Therapeutic effect of NLRP3 inhibition on hearing loss induced by systemic inflammation in a CAPS-associated mouse model

Ji-Hyun Ma,[a,1] Eunju Lee,[b,1] Sung-Hyun Yoon,[b,1] Hyehyun Min,[a,1] Jae Hwan Oh,[a] Inhwa Hwang,[b] Yejin Sung,[c] Ju Hee Ryu,[c] Jinwoong Bok,[a]* and Je-Wook Yu [b]*

[a]Department of Anatomy, Brain Korea 21 Project for Medical Science, Severance Biomedical Science Institute, Yonsei University College of Medicine, Seoul 03722, Republic of Korea
[b]Department of Microbiology and Immunology, Institute for Immunology and Immunological Diseases, Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul 03722, Republic of Korea
[c]Theragnosis Research Center, Biomedical Research Division, Korea Institute of Science and Technology (KIST), Seoul, Republic of Korea

eBioMedicine 2022;82: 104184
Published online xxx
https://doi.org/10.1016/j.ebiom.2022.104184

Summary

Background Cryopyrin-associated periodic syndrome (CAPS) is an inherited autoinflammatory disease caused by a gain-of-function mutation in *NLRP3*. Although CAPS patients frequently suffer from sensorineural hearing loss, it remains unclear whether CAPS-associated mutation in *NLRP3* is associated with the progression of hearing loss.

Methods We generated a mice with conditional expression of CAPS-associated NLRP3 mutant (D301N) in cochlea-resident CX3CR1 macrophages and examined the susceptibility of CAPS mice to inflammation-mediated hearing loss in a local and systemic inflammation context.

Findings Upon lipopolysaccharide (LPS) injection into middle ear cavity, NLRP3 mutant mice exhibited severe cochlear inflammation, inflammasome activation and hearing loss. However, this middle ear injection model induced a considerable hearing loss in control mice and inevitably caused an inflammation-independent hearing loss possibly due to ear tissue damages by injection procedure. Subsequently, we optimized a systemic LPS injection model, which induced a significant hearing loss in NLRP3 mutant mice but not in control mice. Peripheral inflammation induced by a repetitive low dose of LPS injection caused a blood-labyrinth barrier disruption, macrophage infiltration into cochlea and cochlear inflammasome activation in an NLRP3-dependent manner. Interestingly, both cochlea-infiltrating and -resident macrophages contribute to peripheral inflammation-mediated hearing loss of CAPS mice. Furthermore, NLRP3-specific inhibitor, MCC950, as well as an interleukin-1 receptor antagonist significantly alleviated systemic LPS-induced hearing loss and inflammatory phenotypes in NLRP3 mutant mice.

Interpretation Our findings reveal that CAPS-associated NLRP3 mutation is critical for peripheral inflammation-induced hearing loss in our CAPS mice model, and an NLRP3-specific inhibitor can be used to treat inflammation-mediated sensorineural hearing loss.

Funding National Research Foundation of Korea Grant funded by the Korean Government and the Team Science Award of Yonsei University College of Medicine.

Keywords: CAPS; NLRP3 inflammasome; Hearing loss; Cochlear inflammation; Blood-labyrinth barrier disruption; NLRP3 inhibitor

Abbreviations: ABR, auditory brainstem responses; BLB, blood-labyrinth barrier; BMDM, bone marrow-derived macrophage; CAPS, cryopyrin-associated periodic syndrome; CX3CR1, C-X3-C motif chemokine receptor 1; DPOAE, distortion product otoacoustic emission; FITC, fluorescein isothiocyanate; IL-1β, interleukin-1β; IL-1RA, interleukin-1 receptor antagonist; LPS, lipopolysaccharide; NLRP3, NOD-like receptor family, pyrin domain-containing 3; NOMID, neonatal-onset multisystem inflammatory diseases

*Corresponding authors.
E-mail addresses: bokj@yuhs.ac (J. Bok), jewookyu@yuhs.ac (J.-W. Yu).

[1] These authors equally contributed to this paper.

Research in context

Evidence before this study

Hearing loss is one of the most common sensory disorders caused by various factors, including genetic mutations and non-genetic causes such as noise and ototoxic drugs. Recently, inflammation has also been highlighted as a potential contributor to the pathogenesis of hearing loss, despite the previous idea that the inner ear is an immune-privileged organ protected by the blood-labyrinth barrier. Of interest, the NLRP3 inflammasome, an essential player of innate immune sensing primarily expressed in myeloid cells, has been associated with hearing loss. Patients with cryopyrin-associated periodic syndrome (CAPS), a systemic autoinflammatory disease in which gain-of-function mutations in *NLRP3* cause abnormal inflammasome activation and IL-1β secretion, suffer from hearing loss. Furthermore, a recent study reported that hearing loss was exhibited in two families with gain-of-function mutations in *NLRP3*, although they do not present typical key aspects of CAPS symptoms. In addition, hearing loss in these patients was alleviated by treatment with an IL-1 receptor antagonist. These findings suggest that aberrant NLRP3 inflammasome activation is closely associated to the pathogenesis of inflammation-mediated hearing loss. However, it has not been determined whether CAPS-associated *NLRP3* mutation is a direct cause of hearing loss, primarily due to the lack of CAPS-mimicking animal models.

Added value of this study

We generated a CAPS murine model with conditional expression of CAPS-associated NLRP3 mutants in CX3CR1-positive cells, which are reportedly the primary immune cells in the cochlea. Our results show that systemic inflammation induced by intraperitoneal injection of lipopolysaccharide (LPS) caused significant hearing loss in the CAPS mouse model but not in the control group. Mechanistically, LPS-induced peripheral inflammation resulted in blood-labyrinth barrier disruption and monocyte infiltration into the cochlea in the CAPS mouse model. In addition, NLRP3 inflammasome activity in CX3CR1-positive cochlear-resident macrophages and infiltrating macrophages contributes to inflammation-mediated hearing loss in the CAPS mouse. We further demonstrated that an NLRP3-specific inhibitor could significantly ameliorate hearing loss caused by systemic inflammation in the CAPS mouse models.

Implications of all the available evidence

Our findings provide *in vivo* evidence that CAPS-associated *NLRP3* mutations in CX3CR1-positive macrophages are a direct cause of peripheral inflammation-induced hearing loss. Furthermore, our demonstration of the rescue of hearing loss by pharmacological intervention suggests that the CAPS murine model provides a valuable *in vivo* platform for elucidating the pathological mechanisms of inflammation-mediated hearing loss and screening novel therapeutic targets for hearing loss induced by various stress conditions known to cause cochlear inflammation such as noise or ototoxic drugs.

Introduction

Autoinflammatory diseases are characterized by recurrent and periodic severe inflammation persisting for several days up to a week without any signs of microbial infection and self-reactive lymphocytes.[1] Although molecular mechanisms underlying recurrent inflammation in autoinflammatory diseases remains elusive, initial positional cloning studies have led to the identification of several genetic variants, including the *MEFV* gene, which encodes pyrin, and the *CIAS1/NLRP3* gene, which encodes cryopyrin or NOD-like receptor family, pyrin domain-containing 3 (NLRP3) in patients with autoinflammatory diseases.[2,3] Intriguingly, these autoinflammatory disease-associated molecules are critical components of an inflammasome complex, and mutations in pyrin or NLRP3 are closely associated with the dysregulation of inflammasome signaling.[4] Inflammasome sensor molecules such as pyrin and NLRP3 may be present as their inactive monomeric form at a resting state but transform into the active form upon sensing diverse pathogen- or damage-associated factors, subsequently assembling the inflammasome complex with adaptor molecule ASC and procaspase-1.[5–7] This inflammasome assembly causes an immediate activation of caspase-1, which in turn induces the maturation and secretion of leaderless interleukin (IL)-1β.[8] Excessive inflammasome activation and IL-1β production has been closely implicated in the pathogenesis of diverse inflammatory diseases including autoinflammatory syndromes,[9,10] and the blockade of IL-1β signaling was shown to mitigate the symptoms of the related inflammatory diseases.[11,12]

NLRP3 is the most extensively-studied inflammasome sensor molecule owing to its activation by a wide spectrum of stimulators as well as its essential role in several inflammatory and degenerative diseases.[9,13] Multiple gain-of-function mutations in *NLRP3* have been identified in patients diagnosed with autoinflammatory cryopyrin-associated periodic syndromes (CAPS), such as familial cold autoinflammatory syndrome (FCAS), Muckle-Wells syndrome (MWS), and neonatal-onset multisystem inflammatory diseases (NOMID).[14–16] In particular, patients diagnosed with CAPS exhibited a wide range of symptoms with varying degrees of severity: mild (FCAS), moderate (MWS), and severe (NOMID). Patients with severe CAPS-like NOMID frequently showed a sensorineural hearing loss, caused by an impairment in relaying the sound waves in the inner ear into the brain due to a defect in

the organ of Corti or auditory neurons.[17,18] Moreover, anakinra, an IL-1 receptor antagonist, has been shown to mitigate the symptoms of CAPS including hearing loss.[12,19] Furthermore, a recent study demonstrated that NLRP3 missense mutation (R918Q) in two unrelated families caused a DFNA34 hearing loss.[20] These previous findings suggest that excessive NLRP3-mediated inflammasome signaling may be closely associated with the pathophysiology of sensorineural hearing loss under inflammatory circumstances.

Hearing loss is a common manifestation of CAPS and approximately 76% of all NOMID patients suffer from hearing loss.[21] A previous study revealed that CX3CR1-positive resident macrophages in the cochlea express NLRP3.[20] However, it remains to be fully determined whether a gain-of-function NLRP3 mutation observed in CAPS is directly capable of impairing hearing ability in the cochlea. Based on this possibility, the occurrence of inflammasome activation inside the cochlea and the inflammasome-occurring cell types responsible for sensorineural hearing loss require further clarification. In the present study, we generated a mouse model which conditionally expresses NLRP3 (D301N), a critical missense mutation in NOMID, in CX3CR1 cells of mice and examined the hearing ability of these mice under cochlear and systemic inflammation context.

Methods

Mice

C57BL/6 mice (RRID:IMSR_JAX:005304) were obtained from Orient Bio (Seongnam, Korea). *Nlrp3*^{D301NneoR} (RRID:IMSR_JAX:017971) and *Cx3cr1*^{CreER} (RRID:IMSR_JAX:021160) mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and bred at the Yonsei University College of Medicine. All mice were maintained under specific pathogen-free conditions. Given that C57BL/6 strain display progressive hearing loss beginning around 4–6 months of age, hearing experiments were started with 10–13 weeks old mice and all the measurements were completed by 13–16 weeks of age. In addition, mice at a similar age were randomly assigned to each experimental group.

Ethics statement

Protocols for the animal experiments were approved by the Institutional Ethical Committee of Yonsei University College of Medicine (2020-0317). All experiments were performed in accordance with the approved guidelines of the Institutional Ethical Committee.

CX3CR1-specific expression of the NLRP3 (D301N) mutant in mice

To induce the expression of NLRP3 (D301N) mutant, *Nlrp3*^{D301NneoR} mice harboring a loxP-flanked neomycin resistant cassette (reverse orientation) in intron 2 and a point mutation in exon 3 of *Nlrp3* (designated as *Nlrp3*$^{flox/flox}$) were bred to *Cx3cr1*^{CreER} mice. *Cx3cr1*$^{CreER/+}$; *Nlrp3*$^{+/flox}$ mice were intraperitoneally injected with tamoxifen (75 mg/kg) for 5 days to induce the expression of active Cre recombinase, resulting in the excision of the neomycin resistant cassette and the expression of NLRP3 (D301N) in CX3CR1-specific cells, including cochlear macrophages.

Auditory brainstem responses (ABRs)

ABR thresholds were determined in a sound-proofed room using a RZ6 digital signal processing hardware and the BioSigRZ software package (Tucker-Davis Technologies, Alachua, FL, USA). Mice were anesthetized with a mixture of Zoletil (40 mg/kg) and rompun (10 mg/kg) by intraperitoneal injection. Subcutaneous needles were placed into the vertex, contralateral, and ipsilateral. The SigGenRZ software package and the RZ6 digital signal processor were used to generate a calibrated click stimuli (10 μs duration) or tone burst stimuli (5 ms duration) at 6, 12, 18, 24, and 30 kHz, which were delivered to the ear canal through a MF1 multifield 1 magnetic speaker (Tucker–Davis Technologies). The acoustic stimulus intensity was raised in 5 dB SPL increments from 10 to 95 dB SPL. The ABR data were supplied into the RZ6 digital signal processing hardware via a low-impedance Medusa Biological Amplifier System (RA4LI, TDT). The ABR waveforms in response to 512 tone bursts were averaged after the recorded signals were filtered using a 0.5–1 kHz band-pass filter.

Distortion production otoacoustic emissions (DPOAEs)

A combined microphone-speaker system (Tucker–Davis Technologies) was used to measure DPOAEs. The primary stimulus tones were generated using the RZ6 digital signal processor and the SigGenRZ software package and were administered via a custom probe equipped with an ER 10B+ microphone (Etymotic, Elk Grove Village, IL, USA) and MF1 speakers positioned in the ear canal. The frequency ratio (f_2/f_1) of the main tones was set at 1.2 with target frequencies of 6, 12, 18, 24, and 30 kHz. The f_1 and f_2 intensities were adjusted at identical levels (L1 = L2) and raised in 5 dB SPL increments from 20 to 80 dB SPL. The fast Fourier transform (FFT) algorithm was used to calculate the average spectra of the two primaries, the 2 f_1- f_2 distortion products, and the noise floors at each primary tone for each intensity.

Reagents and antibodies

LPS (L4391 for cell culture, L3012 for mice), ATP, MCC950, tamoxifen, and 4-hydroxytamoxifen were purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-F4/80 antibody (Cat# ab6640, RRID:AB_1140040) was obtained from Abcam (Cambridge, United Kingdom).

Cy3- (Cat# 712-165-050, RRID:AB_2340666) and Alexa Fluor 488-conjugated (Cat# 712-545-150, RRID: AB_2340683) anti-rat IgG antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA). FITC-dextran (46944) and TRITC-dextran (T1037) were obtained from Sigma-Aldrich. The IL-1 receptor antagonist (Cat# CYT-203) was purchased from ProSpec (Rehovot, Israel). Anti-mouse caspase-1 (Cat# AG-20B-0042, RRID:AB_2490248) and anti-NLRP3 (Cat# AG-20B-0014, RRID:AB_2490202) antibodies were obtained from AdipoGen Life Sciences (San Diego, CA, USA). Anti-mouse IL-1β antibody (Cat# AF-401-NA, RRID:AB_416684) was obtained from R&D Systems (Minneapolis, MN, USA), and anti-ASC antibody (Cat# SC-22514-R, RRID:AB_2174874) was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Anti-GFP antibody (Cat# NB600-308, RRID:AB _10003058) was obtained from NOVUS Biologicals.

Immunohistochemistry

The cochlea was isolated from the skull, and the stapes and partial cochlear apical bones were removed to allow a fixative solution to flow into the cochlea. The isolated cochlea was fixed in 4% paraformaldehyde overnight at 4 °C and decalcified with 0.2 M ethylenediaminetetraacetic (EDTA) in PBS for 2 days. The cochlear tissue was dehydrated with 30% sucrose in PBS overnight at 4 °C and embedded in Optimal Cutting Temperature (OCT) compound (Tissue-Tek®, Sakura Finetek, Torrance, CA, USA). The cochlear tissues were sectioned into 12-µm-thick slices using a cryostat. Tissue sections were permeabilized with Triton-X (0.3%, 30 min) and blocked with BSA (4%, 1 h) at room temperature (RT). The sections were then incubated with anti-F4/80 antibody in 1% BSA overnight at 4 °C, followed by secondary antibody incubation (Alexa 488 or Cy3-labeled anti-Rat). The sections were further incubated with DAPI (D1306, Invitrogen, Waltham, MA, USA). Finally, cover slips were placed on slides after adding the mounting media (P36934, Invitrogen). Images were captured using a confocal microscope (LSM 780, Zeiss, Oberkochen, Germany) and the ZEN 2011 software. F4/80 positive cells were quantified using the Image J software.

Assay of dextran leakage into cochlea

To visualize dextran leakage from damaged blood vessel, FITC- or TRITC-conjugated dextran (4 kDa) was administered via tail vein 1 h before sacrifice (1 mg). The cochlea was isolated from mice and processed into tissue sections as described in the "immunohistochemistry" section. Subsequently, tissue sections were washed with PBS and incubated with DAPI. Cover slips were placed on the slides after adding the mounting media. Images were observed using a confocal microscope. Dextran leakage was analyzed using the Image J software.

Detection of active caspase-1 in the cochlea

Cy5.5- and BHQ-3-conjugated caspase-1-activatable probe was synthesized according to a previous study.[22] To detect active caspase-1 in the cochlea, the caspase-1-specific probe (100 µg/mice) was intravenously injected via tail vein 1 h before sacrifice of mice. For *ex vivo* imaging, cochlea tissue was collected from sacrificed mice after transcardial perfusion with PBS. Cy5.5 fluorescence, produced only in the presence of active caspase-1, in the isolated cochlea was analyzed using an IVIS spectrum In Vivo imaging system (PerkinElmer, Waltham, MA, USA).

Cell cultures

Mouse bone marrow cells were isolated from femurs and tibias of mice and differentiated into BMDMs as previously described.[23] To validate the purity of BMDMs, cells were stained with antibodies targeting macrophage markers, CD11b (Invitrogen, Cat# 12-0112-82, RRID:AB_2734869) and F4/80 (Invitrogen, Cat# 17-4801-82, RRID:AB_2784648), and then analyzed by flow cytometry (purity, >95%). All BMDMs were maintained in L929-conditioned DMEM supplemented with 10% FBS and antibiotics.

Immunoblot analysis

Cells were lysed in a buffer containing 20 mM HEPES (pH 7·5), 0.5% Nonidet P-40, 50 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, and protease inhibitors. Soluble lysates were fractionated by SDS-PAGE and then transferred to PVDF membranes. In some experiments, cell culture supernatants were precipitated using methanol/chloroform as described previously[24] and then immunoblotted. All blots shown are representative images of at least three-independent experiments.

Assay of inflammasome/caspase-1 activation

To induce a conventional NLRP3 inflammasome activation, BMDMs were primed with LPS (0.25 µg/mL, 3 h), followed with ATP treatment (2∼2.5 mM, 30−45 min). Inflammasome activation was determined by the presence of active caspase-1 p20 and active IL-1β in the supernatant of cultured cell using immunoblots and by quantification of extracellular IL-1β using ELISA.

Statistical analysis

All graphed results are expressed as means ± S.E.M. Statistical comparisons were made using two-tailed student *t*-test with Welch's correction or one- or two-way analysis of variance (ANOVA) with Tukey's or Bonferroni's correction for fluorescent images and

IL-1β ELISA, and using one- or two-way ANOVA with Tukey's or Bonferroni's correction for hearing tests (ABR and DPOAE). All statistical analysis was conducted using PRISM 8.0 (GraphPad, San Diego, CA, USA). When required, Shapiro-Wilk and Kolmogorov-Smirnov were used to test data for normality. Statistical significance is indicated in the figures as follows: n.s., non-significant ($P > 0.05$), *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. All the P values and statistical analyses used in our study are listed in Supplementary Table 1. The number of animals per experimental group were determined in relation to statistical difference observed in our previous studies, and specified in the figure legends. Mouse hearing tests were conducted with at least five different animals. All hearing tests in this study exhibited statistical power between 0.8~1.0 (IBM SPSS statistics 22). All other analyses were conducted with at least three independent experiments or samples to minimize statistical error.

Role of the funding source

The funders did not have any role in the design of the study; the collection, analysis, or interpretation of the data; writing of the report; or the decision to submit the paper for publication.

Results

Generation of CAPS-associated mice conditionally expressing NLRP3 mutant in CX3CR1 cells

To observe the role of CAPS mutation in inflammation-induced hearing loss, we first generated a mouse model that conditionally expresses NLRP3 mutant (D301N), which corresponds to human D303N mutation identified in the NOMID patients,[25,26] in CX3CR1-positive monocytes or macrophages (Figure 1a). Tamoxifen (TAM) treatment caused a deletion of floxed neomycin cassette in CX3CR1^{+} (YFP^{+}) cells alone, which led to an expression of NLRP3 (D301N) in NLRP3 mutant mice (*Cx3cr1*$^{creER/+}$; *Nlrp3*$^{+/flox}$) but not in control mice (*Cx3cr1*$^{creER/+}$; *Nlrp3*$^{+/+}$) (Figures 1a-c). After tamoxifen treatment, YFP^{+} cells obtained from mutant mice exhibited a complete deletion of neomycin cassette (Figure 1d). Next, we checked whether NLRP3 mutant-expressing macrophages are more responsive to NLRP3 inflammasome agonist than wild type NLRP3-harboring macrophages. Inflammasome activation is normally assessed by the presence of cleaved caspase-1 (p20) and mature interleukin-1β (IL-1β) in the supernatants of cultured cells. LPS plus ATP stimulation, a well-known NLRP3 activator, caused a strong caspase-1 and IL-1β cleavage in control and mutant mouse bone marrow-derived macrophages (BMDMs) irrespective of tamoxifen treatment (Figure 1e). However, tamoxifen-induced expression of mutant NLRP3 (D301N) in one allele of mouse BMDMs exhibited a robust caspase-1 and IL-1β cleavage in response to LPS stimulation alone, while control BMDMs harboring a wild-type NLRP3 or tamoxifen-untreated mutant BMDMs showed no response in this condition (Figure 1e). Thus, we employed these mice expressing gain-of-function NLRP3 mutant in CX3CR1 cells following tamoxifen treatment as an auto-inflammatory disease model. Further, CX3CR1+ macrophages were indeed observed in the cochlea of NLRP3 mutant mice under resting and LPS-mediated inflammatory condition (Figure 1f). The population of CX3CR1 cells were approximately 60% of F4/80-positive macrophages in the cochlea of LPS-treated NLRP3 mutant mice (Figure 1g and S1).

LPS injection into the middle ear cavity drives monocyte infiltration and inflammasome activation in the cochlea of NLRP3 mutant mice

To induce cochlear inflammation-mediated hearing loss, we optimized a direct lipopolysaccharide (LPS) injection model into the middle ear cavity (Figure 2a). PBS injection into the other side (middle ear cavity of the right ear) was used as a control for the injection procedure (Figure 2a). Next, we examined whether the middle ear LPS-injection induced the infiltration of myeloid cells into the cochlea. We analyzed monocyte/macrophage populations in the cochlea using a pan-macrophage marker, F4/80 staining. Resident F4/80+ macrophages were detected in the cochlea irrespective of the LPS injection to a certain degree (Figure 2b). The population of F4/80+ macrophages was significantly increased by LPS injection in the cochlea of NLRP3 mutant-expressing mice (P=0.02; Figure 2c). In control mice, we found increased number of macrophages that did not reach statistical significance (P=0.07). Also, there was no significant difference in the cochlear F4/80+ macrophages between LPS-treated control and mutant mice (P=0.33; Figure 2c). Since LPS administration to middle ear cavity caused myeloid cell infiltration into the cochlea, we checked the permeability of blood-labyrinth barrier by measuring the leakage of FITC-labeled dextran, intravenously injected into the cochlea 1 h before sacrifice. LPS injection in control mice caused only a slight insignificant increase in dextran fluorescence compared to PBS injection (P=0.07; Figures 2d, e). In contrast, LPS injection in mutant mice significantly increased dextran fluorescence in the whole cochlea, including organ of Corti, stria vascularis, and spiral ganglia (P<0.001; Figures 2d, e). These findings indicate that LPS injection into the middle ear cavity resulted in a more severe proinflammatory condition in NLRP3 mutant-expressing cochlea than in control cochlea.

Subsequently, to confirm whether middle ear LPS administration induces inflammasome activation inside cochlea, we isolated cochlea fraction. Robust cleavage of IL-1β, the most reliable indicator of inflammasome

a

	Genotype	NLRP3 expression			
		No tamoxifen		tamoxifen	
		CX3CR1 cells	Other cells	CX3CR1 cells	Other cells
Control	*Cx3cr1* $^{creER/+}$ *Nlrp3* $^{+/+}$	WT/WT	WT/WT	WT/WT	WT/WT
Mutant	*Cx3cr1* $^{creER/+}$ *Nlrp3* $^{+/flox}$	WT/ -	WT/ -	WT/**D301N**	WT/ -

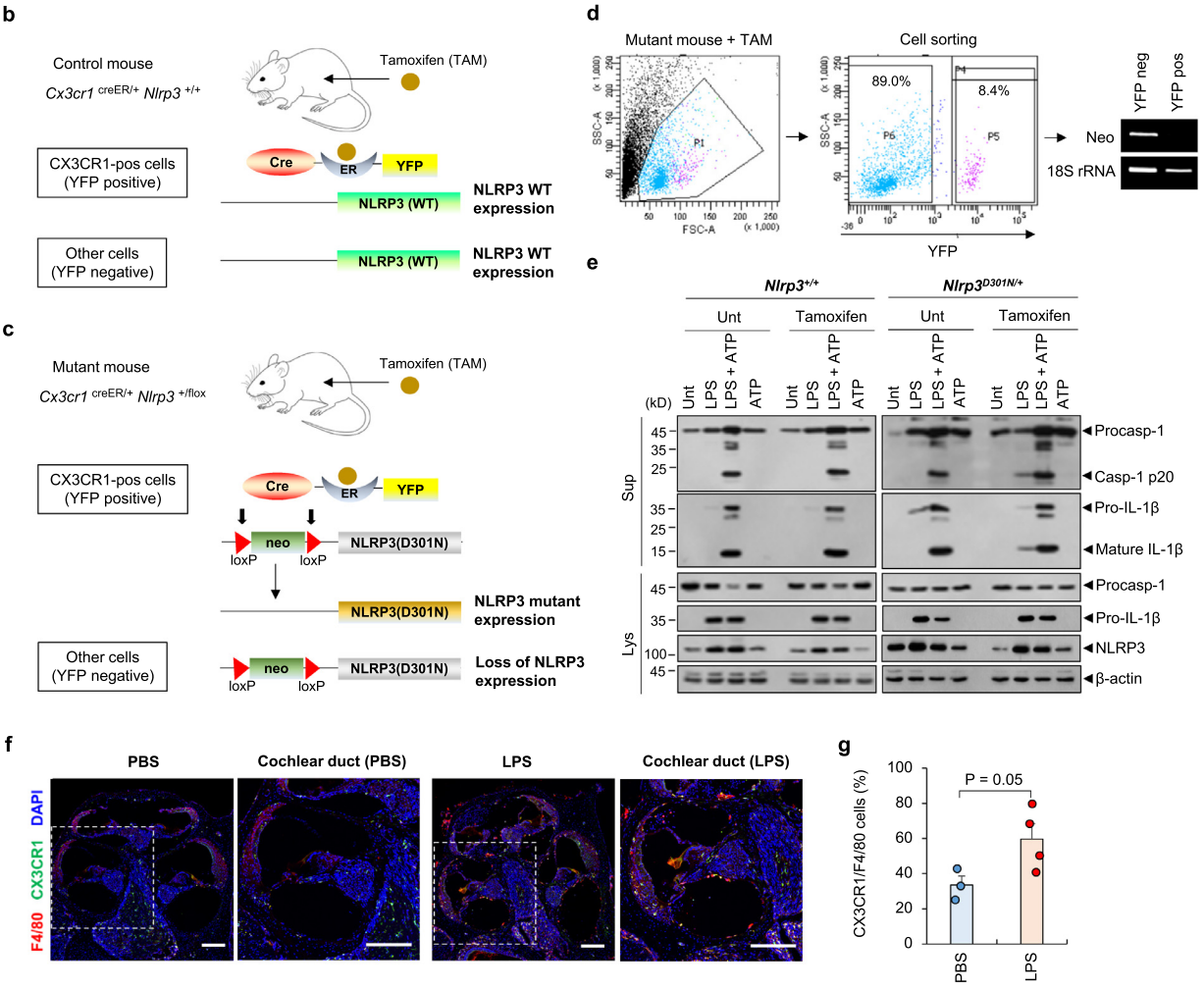


Figure 1. Generation of CAPS-related mice conditionally expressing NLRP3 mutant in CX3CR1 cells. (**a**) Summary of *Nlrp3* gene expression in CX3CR1 cells and other cell types of control and NLRP3 mutant mice upon tamoxifen treatment. (**b, c**) Schematic diagram of tamoxifen-induced deletion of neomycin cassette and NLRP3 mutant expression in CX3CR1 cells of NLRP3 mutant mice. (**d**) Flow cytometric analysis of brain cells obtained from NLRP3 mutant (*Cx3cr1*$^{creER/+}$; *Nlrp3*$^{+/flox}$) mice following tamoxifen intraperitoneal injection (75 mg/kg for 5 days). CX3CR1-YFP^{+} or CX3CR1-YFP^{-} cells were collected using a FACS Aria III cell sorter (BD), and DNA was extracted from each cell and assayed by RT-PCR using a probe detecting neomycin and 18S rRNA. (**e**) Immunoblots from control (*Cx3cr1*$^{creER/+}$; *Nlrp3*$^{+/+}$) and mutant (*Cx3cr1*$^{creER/+}$; *Nlrp3*$^{+/flox}$) mouse BMDMs untreated (Unt) or incubated with 4-hydroxytamoxifen (10 μM, 24 h), followed by treatment with LPS alone (0.5 μg/ml, 6 h), ATP alone (2.5 mM, 30 min), or LPS + ATP. Cell culture supernatants (Sup) or cell lysates (Lys) were immunoblotted with the indicated antibodies. (**f**) Representative immunofluorescence image of cochlea sections in PBS- or LPS-treated *Nlrp3* mutant mice after staining with anti-F4/80 (red) and anti-GFP antibody detecting YFP+ cells (green). Scale bars, 200 μm. (**g**) Quantification of YFP+ cells among F4/80+ cells as calculated by Zen software in the confocal images as shown in (f). Data were analyzed using unpaired *t* test. (*n* = 3 or 4).

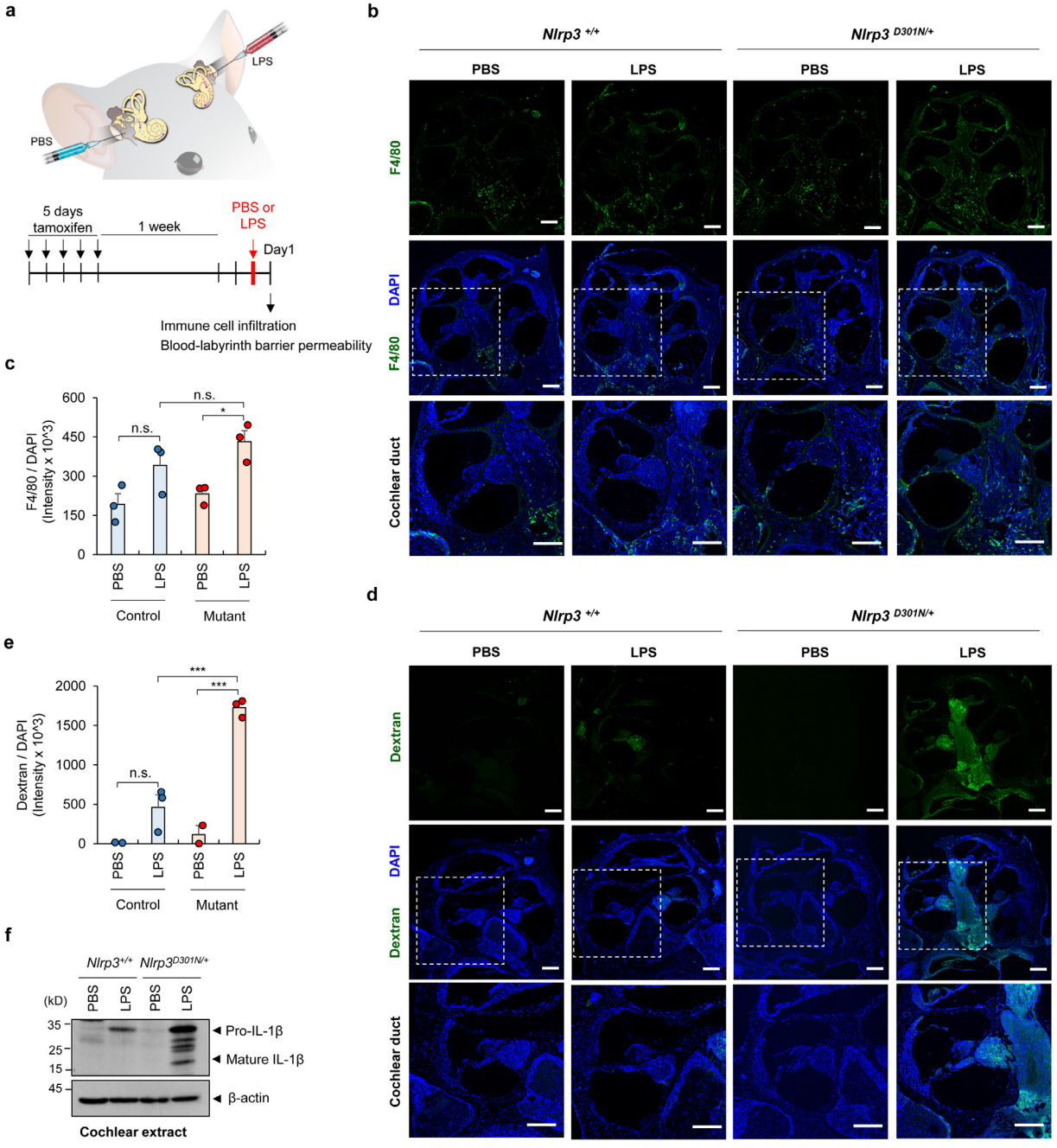


Figure 2. LPS injection into middle ear cavity causes macrophage infiltration into cochlea and blood-labyrinth barrier disruption. (**a**) Schematic diagram (upper panel) and injection schedule (lower panel) for LPS injection into the middle ear cavity. Mice were intraperitoneally injected with tamoxifen (75 mg/kg) once a day for five consecutive days and then were allowed to rest for one week before LPS injection into the middle ear cavity. The left ear was injected with LPS (25 µg per mice) and the right ear was injected with PBS as a control for the injection procedure. (**b**) Representative immunofluorescence image of cochlea sections in PBS- or LPS-treated control and *Nlrp3* mutant mice after staining with anti-F4/80 antibody (green). DAPI represents nuclear signal (blue). Scale bars, 200 µm. (**c**) Quantification of F4/80-positive cells per DAPI in the confocal images as shown in (b). ($n = 3$) (**d**) Representative immunofluorescence image of cochlea sections of PBS- or LPS-treated control and *Nlrp3* mutant mice injected with FITC-labeled dextran. DAPI represents nuclear signal (blue). Scale bars, 200 µm. (**e**) Quantification of dextran-containing cells per DAPI in the confocal images as shown in (d). ($n = 3$) (**f**) Immunoblots from cochlea tissue extracts of PBS- or LPS-treated control and NLRP3 mutant mice. Data were analyzed using two-way ANOVA with Bonferroni corrections (*$P < 0.05$, ***$P < 0.001$, n.s. non-significant).

activation, was clearly detected in the cochlear extract of NLRP3 mutant-expressing mice upon LPS injection, but not in those of control mice, as observed by immunoblotting (Figure 2f). These results indicate that middle ear LPS injection triggered a specific inflammasome activation in the cochlea of CAPS-associated NLRP3 mutant mice.

Hearing loss model by LPS injection into middle ear cavity

Next, we checked the vulnerability of the CAPS mouse to cochlear inflammation-triggered hearing loss in direct LPS injection into middle ear cavity (Figure 3a). Hearing function was examined by measuring the thresholds of auditory brainstem responses (ABRs) in response to the click stimuli of broadband mixed sounds or pure tone stimuli at individual frequencies. ABR thresholds were examined in the left and right ears injected with LPS and PBS, respectively, in control or mutant mice 1 day before injection and 1 and 7 days after injection (Figure 3a). ABR thresholds for click stimuli were significantly elevated at day 1 post-injection in LPS-injected ears compared to PBS-injected ears in both control and mutant mice ($P<0.001$; Figure 3b), indicating that LPS injection into the middle ear cavity causes hearing loss in both control and mutant mice. However, at day 7 post-injection, the ABR thresholds were increased in PBS-injected ears and decreased in LPS-injected ears, showing no significant difference between LPS- and PBS-injected ears in both control and mutant mice ($P>0.99$; Figure 3b). Similar changes were observed for ABR thresholds of pure tone stimuli, which were significantly increased at most frequencies at day 1 post-injection with LPS compared to PBS injection in both control (Figure S2a) and mutant (Figure S2b) mice. However, at day 7 post-injection, ABR thresholds in LPS-injected ears were not significantly different from those in PBS-injected ears at all frequencies in in both control (Figure S2a) and mutant (Figure S2b) mice. These results indicate that LPS injection into the middle ear cavity causes acute hearing loss (at day 1 post-injection), which is alleviated after 7 days in both control and mutant mice.

We subsequently examined whether the changes in hearing sensitivity assessed by ABR threshold shifts are associated with defective outer hair cell function by measuring the thresholds of distortion product otoacoustic emissions (DPOAEs), which reflect cochlear amplifier function of outer hair cells. Similar to ABR threshold changes, DPOAE thresholds are significantly elevated at all frequencies at day 1 post-injection with LPS compared to PBS in both control (Figure S2c) and mutant mice (Figure S2d). DPOAE thresholds decreased in LPS-injected ears at day 7 post-injection and became similar to those in PBS-injected ears in both control and mutant mice (Figure S2c, S2d). These results suggest that the changes in hearing sensitivity caused by the middle ear cavity injection are closely associated with defective outer hair cell function.

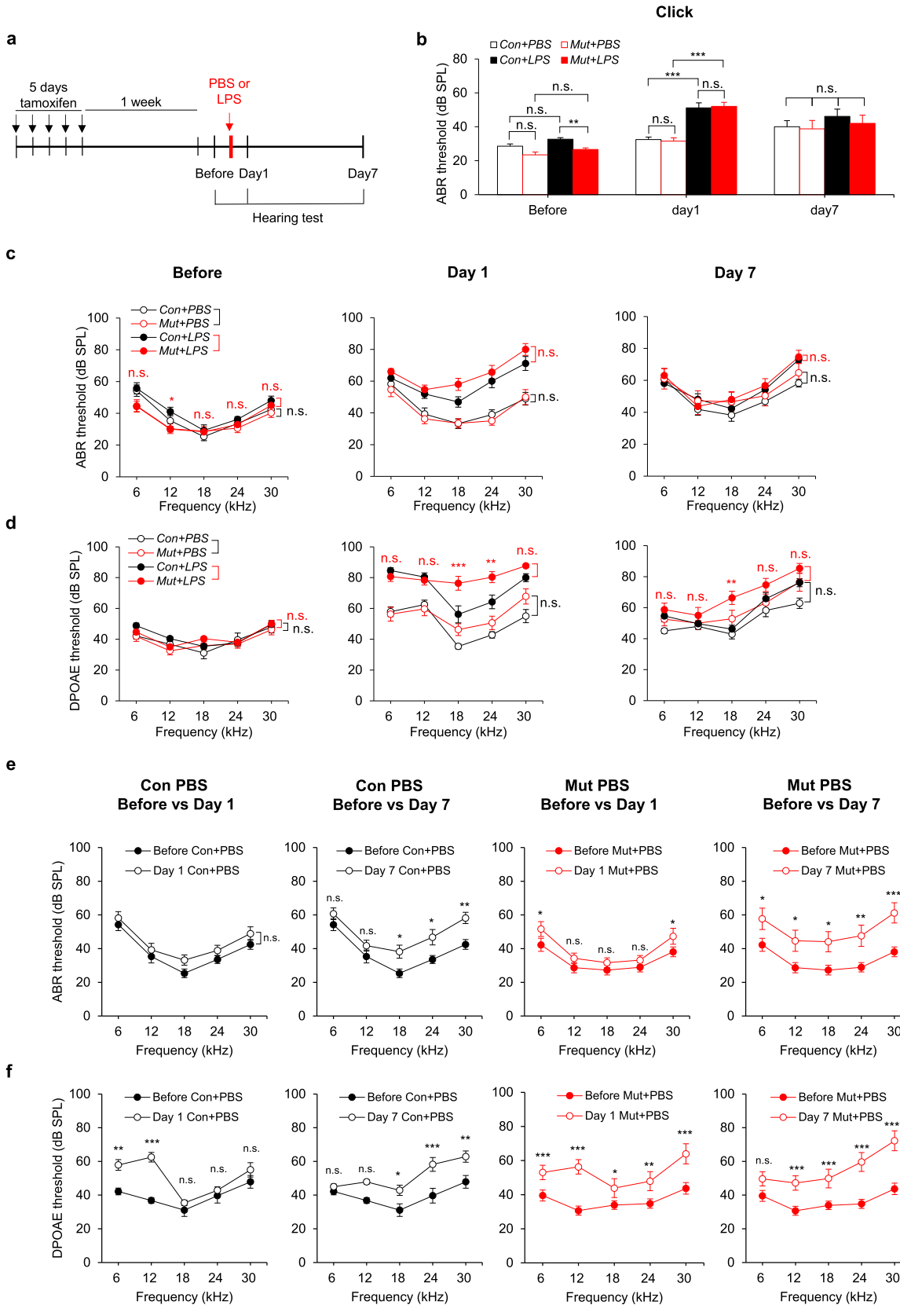
Since LPS injection into the middle ear cavity caused hearing loss not only in mutants but also in control mice, we further analyzed our ABR and DPOAE results to check whether hearing loss induced by LPS injection was more severe in mutant mice compared to controls. Contrary to the results of dextran leakage and inflammasome activation (Figure 2e, 2f), there was no significant difference in ABR thresholds at all frequencies between LPS-injected control and mutant mice at days 1 and 7 post-injection (Figure 3c). However, the elevation of DPOAE thresholds by LPS injection were significantly higher in some frequencies in mutants compared to controls at days 1 and 7 post-injection (Figure 3d). In contrast to LPS-injected groups, DPOAE thresholds were not significantly different between PBS-injected control and mutant mice at days 1 and 7 post-injection (Figure 3d).

Collectively, these results indicate that LPS injection into the middle ear cavity induces acute hearing loss in both control and mutant mice. However, since PBS injection into the middle ear cavity also caused significant elevation of ABR and DPOAE thresholds at several frequencies at day 7 post injection (Figures 3e, 3f and S3), the hearing loss appears to be attributed to middle ear injection procedures, which cause damages in ear tissues such as tympanic membrane puncture.

Hearing loss model by systemic LPS injection

Our results indicate that even though hearing loss is induced by LPS injection into the middle ear cavity, the severity of hearing loss did not differ significantly between control and mutant mice, suggesting that hearing loss is aggravated by damages that occur in the outer and middle ears during injection procedures such as tympanic membrane puncture and middle ear cavity overflow. Thus, we developed a peripheral inflammation-induced hearing loss model by intraperitoneal LPS injection, which does not directly damage outer and middle ear structures. After testing various injection schemes, we adopted a repetitive LPS injection scheme: three times every other day with different concentrations (Figure 4a). Unlike the middle ear cavity injection, systemic LPS injection resulted in a significant elevation of ABR and DPOAE thresholds only in mutant mice but not in wild type mice (Figures 4b-c).

Next, we checked whether systemic LPS injection can trigger the infiltration of myeloid cells into cochlea. LPS-mediated peripheral inflammation caused a marked increase in F4/80 macrophages in the cochlea of CX3CR1-specific NLRP3 mutant mice ($P<0.001$) but not in the control mice ($P=0.08$; Figure 4d-e). Subsequently, we examined whether systemic LPS injection-mediated hearing loss in our scheme is associated with


a
5 days
tamoxifen
1 week
PBS or
LPS
Before
Day1
Day7
Hearing test
b
Click
Con+PBS
Mut+PBS
Con+LPS
Mut+LPS
ABR threshold (dB SPL)
Before
day1
day7
c
Before
Day 1
Day 7
ABR threshold (dB SPL)
Frequency (kHz)
d
DPOAE threshold (dB SPL)
Frequency (kHz)
e
Con PBS
Before vs Day 1
Con PBS
Before vs Day 7
Mut PBS
Before vs Day 1
Mut PBS
Before vs Day 7
Before Con+PBS
Day 1 Con+PBS
Day 7 Con+PBS
Before Mut+PBS
Day 1 Mut+PBS
Day 7 Mut+PBS
ABR threshold (dB SPL)
Frequency (kHz)
f
DPOAE threshold (dB SPL)
Frequency (kHz)

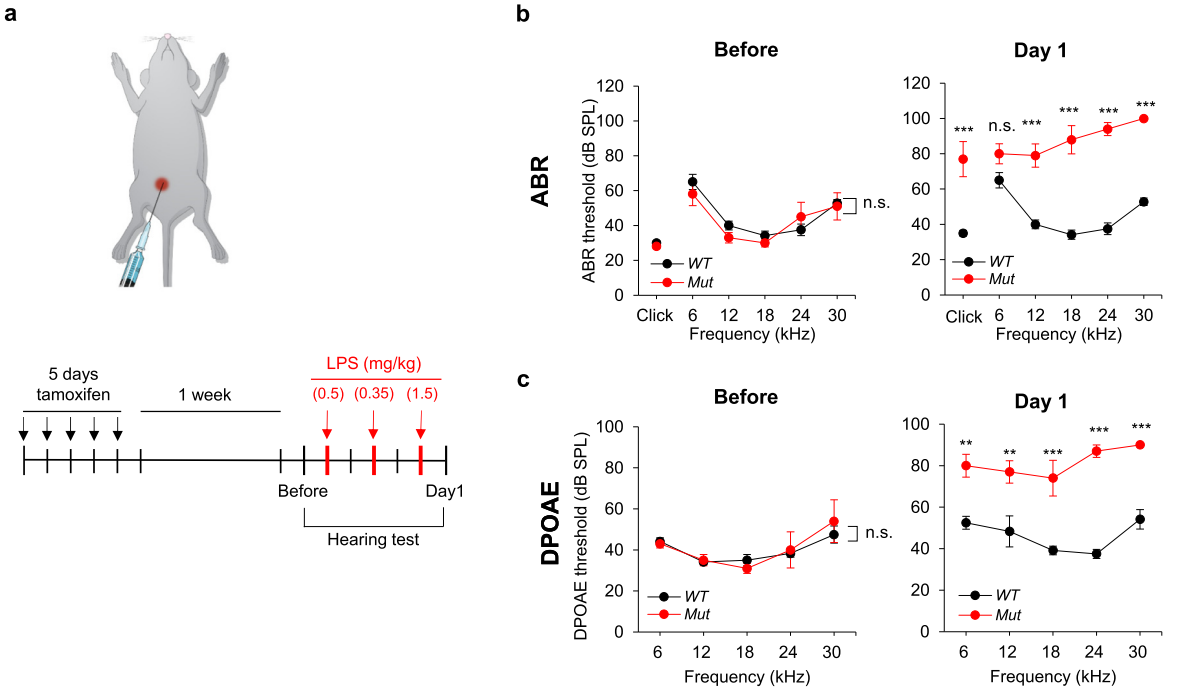
caspase-1-inflammasome activation inside cochlea. *In vivo* caspase-1 activity inside the mouse cochlea was measured using a selective caspase-1-activatable probe, which emits Cy5.5 fluorescence only in the presence of active caspase-1.[22] To exclude false signals from surrounding tissues, we isolated a whole cochlea from control and mutant mice and measured fluorescence using an IVIS intravital imaging system. We observed that systemic LPS injection led to a robust caspase-1 activation in the cochlea of NLRP3 mutant mice, which was much less in control mice (Figure 4f). The caspase-1 fluorescence intensity was significantly higher in LPS-injected mutant mice than LPS-injected control mice (P=0.02; Figure 4g), indicating that the increase in caspase-1 activity is more robust in mutant mice due to excessive NLRP3 inflammasome activation. Consistent with this result, systemic inflammation caused a robust IL-1β maturation in the cochlea of NLRP3 mutant mice but not of control mice (Figure 4h). These results indicate that peripheral inflammation by systemic LPS injection can trigger a robust inflammasome activation in the cochlea in an NLRP3-dependent manner.

