The Journal of Immunology

Activation of Lipopolysaccharide–TLR4 Signaling Accelerates the Ototoxic Potential of Cisplatin in Mice

Gi-Su Oh, Hyung-Jin Kim, Jae-Hyuck Choi, AiHua Shen, Chang-Hoi Kim, Se-Jin Kim, Sae-Ron Shin, Seung-Heon Hong, Yunha Kim, Channy Park, Sung-Joong Lee, Shizuo Akira, Raekil Park and Hong-Seob So

J Immunol 2011; 186:1140-1150; Prepublished online 8 December 2010;
doi: 10.4049/jimmunol.1002183
http://www.jimmunol.org/content/186/2/1140

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Activation of Lipopolysaccharide–TLR4 Signaling Accelerates the Ototoxic Potential of Cisplatin in Mice

Gi-Su Oh,*,[1] Hyung-Jin Kim,*,[1] Jae-Hyuck Choi,[†] AiHua Shen,* Chang-Hoi Kim,* Se-Jin Kim,* Sae-Ron Shin,[‡] