were significantly higher than in 0.1 and 1 mg/kg LPS-treated mice at both time points ($P < 0.05$). Elevated serum GTTR concentrations in 10 mg/kg LPS-treated mice showed borderline significance at 1 hour compared to 0.1 and 1 mg/kg LPS-treated mice ($P = 0.087$, 0.053 , respectively), and variable significance at 3 hours ($P = 0.27$, 0.028 , respectively; see also Table S2; Mann-Whitney U test; $*P < 0.05$; $**P < 0.01$; $***P < 0.005$; N in Table S1). (B, D) Using ELISA, serum concentrations of GTTR or gentamicin were not statistically different between DPBS-treated or 1 mg/kg LPS-treated mice at 1 or 3 hours after injection. (C, E) Cochlear concentrations of GTTR or gentamicin (GT) were significantly increased in 1 mg/kg LPS-treated mice compared to controls at 1 or 3 hours after injection ($*P < 0.05$; N=4/group; Student's unpaired t-test). Error bars=s.e.m; au, arbitrary units.

Fig. 3. LPS does not alter BLB permeability but vasodilates basal strial capillaries

(A) The relative mean intensities of hTR fluorescence in marginal cell (MC), intra-strial tissue (IS), basal cell (BC) and spiral ligament (SL) layers from P6 pups were significantly elevated compared to the same ROIs in adult mice. There was no difference in hTR fluorescence of lateral wall ROIs between DPBS-treated and LPS-treated adult mice ($*P < 0.05$, $***P < 0.001$; $N = 6$ cochleae/group; error bars = s.d.; 2-way ANOVA with Bonferroni post-hoc tests). Absolute fluorescent intensities are shown in fig. S5A. (B, C) In P6 mice, the lumen of strial capillaries, revealed by phalloidin labeling, was larger than in adult DPBS-treated mice (endothelial cells indicated by white arrowheads). (D) Twenty-five hours after LPS treatment, a subpopulation of strial capillaries were dilated compared to DPBS-treated mice (C). Scale bar, 20 μm . (E) Strial capillary diameters in P6 mice were wider than in DPBS-treated adults (see also Table 1). LPS-treated adult mice had a subset of dilated strial capillaries, resulting in a bimodal distribution. (F) LPS also dilated a subset of strial capillaries in C3H/HeOuJ mice compared to DPBS-treated C3H/HeOuJ mice. (G) In TLR4-hyporesponsive C3H/HeJ mice, LPS dilated fewer strial capillaries compared to LPS-treated control C3H/HeOuJ mice (F), resulting in an asymmetrical bimodal distribution. A Gaussian regression curve fit was applied to obtain the bimodal peak means in Table 1.

Fig. 4. Low dose LPS induced major inflammatory responses in serum and cochleae within 6 hours

(A) Significant increases in selected serum inflammatory proteins were observed 6 hours after LPS ($\pm$ gentamicin) injection compared to DPBS-treated mice ($\pm$ gentamicin; N=10/cohort). (B) Significant increases in cochlear inflammatory proteins were observed 6 hours after LPS ($\pm$ gentamicin) injection for TNF α , IL-1 α , IL-6, IL-8, MIP-1 α and MIP-2 α (but not IL-1 β and IL-10) compared to DPBS-treated mice ($\pm$ gentamicin; N=5/cohort; 4 cochleae/sample; measured in duplicate; *significant difference after 1-way ANOVA with Bonferroni multiple comparison correction and a family-wise 95% confidence level; error bars, 95% confidence intervals [CI] derived from Student's t-tests). (C) Significant increases

in cochlear mRNA for selected inflammatory markers were observed 6 hours after LPS ($\pm$ gentamicin) injection when normalized to DPBS-treated mice (N=6/cohort; 2 cochleae/sample; *significant difference if the 95% CI does not overlap with 1 [*i.e.*, DPBS-treated mice baseline]). Gentamicin did not modulate serum or cochlear expression of inflammatory proteins or mRNA for inflammatory markers.

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Fig. 5. TLR4-mediated cochlear inflammatory markers are attenuated in C3H/HeJ mice
(A) All selected acute phase inflammatory proteins (except for IL-10) were significantly elevated in cochleae from LPS-treated C57BL/6 and C3H/HeOuJ mice compared to DPBS-treated mice of the same strain. Several inflammatory proteins (TNF α , IL-6, IL-8, MIP-1 α , MIP-2 α) were more elevated in C57BL/6 compared to C3H/HeOuJ mice after LPS. In TLR4-hyporesponsive cochleae from C3H/HeJ mice, only a subset of inflammatory proteins (IL-1 α , IL-6, IL-8, MIP-1 α) were elevated after LPS, with small differences between the means for TNF α and IL-10. Expression of predominantly later-expressing inflammatory markers (IL-1 α , IL-6, IL-8, MIP-1 α) was significantly attenuated in LPS-treated C3H/HeJ mice compared to LPS-treated C3H/HeOuJ and C57BL/6 mice (N=4/cohort; 6 cochleae from 3 mice/sample) **(B)** In C57BL/6 and C3H/HeOuJ mice, significant increases were observed in cochlear expression of *Il-1 β* , *Il-6*, *Il-8*, *Il-10*, *Mip-1 α* and *Mip-2 α* mRNA after LPS treatment when normalized to DPBS-treated mice. These increases were attenuated for *Il-8*, *Mip-1 α* and *Mip-2 α* in LPS-treated C3H/HeJ mice compared to LPS-treated C3H/HeOuJ mice. *Il-10* mRNA expression was significantly higher in C3H/HeJ mice compared to C3H/HeOuJ and C57BL/6 mice (N=4/cohort; 2 cochleae from 1 mouse/sample). Error bars, 95% CI derived from Student's t-tests; *significant difference compared to C3H/HeOuJ mice after 1-way ANOVA with Dunnett's post-hoc tests and a family-wise 95% confidence level). See also fig. S6.

C3H/HeOuJ vs C3H/HeJ fold change in GTTR fluorescence 24 hours post-LPS treatment

Fig. 6. LPS-induced GTTR uptake by lateral wall cells is attenuated in TLR4-hyporesponsive C3H/HeJ mice

The fold change in GTTR intensity in LPS-treated mice over DPBS-treated mice is shown. GTTR fluorescence was significantly enhanced in strial marginal (MC), intermediate (IC) and basal (BC) cells, as well as fibrocytes (FC) of LPS-treated C3H/HeOuJ mice compared to that in DPBS-treated C3H/HeOuJ mice. LPS also significantly enhanced GTTR fluorescence intensities in strial cells (but not fibrocytes) in LPS-treated C3H/HeJ mice compared to that in DPBS-treated C3H/HeJ mice. Note that LPS-induced GTTR uptake was significantly attenuated ($P < 0.05$) in marginal cells, intermediate cells and fibrocytes in C3H/HeJ mice compared to C3H/HeOuJ mice. (* $P < 0.05$; $N = 8$ /bar; error bars = 95% CI derived from Student's *t*-tests; significance was determined if 95% CI did not overlap with 1; # $P < 0.05$; unpaired 1-way *t*-test between strains; see fig. S8 for raw data).

Fig. 7. Chronic endotoxemia potentiates kanamycin ototoxicity

(A) Three weeks after chronic LPS (or DPBS) exposure $\pm$ kanamycin (see fig. S13), ABR threshold shifts for LPS-only mice (N=5) were not different from DPBS-treated mice (N=4). Kanamycin alone (N=5) induced a small but significant PTS at only 32 kHz (** $P<0.01$) compared to DPBS-treated mice. Mice that received LPS+kanamycin (N=6) had significant PTS at 16, 24 (## $P<0.01$) and 32 kHz (# $P<0.05$) compared to mice treated with kanamycin, DPBS or LPS only (** $P<0.01$). Mice receiving LPS+kanamycin also had significant PTS at 12 kHz compared to mice treated with DPBS or LPS only ($\phi P<0.05$ and $P<0.01$, respectively), or LPS-only mice at 8 kHz ($\dagger P<0.05$). Error bars=s.d. All statistical results were produced using 2-way ANOVA with Bonferroni post-hoc correction with 95% family-wise confidence intervals. (B) Cytocochleogram for mice in (A) revealed that OHC loss in the basal cochlear regions was greater and over a wider frequency range in LPS+kanamycin-treated mice compared to mice treated with LPS, DPBS, or kanamycin. Mean cochlear length = 6.84 (± 0.79 , s.d.) mm. Error bars, 95% CI derived from Student's t-tests. See also figs. S9–S11, and tables S5, S7 for statistical comparisons using 2-way ANOVA Bonferroni post-hoc correction with 95% family-wise confidence intervals.

Table 1

Vasodilation of strial capillaries by LPS.

Mouse	LPS	Mean diameter (±s.d.) in μm	Mean bimodal diameter (±s.d.) in μm [% of N]	N (number of mice)	Significance (vs in-strain DPBS-treated mice)
Basal coils C57BL/6 P6 pups	–	13.8 (±1.5)		300 (3)	***
C57BL/6 adults	–	5.1 (±1.0)		300 (3)	
C57BL/6 adults	+	5.3 (±0.9)	8.6 (±1.1) [45.3%]	300 (3)	***
C3H/HeOuJ adults	–	5.3 (±0.8)		480 (4)	
C3H/HeOuJ adults	+	5.7 (±0.8)	8.6 (±0.9) [49.7%]	480 (4)	***
C3H/HeJ adults	–	5.3 (±0.9)		480 (4)	
C3H/HeJ adults	+	5.4 (±0.8)	8.1 (±0.9) [22.1%]	480 (4)	***
Apical coils C57BL/6 adults	–	5.0 (±0.7)		480 (4)	
C57BL/6 adults	+	5.6 (±0.6)	8.1 (±1.0) [59.8%]	480 (4)	***

In basal coils, strial capillary diameters in P6 pups were significantly larger than in DPBS-treated C57BL/6 adult mice. LPS dilated a subset (45%) of strial capillaries in adult mice. The population of capillary diameters in LPS-treated C57BL/6 mice was significantly greater compared to DPBS-treated C57BL/6 mice ($P<0.0001$). LPS also dilated a subset (50%) of strial capillaries in C3H/HeOuJ mice. In TLR4-hyporesponsive C3H/HeJ mice, LPS dilated a smaller subset (22%) of strial capillaries. The population of capillary diameters in LPS-treated C3H/HeJ mice was significantly greater compared to DPBS-treated C3H/HeJ mice ($P<0.0001$). The population of capillary diameters in LPS-treated C3H/HeJ mice was significantly smaller compared to LPS-treated C3H/HeOuJ mice ($P<0.0001$; 1-way ANOVA with Tukey post-hoc tests). In apical coils, LPS dilated a subset (45%) of strial capillaries in C57BL/6 mice. The population of apical strial capillary diameters in LPS-treated C57BL/6 mice was significantly greater compared to DPBS-treated C57BL/6 adult mice ($P<0.0001$). A Gaussian regression curve fit was applied to obtain the peak means of the bimodal distributions in Fig. 3.

Table 2

Effect of LPS on serum concentrations of histamine and serotonin.

LPS dose (mg/kg)	Histamine (ng/ml $\pm$ s.e.m.)	Serotonin (ng/ml $\pm$ s.e.m.)
0	33 ($\pm$ 2)	2770 ($\pm$ 100)
0.1	25 ($\pm$ 3)	2135 ($\pm$ 285) *
1.0	24 ($\pm$ 4)	579 ($\pm$ 157) **
2.5	28 ($\pm$ 3)	830 ($\pm$ 246) **
10	36 ($\pm$ 6)	837 ($\pm$ 329) **

Serum histamine concentrations were not affected by increasing doses of LPS. Serum serotonin concentrations were significantly decreased at all LPS doses compared to DPBS-treated mice

* $P < 0.05$,

** $P < 0.001$;

Mann-Whitney U test; N in Table S1 at 3 hours after GTTR injection.