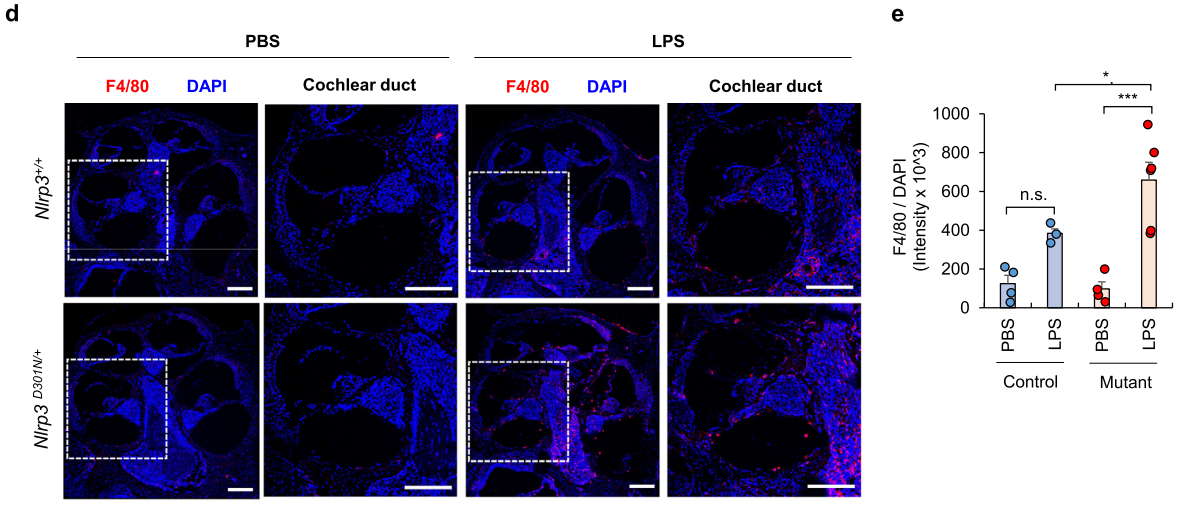
Cochlea-resident and infiltrating macrophages contribute to hearing loss induced by systemic LPS injection in NLRP3 mutant mice

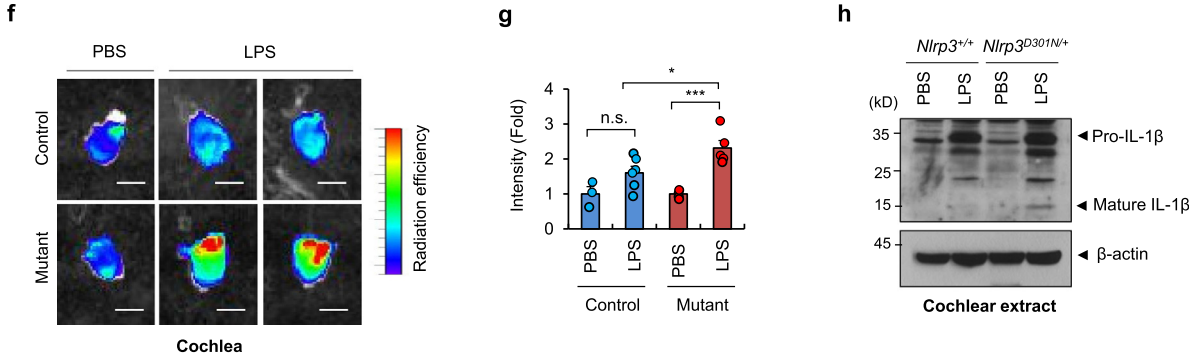
Next, we determined whether cochlea-resident macrophages or infiltrating monocytes/macrophages contributed to hearing loss induced by systemic LPS injection. Cochlea-resident macrophages are considered yolk sac- or fetal liver-derived cells, which have a half-life longer than one month.[27,28] In contrast, cochlea-infiltrating monocytes are bone marrow-derived cells, which can survive for less than a month.[29] Thus, we expected that 1−2 weeks after tamoxifen treatment, mutant forms of NLRP3 protein would be expressed in both cochlea-resident and infiltrating macrophages. In contrast, 4−5 weeks after tamoxifen treatment, long-lived cochlea-resident macrophages would still survive and continue to express the mutant NLRP3 protein, whereas short-lived monocytes would die and newly born infiltrating monocytes would express only wild-type NLRP3 protein. On this basis, we investigated the relative contribution of resident and infiltrating macrophages to NLRP3-inflammasome-induced hearing loss by comparing the severity of hearing loss in systemic LPS-injected mutant mice, 2 or 5 weeks after tamoxifen treatment (Figure 5a).

On measuring ABR thresholds for click stimuli broadband mixed sounds 2 weeks after tamoxifen treatment, ABR thresholds of LPS-treated group were significantly increased at day 1 post-injection compared to PBS-treated group (P<0.001), and the increased ABR thresholds persisted until day 7 post-injection (P=0.004; Figure 5b). We observed similar results 5 weeks after tamoxifen treatment, in which ABR thresholds for click stimuli were significantly increased at days 1 (P=0.002) and days 7 (P=0.05) post injection with LPS (Figure 5b). In contrast to click stimuli, ABR thresholds for pure tone stimuli at individual frequencies exhibited different results 2 and 5 weeks after tamoxifen treatment. Two weeks after tamoxifen treatment, ABR thresholds were significantly elevated at all frequencies at day 1 post-injection with LPS (Figure S4a). Although hearing function was slightly recovered after 7 days, the threshold shifts remained significant at all frequencies (Figure S4a). Five weeks after tamoxifen treatment, ABR threshold shifts were significant only at high frequencies such as 18, 24, and 30 kHz but not at lower frequencies such as 6 and 12 kHz at day 1 post-injection with LPS (Figure S4a). ABR thresholds were dramatically decreased after seven days, although threshold shifts at high frequencies were still significant (Figure S4a). DPOAE measurements also showed similar results as those of ABRs; DPOAE thresholds were significantly elevated at day 1 post injection with LPS both 2 and 5 weeks after tamoxifen treatment. Seven days after LPS injection, DPOAE threshold shifts remained significant at all frequencies 2 weeks after tamoxifen injection, while there were partial recoveries 5 weeks after tamoxifen injection at lower but not higher frequencies (Figure S4b). Thus, hearing loss caused by systemic LPS injection appears to be more severe at 2 weeks of tamoxifen injection than 5 weeks. However, when elevated levels of ABR and DPOAE thresholds were compared between 2 and 5 weeks, the differences did not reach statistical significance, although the threshold elevations were generally greater at most frequencies at 2 weeks than 5 weeks (Figures 5c, 5d).

Figure 3. LPS injection into middle ear cavity causes hearing loss in control and NLRP3 mutant-expressing mice. (**a**) Schedule for LPS middle ear injection model. Hearing function was analyzed 1 day before middle ear injection (Before) and on days 1 and 7 after LPS injection (Day 1 and Day 7). (**b**) ABR thresholds in response to click stimuli of broadband mixed sounds in control mice injected with PBS or LPS and in mutant mice injected with PBS or LPS. (**c**) Comparisons of ABR thresholds in response to pure tone stimuli at individual frequencies between PBS- or LPS-injected control and mutant mice. (**d**) DPOAE thresholds in response to the primary tones set at a frequency ratio (f_2/f_1) of 1.2 with target frequencies at 6, 12, 18, 24, and 30 kHz. **(e, f)** ABR (e) or DPOAE (f) thresholds for pure tone stimuli were re-plotted to compare observations before and 1 day after PBS injection and those before and 7 days after PBS injection. (b-f) control mice injected with PBS (n = 14) or LPS (n = 13); mutant mice injected with PBS (n = 16) or LPS (n = 15). Values and error bars reflect means ± S.E.M. Statistical comparisons were determined using two-way ANOVA with Bonferroni corrections for multiple comparisons (*P < 0.05, **P < 0.01, ***P < 0.001, n.s. non-significant).


a
5 days
tamoxifen
1 week
LPS (mg/kg)
(0.5) (0.35) (1.5)
Before
Day1
Hearing test
b
Before
Day 1
ABR
ABR threshold (dB SPL)
WT
Mut
n.s.
***
Click 6 12 18 24 30
Frequency (kHz)
c
DPOAE
DPOAE threshold (dB SPL)
**


d
PBS
LPS
F4/80
DAPI
Cochlear duct
Nlrp3+/+
Nlrp3 D301N/+
e
F4/80 / DAPI
(Intensity x 10^3)
n.s.
*
***
PBS
LPS
Control
Mutant


f
PBS
LPS
Control
Mutant
Radiation efficiency
Cochlea
g
Intensity (Fold)
n.s.
*
***
Control
Mutant
h
Nlrp3+/+
Nlrp3D301N/+
(kD)
35
25
15
45
Pro-IL-1β
Mature IL-1β
β-actin
Cochlear extract

Then, we assessed myeloid cell infiltration into cochlea and dextran leakage in systemic LPS-treated mice with 2 or 5 weeks interval after tamoxifen treatment. The population of F4/80+ cochlear macrophages in LPS-treated mice at 2 weeks after tamoxifen treatment was significantly higher than those with 5 weeks interval (P=0.01; Figures 5e, 5f and S5a). Consistent with these findings, systemic LPS-induced dextran leakage from 2 weeks interval was significantly higher than 5 weeks interval (P=0.005; Figure 5e, 5g and S5b). These results suggest that myeloid cell infiltration and blood-labyrinth barrier disruption are more severe at 2 weeks after tamoxifen treatment when both resident and infiltrating macrophages express mutant NLRP3 proteins than 5 weeks when only resident macrophages harbor mutant NLRP3 proteins.

Collectively, these results suggest that although the infiltration of macrophages into the cochlea is a key feature observed in systemic LPS-injected mutant mice, both infiltrating and resident macrophages contribute to hearing loss induced by excessive activation of NLRP3 inflammasome.

Blockade of NLRP3 inflammasome signaling attenuates systemic LPS-induced cochlear inflammation

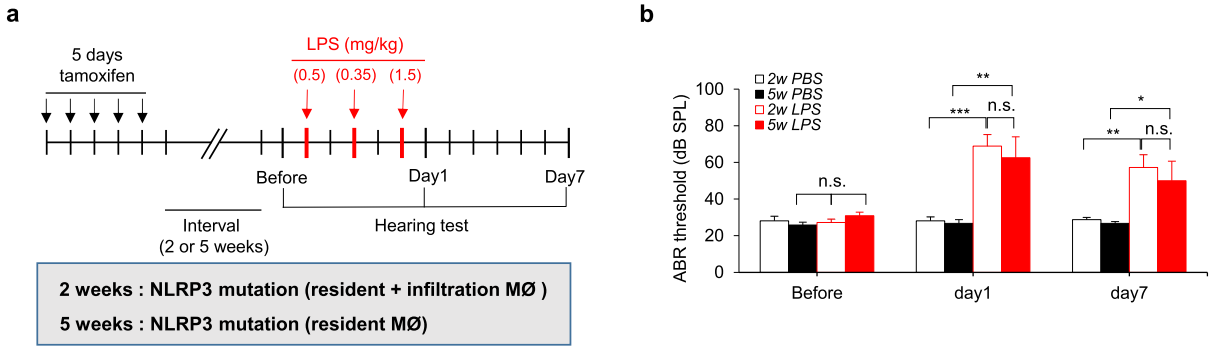
Next, we examined whether the blockade of NLRP3 inflammasome signaling can ameliorate systemic LPS-induced cochlear inflammation using an NLRP3-specific inhibitor MCC950, which directly blocks NACHT domain of NLRP3,[30] and an IL-1 receptor antagonist (IL-1RA), which inhibits the binding of IL-1β (a major inflammasome product) to IL-1 receptor. MCC950 treatment clearly abrogated LPS- and LPS/ATP-triggered caspase-1 activation in NLRP3 mutant-expressing BMDMs (Figure 6a). Further, intraperitoneal injection of MCC950 significantly reduced IL-1β level in the serum of NLRP3 mutant mice when challenged with systemic LPS injection (P=0.02; Figure 6b). Systemic LPS injection resulted in weight loss in both control and NLRP3 mutant mice, with the mutant mice displaying a more severe phenotype than the control mice (Figure S6a). Both IL-1RA and MCC950 significantly alleviated systemic LPS-induced weight loss in NLRP3 mutant mice (Figure S6b).

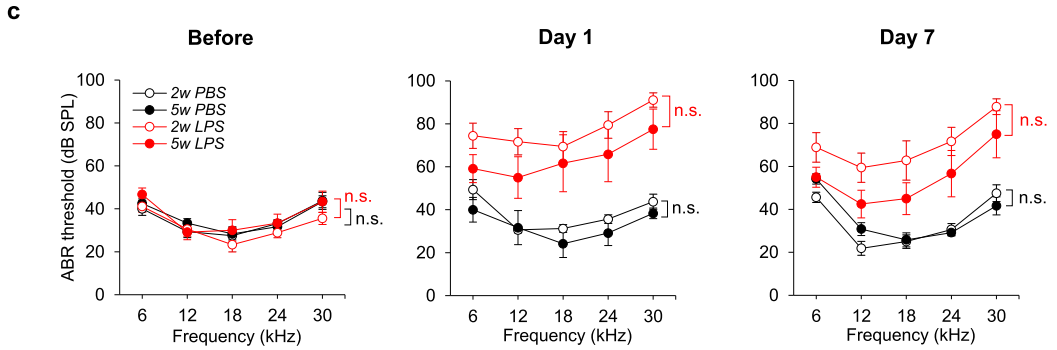
Subsequently, we checked the efficacy of MCC950 and IL-1RA on the systemic LPS injection-induced cochlear inflammatory phenomenon such as monocyte infiltration and blood-labyrinth barrier disruption. IL-1RA or MCC950 was administered daily during the systemic LPS injection period (Figure 6c). The fluorescent intensity of F4/80 representing the macrophage population, which was remarkably increased by systemic LPS injection, was significantly decreased by IL-1RA or MCC950 treatment (P<0.001; Figures 6d, e & S7a). Consistently, the blockade of NLRP3 inflammasome signaling significantly reduced systemic LPS-driven leakage of rhodamine-dextran into the cochlea of NLRP3 mutant mice (P=0.004, IL-1RA; P<0.001, MCC950; Figures 6f, g & S7b). These results indicate that inflammasome-dependent IL-1β production plays a critical role in systemic inflammation-induced blood-labyrinth barrier disruption and subsequent macrophage infiltration into the cochlea.

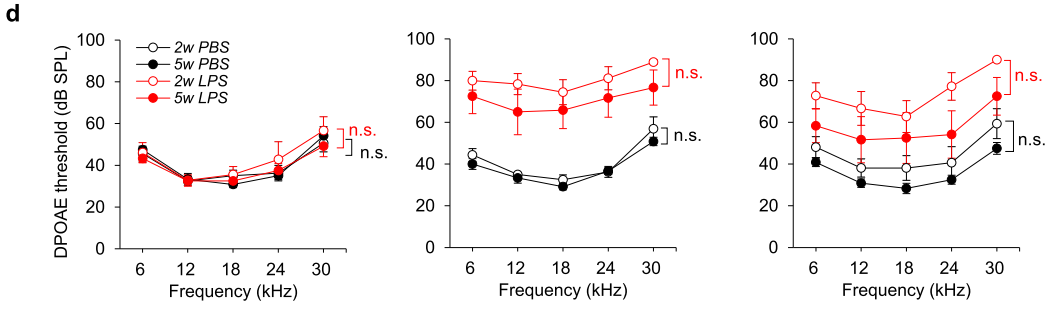
Blockade of NLRP3 inflammasome signaling attenuates systemic LPS-induced hearing loss in NLRP3 mutant-expressing mice

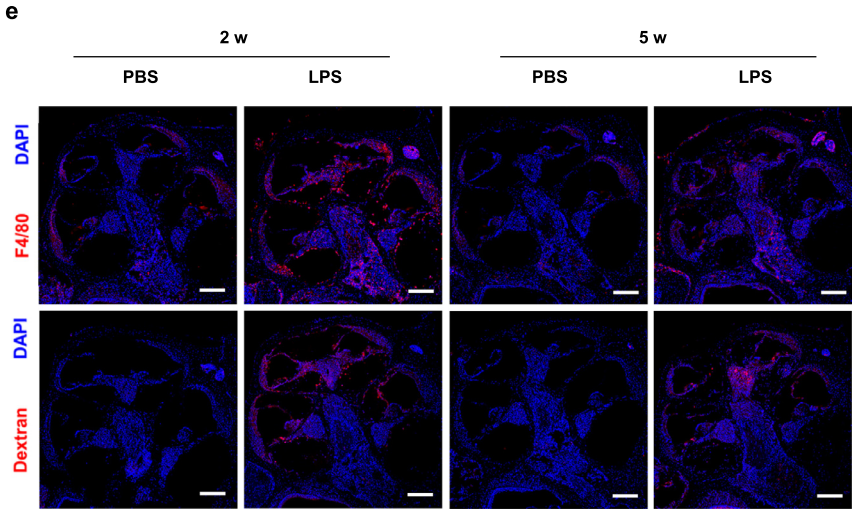
Next, we checked whether the selective inhibition of NLRP3 by MCC950 treatment can alleviate hearing loss induced by systemic LPS injection. IL-1RA, a well-known medication for CAPS,[19] was used as a positive control to compare the therapeutic effect of MCC950. Similar to the scheme as shown in Figure 6c, IL-1RA or MCC950 was administered daily during the systemic LPS injection period and hearing function was measured 1 day and 7 days after final LPS injection (Figure 7a). ABR thresholds for click stimuli of

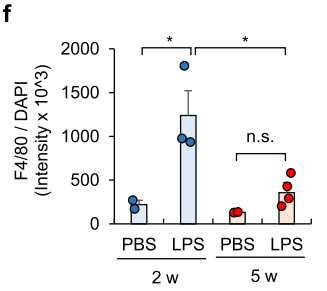
Figure 4. Systemic LPS injection causes severe hearing loss in NLRP3 mutant mice associated with inflammasome activation. (**a**) Schematic diagram and injection scheme for LPS-mediated systemic inflammation. Mice were administered with tamoxifen via IP-injection for 5 days, allowed to rest for a week, and then intraperitoneally injected with LPS three times every other day with different concentrations. (**b**) ABR thresholds in response to click stimuli and pure tone stimuli at individual frequencies in control and mutant mice before and 1 day after LPS injection. (**c**) DPOAE thresholds before and 1 day after LPS injection. Values and error bars reflect means ± S.E.M. (b, c) control WT mice (n = 4, 8 ears); mutant mice (n = 3, 6 ears). (b, c) Statistical comparisons were determined using two-way ANOVA with Bonferroni corrections for multiple comparisons (**P < 0.01, ***P < 0.001, n.s. non-significant). (**d**) Representative immunofluorescence image of cochlea sections in PBS- or LPS-treated control and *Nlrp3* mutant mice after staining with anti-F4/80 antibody (red). DAPI represents nuclear signal (blue). Scale bars, 200 µm. (**e**) Quantification of F4/80-positive cells per DAPI in the confocal images as shown in (d). control mice injected with PBS (n = 4) or LPS (n = 3); mutant mice injected with PBS (n = 4) or LPS (n = 6). (**f**) *Ex vivo* imaging of active caspase-1 in the cochlea tissue of control and NLRP3 mutant mice injected with caspase-1-activatable probe. Scale bars, 2 mm. (**g**) Quantification of caspase-1-dependent fluorescence as in (f). control mice injected with PBS (n = 3) or LPS (n = 6); mutant mice injected with PBS (n = 5) or LPS (n = 5). (e, g) Data were analyzed using two-way ANOVA with Bonferroni corrections (*P < 0.05, ***P < 0.001, n.s. non-significant). (**h**) Immunoblots from cochlea tissue extracts of PBS- or LPS-treated control and NLRP3 mutant mice.

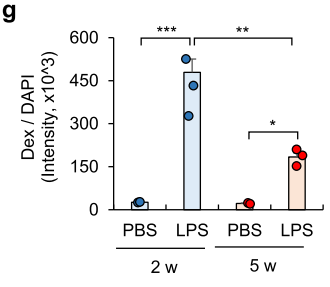

a
5 days
tamoxifen
LPS (mg/kg)
(0.5) (0.35) (1.5)
Before
Day1
Day7
Interval
(2 or 5 weeks)
Hearing test
2 weeks : NLRP3 mutation (resident + infiltration MØ)
5 weeks : NLRP3 mutation (resident MØ)
b
2w PBS
5w PBS
2w LPS
5w LPS
ABR threshold (dB SPL)
n.s.
Before
day1
day7


c
Before
Day 1
Day 7
2w PBS
5w PBS
2w LPS
5w LPS
ABR threshold (dB SPL)
Frequency (kHz)
n.s.


d
2w PBS
5w PBS
2w LPS
5w LPS
DPOAE threshold (dB SPL)
Frequency (kHz)
n.s.


e
2 w
5 w
PBS
LPS
DAPI
F4/80
Dextran


f
F4/80 / DAPI
(Intensity x 10^3)
n.s.
PBS
LPS
2 w
5 w


g
Dex / DAPI
(Intensity, x10^3)
PBS
LPS
2 w
5 w

broadband mixed sounds, which were elevated by LPS injection, were slightly decreased following IL-1RA treatment at days 1 and 7 post-injection, but this decrease was not statistically significant (P=0.42, day 1; P=0.15, day 7; Figure 7b). In contrast, MCC950 treatment significantly reduced ABR thresholds for click stimuli elevated by LPS injection at day 1 post-injection (P=0.008), and this therapeutic effect on hearing loss was maintained until day 7 post-injection (P=0.05; Figure 7b).

The therapeutic effects of IL-1RA and MCC950 were also examined for individual frequencies. IL-1RA treatment significantly reduced ABR thresholds elevated by systemic LPS injection at all frequencies except for 6 kHz at day 1 post injection (Figure 7c, green asterisks). ABR thresholds were further reduced at day 7 post-injection to a level, which was not significantly different from PBS-injected controls at some but not all frequencies (Figure 7c). DPOAE thresholds elevated by LPS were also decreased by IL-1RA treatment to the control level at some but not all frequencies at day 7 post-injection (Figure 7d). These results indicate that inhibiting IL-1β binding to its receptor can alleviate hearing loss induced by excessive inflammasome activation, but not to control levels at all frequencies. MCC950 treatment also alleviated hearing loss induced by LPS-injection at day 1 post injection at some frequencies (Figure 7c). However, at day 7 post-injection, MCC950 treatment further reduced ABR thresholds at all frequencies to levels not significantly different from PBS-injected controls (Figure 7c). DPOAE thresholds were also reduced at most frequencies by MCC950 treatment to levels not significantly different from PBS-injected controls at day 7 post-injection (Figure 7d). These results suggest that MCC950 treatment can effectively alleviate hearing loss induced by excessive NLRP3 inflammasome activation.

Discussion

Inner ear was previously considered an immune-privileged organ protected by the blood-labyrinth barrier.[31] However, recent studies have challenged this concept by revealing the presence of cochlea-resident macrophages and antigen-specific immune responses in the inner ear.[32,33] In this context, cochlear inflammation and immune response are thought to be major contributors of hearing impairment under certain circumstances. Sensorineural hearing loss is a common recurrent symptom of CAPS, a group of autoinflammatory diseases caused by an autosomal dominant mutation in NLRP3.[17–19] However, there are no biochemical studies demonstrating a direct association between CAPS-related NLRP3 inflammasome and sensorineural hearing loss. It is thus useful to investigate whether a gain-of-function NLRP3 mutation can impair hearing ability in the cochlea.

Unfortunately, there are no proper mouse models to study the pathogenesis of CAPS-associated hearing loss. Expression of CAPS-associated NLRP3 mutant in myeloid cells using LysM-cre mice caused growth retardation and lethality of NLRP3 mutant knock-in mice 2–3 weeks after birth,[34,35] which precludes proper hearing function tests. To avoid deleterious effects of NLRP3 mutation during the development process, we generated a mouse model that conditionally expresses NLRP3 D301N mutant, observed in NOMID patients, in CX3CR1-positive cells upon tamoxifen injection. CX3CR1+ macrophages were reported to be a major immune cell population in the cochlea.[36] Thus, CX3CR1-targeted expression of NLRP3 mutant can provide a useful model to clarify the underlying molecular pathological mechanism through which the CAPS-associated NLRP3 mutation causes an inflammation-mediated sensorineural hearing loss.

Inflammation has been considered a crucial factor for mediating sensorineural hearing loss.[37,38] However, the pathological mechanism underlying inflammation-mediated hearing loss remains unclear. Previous studies showed that inflammation can mediate functional and structural changes in the inner ear, including sensory hair cell damage and fluid imbalance of inner ear.[37,39–41] In the present study, we clearly demonstrated that inflammasome activity is critical for the increased vascular permeability of blood-labyrinth barrier and the subsequent infiltration of myeloid cells under local and systemic inflammatory conditions. We

Figure 5. Comparisons of peripheral inflammation-induced hearing loss in NLRP3 mutant mice 2 and 5 weeks after tamoxifen treatment. (**a**) Experimental schemes to examine the contribution of resident and infiltrating macrophages in inflammasome-induced hearing loss. (**b**) ABR thresholds in response to click stimuli at 2-weeks post-injection with PBS (n = 4) and LPS (n = 5) and at 5-weeks post-injection with PBS (n = 3) and LPS (n = 3). (**c**) ABR thresholds in response to pure tone stimuli at individual frequencies at 2- and 5-weeks post-injection with PBS or LPS. (**d**) DPOAE thresholds at 2- and 5-weeks post-injection with PBS or LPS. Values and error bars reflect means ± S.E.M. (b -d) 2-weeks post-injection with PBS (n = 4, 8 ears) or LPS (n = 5, 9 ears) and 5-weeks post-injection with PBS (n = 3, 6 ears) and LPS (n = 3, 6 ears). (**e**) Representative immunofluorescence image of cochlea sections in PBS- or LPS-treated *Nlrp3* mutant mice 2 or 5 weeks after tamoxifen injection. Cochlea sections were stained with anti-F4/80 antibody (upper panel). Mice were injected with TRITC-dextran 1 h before sacrifice. DAPI represents nuclear signal (blue). Scale bars, 200 µm. (**f, g**) Quantification of F4/80-positive cells (f) or dextran-containing cells (g) per DAPI in the confocal images as shown in (e). (e-g) control mice injected with PBS (n = 2) or LPS (n = 3); mutant mice injected with PBS (n = 2) or LPS (n = 3). Statistical comparisons were determined using two-way ANOVA with Bonferroni corrections for multiple comparisons (*P < 0.05, **P < 0.01, ***P < 0.001, n.s. non-significant).

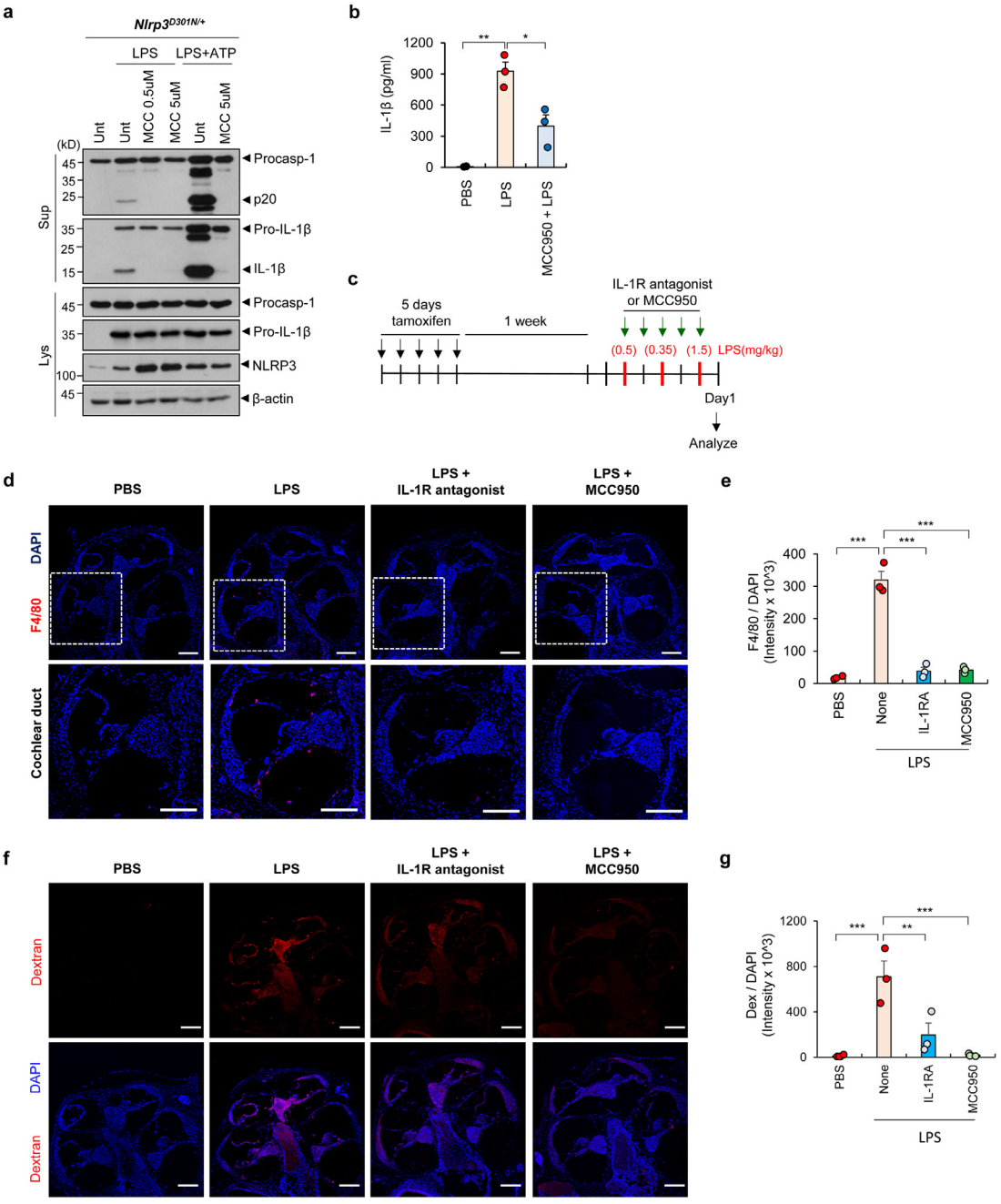
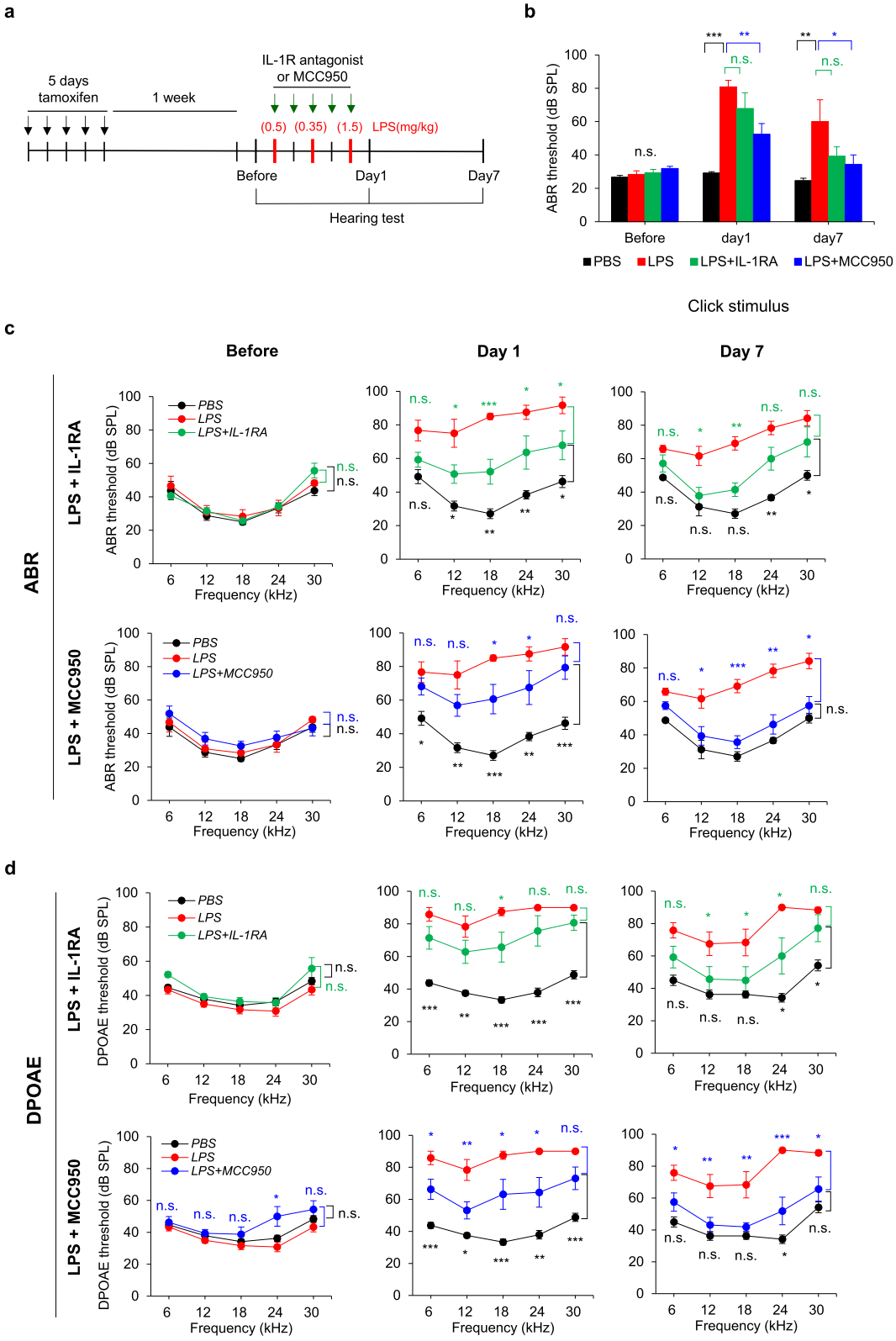


Figure 6. NLRP3 inflammasome inhibitors mitigate systemic LPS injection-mediated macrophage infiltration and blood-labyrinth barrier disruption in NLRP3 mutant mice. (**a**) Immunoblots from *Nlrp3*$^{D301N/+}$ BMDMs treated with 4-hydroxytamoxifen (10 μM, 24 h), followed by the treatment with LPS alone (0.25 μg/mL, 6 h) or LPS plus ATP (2.5 mM, 30 min) in the presence of MCC950 (0.5 μM or 5 μM). (**b**) Quantification of IL-1β in the serum of *Nlrp3*$^{D301N/+}$ mice injected with LPS (0.75 mg/kg, 3 h) and MCC950 (10 mg/kg, 30 min pre-treatment before LPS). (n = 3) (**c**) An experimental scheme to examine the therapeutic effects of IL-1RA and MCC950 on systemic LPS injection-mediated inflammatory phenotypes. (**d**) Representative immunofluorescence images of cochlea tissue in PBS- or LPS-treated *Nlrp3* mutant mice administered with MCC950 (10 mg/kg) or IL-1RA (10 mg/kg) after staining with anti-F4/80 antibody (red). Scale bars, 200 μm. (**e**) Quantification of F4/80 positive cells per DAPI in the confocal images as shown in (d). (n = 3) (**f**) Representative immunofluorescence image of cochlea sections of PBS- or LPS-treated control and *Nlrp3* mutant mice injected with TRITC-dextran. DAPI represents nuclear signal (blue). Scale bars, 200 μm. (**g**) Quantification of dextran-containing cells per DAPI in the confocal images as shown in (f). PBS (n = 4), LPS (n = 3), LPS + IL-1RA (n = 3), and LPS + MCC950 (n = 4). Dextran leakage assay in the cochlea tissue using TRITC-labelled Dextran (1 mg/mouse, 1 h pre-injection, i.v.). Statistical comparisons were determined using one-way ANOVA with Tukey's corrections for multiple comparisons (**$P < 0.01$, ***$P < 0.001$).


a
5 days
tamoxifen
1 week
IL-1R antagonist
or MCC950
(0.5) (0.35) (1.5) LPS(mg/kg)
Before
Day1
Day7
Hearing test
b
ABR threshold (dB SPL)
Before
day1
day7
PBS
LPS
LPS+IL-1RA
LPS+MCC950
Click stimulus
c
Before
Day 1
Day 7
ABR
LPS + IL-1RA
LPS + MCC950
PBS
LPS
LPS+IL-1RA
LPS+MCC950
ABR threshold (dB SPL)
Frequency (kHz)
d
DPOAE
DPOAE threshold (dB SPL)
n.s.

employed two-independent routes (middle ear cavity and intraperitoneal injections) of LPS administration into mice to mimic local and systemic inflammation, respectively. Regardless of the injection route, LPS administration triggered a robust inflammasome activation in the cochlea and profound hearing loss. However, direct middle ear injection method caused LPS-independent hearing impairment after 7 days of injection, most likely due to structural damages that occurred during the injection procedure. Thus, we conclude that systemic LPS administration is a more appropriate model for studying the inflammasome-associated hearing loss phenomenon.

Given that IL-1RA can mitigate the symptoms of CAPS to a certain degree,[12] inflammasome activation may be a key phenomenon for the CAPS pathogenesis. Previous study showed that NLRP3 is expressed in CX3CR1-expressing macrophages in the cochlea.[20] However, it remained elusive whether the inflammasome activation really occurs inside the cochlea under physiological inflammatory conditions. In this context, our results here provide the first *in vivo* evidence for caspase-1 and inflammasome activation induced by the NLRP3 mutant in CX3CR1 macrophages of cochlea in response to repetitive peripheral inflammation. It will be interesting to elucidate whether physiological conditions other than peripheral inflammation such as noise stress and aging also drive cochlear inflammasome activation.

Our results also indicate that hyperactivation of inflammasome signaling by gain-of-function NLRP3 mutation facilitates the impairment of blood-labyrinth barrier and the infiltration of monocytes in response to peripheral inflammation. Furthermore, our data present that NLRP3 mutant-expressing infiltrating cells significantly contribute to peripheral inflammation-induced hearing loss. Therefore, we can infer that excessive NLRP3 inflammasome activity in CX3CR1-positive infiltrating monocytes as well as cochlea-resident macrophages might damage hearing ability via inflammasome-dependent mediators including IL-1β. Particularly, NLRP3-specific inhibitor MCC950 completely blocked the leakage of dextran protein from the circulation under peripheral inflammation condition. In contrast, a recent study proposed that MCC950 failed to inhibit CAPS-associated NLRP3 mutant-mediated caspase-1 and IL-1β processing.[42] However, our data clearly indicate that MCC950 remarkably attenuated LPS or LPS/ATP-induced activation in NLRP3 (D301N)-expressing BMDMs and significantly reduced systemic LPS-induced events in NLRP3 mutant mice. Supporting our findings, Tapia-Abellan et al. showed that MCC950 can close the open conformation of active NLRP3 and block the subsequent activation of autoinflammatory-related NLRP3 mutants.[30] It would be interesting to study whether MCC950 efficacy varies depending on the NLRP3 mutation in a future study. Nevertheless, MCC950 showed more efficient therapeutic potential than IL-1RA in systemic LPS-driven hearing loss. These results suggest that inflammasome-derived mediators other than IL-1β possibly contribute to the hearing impairment induced by peripheral LPS administration.

Despite our efforts to clarify the role of NLRP3 mutants in the peripheral inflammation-induced hearing loss, how inflammasome activation in CX3CR1 cells causes the impairment of outer hair cells or blood-labyrinth barriers and the subsequent hearing loss remains to be fully understood. Furthermore, as we detected systemic changes such as weight loss in NLRP3 mutant mice by systemic LPS injection, we cannot rule out the possibility that other systemic effect such as central nervous system (CNS) defects may impair hearing ability in our model. However, our DPOAE results, which specifically reflect the function of outer hair cells in the inner ear, are closely correlated with ABR results, which reflect hearing sensitivity. Thus, we believe that hearing loss observed in the LPS injected mice is mainly, if not all, due to defective cochlear amplifier function of outer hair cells in the inner ear rather than other systemic effects.

In conclusion, our study provides the first *in vivo* model to mimic hearing loss due to excessive inflammasome activation observed in human patients carrying NLRP3 gain-of-function mutations. Our results validate the practicality of our mouse model by confirming that inhibition of IL-1β receptors can alleviate hearing loss caused by systemic inflammation, and further suggest a more direct and potent therapeutic modality of targeting NLRP3-mediated inflammasome itself.

Contributors

JHM and EL designed, performed, and analyzed the experiments. SHY, HM, JHO and IH performed the experiments and analyzed the data. YS and JHR

Figure 7. NLRP3 inflammasome inhibitors alleviate hearing loss of NLRP3 mutant mice upon systemic LPS injection. (**a**) An experimental scheme to examine the therapeutic effects of IL-1RA and MCC950 on hearing loss induced by systemic inflammasome activation. (**b**) ABR thresholds in response to click stimuli of broadband mixed sounds in mutant mice injected with PBS (n = 7), LPS (n = 3), LPS + IL-1RA (10 mg/kg) (n = 4), and LPS + MCC950 (10 mg/kg) (n = 4). (**c**) ABR thresholds in response to pure tone stimuli at individual frequencies in mutant mice injected with PBS, LPS, LPS + IL-1RA or LPS + MCC950. (**d**) DPOAE thresholds in response to the primary tones set at a frequency ratio (f_2/f_1) of 1.2 with target frequencies. (b-d) PBS (n = 7, 12 ears), LPS (n = 3, 6 ears), LPS + IL-1RA (n = 4, 7 ears), and LPS + MCC950 (n = 4, 8 ears). Values and error bars reflect means ± S.E.M. Statistical comparisons were determined using one-way ANOVA with Tukey's corrections for multiple comparisons (*P < 0.05, **P < 0.01, ***P < 0.001, n.s. non-significant). Green symbols indicate statistical significance at each frequency between LPS and LPS + IL-1RA, black symbols indicate statistical significance between PBS and LPS + IL-1RA or between PBS and LPS + MCC950 and blue symbols indicate statistical significance between LPS and LPS + MCC950.

generated caspase-1-activatable probe. JB and JWY conceived and supervised the entire project. JHM, EL, SHY, HM, JB and JWY wrote the manuscript, and have accessed and verified the data. JHM, EL, JB and JWY were responsible for decision to submit the manuscript. All authors read and approved the final version of the manuscript.

Data sharing statement

The data generated during this study are made available from the corresponding author upon reasonable request.

Declaration of interests

The authors declare no conflicts of interest.

Acknowledgements

This work was supported by the National Research Foundation of Korea Grant funded by the Korean Government (2020R1A2B5B02001823 and 2020R1A4A1019009 to J-WY; 2014M3A9D5A01073865 and 2016R1A5A2008630 to JB) and by the Team Science Award of Yonsei University College of Medicine (6−2021−0004 to JB). We would like to thank Dong-Su Jang, MID, (Medical Illustration & Design), a part of the Medical Research Support Services of Yonsei University College of Medicine, for all artistic support related to this work, and Dr Ji Soo Chae (Perkin Elmer) for technical support with respect to the IVIS imaging system.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2022.104184.

References

1 Masters SL, Simon A, Aksentijevich I, Kastner DL. Horror autoinflammaticus: the molecular pathophysiology of autoinflammatory disease (*). *Annu Rev Immunol*. 2009;27:621–668.
2 Balow Jr. JE, Shelton DA, Orsborn A, et al. A high-resolution genetic map of the familial Mediterranean fever candidate region allows identification of haplotype-sharing among ethnic groups. *Genomics*. 1997;44(3):280–291.
3 Hoffman HM, Mueller JL, Broide DH, Wanderer AA, Kolodner RD. Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle-Wells syndrome. *Nat Genet*. 2001;29(3):301–305.
4 Heymann MC, Rosen-Wolff A. Contribution of the inflammasomes to autoinflammatory diseases and recent mouse models as research tools. *Clin Immunol*. 2013;147(3):175–184.
5 Yu JW, Fernandes-Alnemri T, Datta P, et al. Pyrin activates the ASC pyroptosome in response to engagement by autoinflammatory PSTPIP1 mutants. *Mol Cell*. 2007;28(2):214–227.
6 Mariathasan S, Weiss DS, Newton K, et al. Cryopyrin activates the inflammasome in response to toxins and ATP. *Nature*. 2006;440(7081):228–232.
7 Kanneganti TD, Ozoren N, Body-Malapel M, et al. Bacterial RNA and small antiviral compounds activate caspase-1 through cryopyrin/Nalp3. *Nature*. 2006;440(7081):233–236.
8 Schroder K, Tschopp J. The inflammasomes. *Cell*. 2010;140(6):821–832.
9 Mangan MSJ, Olhava EJ, Roush WR, Seidel HM, Glick GD, Latz E. Targeting the NLRP3 inflammasome in inflammatory diseases. *Nat Rev Drug Discov*. 2018;17(8):588–606.
10 Heneka MT, McManus RM, Latz E. Inflammasome signalling in brain function and neurodegenerative disease. *Nat Rev Neurosci*. 2018;19(10):610–621.
11 Jesus AA, Goldbach-Mansky R. IL-1 blockade in autoinflammatory syndromes. *Annu Rev Med*. 2014;65:223–244.
12 Harrison SR, McGonagle D, Nizam S, et al. Anakinra as a diagnostic challenge and treatment option for systemic autoinflammatory disorders of undefined etiology. *JCI Insight*. 2016;1(6):e86336.
13 Yu JW, Lee MS. Mitochondria and the NLRP3 inflammasome: physiological and pathological relevance. *Arch Pharm Res*. 2016;39(11):1503–1518.
14 Neven B, Callebaut I, Prieur AM, et al. Molecular basis of the spectral expression of CIAS1 mutations associated with phagocytic cell-mediated autoinflammatory disorders CINCA/NOMID, MWS, and FCU. *Blood*. 2004;103(7):2809–2815.
15 McDermott MF, Tschopp J. From inflammasomes to fevers, crystals and hypertension: how basic research explains inflammatory diseases. *Trends Mol Med*. 2007;13(9):381–388.
16 Welzel T, Kuemmerle-Deschner JB. Diagnosis and management of the cryopyrin-associated periodic syndromes (CAPS): what do we know today? *J Clin Med*. 2021;10(1):128.
17 Ahmadi N, Brewer CC, Zalewski C, et al. Cryopyrin-associated periodic syndromes: otolaryngologic and audiologic manifestations. *Otolaryngol Head Neck Surg*. 2011;145(2):295–302.
18 Kuemmerle-Deschner JB, Koitschev A, et al. Early detection of sensorineural hearing loss in Muckle−Wells-syndrome. *Pediatr Rheumatol Online J*. 2015;13(1):43.
19 Vambutas A, Lesser M, Mullooly V, et al. Early efficacy trial of anakinra in corticosteroid-resistant autoimmune inner ear disease. *J Clin Invest*. 2014;124(9):4115–4122.
20 Nakanishi H, Kawashima Y, Kurima K, et al. NLRP3 mutation and cochlear autoinflammation cause syndromic and nonsyndromic hearing loss DFNA34 responsive to anakinra therapy. *Proc Natl Acad Sci USA*. 2017;114(37):E7766–E7775.
21 Nakanishi H, Prakash P, Ito T, et al. Genetic hearing loss associated with autoinflammation. *Front Neurol*. 2020;11:141.
22 Ko YJ, Lee JW, Yang EJ, et al. Non-invasive in vivo imaging of caspase-1 activation enables rapid and spatiotemporal detection of acute and chronic inflammatory disorders. *Biomaterials*. 2020;226:119543.
23 Fernandes-Alnemri T, Yu JW, et al. The AIM2 inflammasome is critical for innate immunity to Francisella tularensis. *Nat Immunol*. 2010;11(5):385–393.
24 Fernandes-Alnemri T, Yu JW, Datta P, Wu J, Alnemri ES. AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature*. 2009;458(7237):509–513.
25 Feldmann J, Prieur AM, Quartier P, et al. Chronic infantile neurological cutaneous and articular syndrome is caused by mutations in CIAS1, a gene highly expressed in polymorphonuclear cells and chondrocytes. *Am J Hum Genet*. 2002;71(1):198–203.
26 Aksentijevich I, Nowak M, Mallah M, et al. De novo CIAS1 mutations, cytokine activation, and evidence for genetic heterogeneity in patients with neonatal-onset multisystem inflammatory disease (NOMID): a new member of the expanding family of pyrin-associated autoinflammatory diseases. *Arthritis Rheum*. 2002;46(12):3340–3348.
27 Hough K, Verschuur CA, Cunningham C, Newman TA. Macrophages in the cochlea; an immunological link between risk factors and progressive hearing loss. *Glia*. 2022;70(2):219–238.
28 Kishimoto I, Okano T, Nishimura K, Motohashi T, Omori K. Early development of resident macrophages in the mouse cochlea depends on yolk sac hematopoiesis. *Front Neurol*. 2019;10:1115.
29 Yona S, Kim KW, Wolf Y, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity*. 2013;38(1):79–91.
30 Tapia-Abellan A, Angosto-Bazarra D, Martinez-Banaclocha H, et al. MCC950 closes the active conformation of NLRP3 to an inactive state. *Nat Chem Biol*. 2019;15(6):560–564.
31 Okano T. Immune system of the inner ear as a novel therapeutic target for sensorineural hearing loss. *Front Pharmacol*. 2014;5:205.
32 Fujioka M, Okano H, Ogawa K. Inflammatory and immune responses in the cochlea: potential therapeutic targets for sensorineural hearing loss. *Front Pharmacol*. 2014;5:287.
33 Bae SH, Yoo JE, Choe YH, et al. Neutrophils infiltrate into the spiral ligament but not the stria vascularis in the cochlea during

lipopolysaccharide-induced inflammation. *Theranostics*. 2021;11 (6):2522–2533.
34 Wree A, Eguchi A, McGeough MD, et al. NLRP3 inflammasome activation results in hepatocyte pyroptosis, liver inflammation, and fibrosis in mice. *Hepatology*. 2014;59(3):898–910.
35 Brydges SD, Mueller JL, McGeough MD, et al. Inflammasome-mediated disease animal models reveal roles for innate but not adaptive immunity. *Immunity*. 2009;30(6):875–887.
36 Sato E, Shick HE, Ransohoff RM, Hirose K. Expression of fractalkine receptor CX3CR1 on cochlear macrophages influences survival of hair cells following ototoxic injury. *J Assoc Res Otolaryngol*. 2010;11(2):223–234.
37 Hirose K, Hartsock JJ, Johnson S, Santi P, Salt AN. Systemic lipopolysaccharide compromises the blood-labyrinth barrier and increases entry of serum fluorescein into the perilymph. *J Assoc Res Otolaryngol*. 2014;15(5):707–719.
38 Jiang M, Li H, Johnson A, et al. Inflammation up-regulates cochlear expression of TRPV1 to potentiate drug-induced hearing loss. *Sci Adv*. 2019;5(7):eaaw1836.
39 Vethanayagam RR, Yang W, Dong Y, Hu BH. Toll-like receptor 4 modulates the cochlear immune response to acoustic injury. *Cell Death Dis*. 2016;7(6):e2245.
40 Frye MD, Yang W, Zhang C, Xiong B, Hu BH. Dynamic activation of basilar membrane macrophages in response to chronic sensory cell degeneration in aging mouse cochleae. *Hear Res*. 2017;344:125–134.
41 Zhang F, Zhang J, Neng L, Shi X. Characterization and inflammatory response of perivascular-resident macrophage-like melanocytes in the vestibular system. *J Assoc Res Otolaryngol*. 2013;14(5):635–643.
42 Vande Walle L, Stowe IB, Sacha P, et al. MCC950/CRID3 potently targets the NACHT domain of wild-type NLRP3 but not disease-associated mutants for inflammasome inhibition. *PLoS Biol*. 2019;17(9):e3000354.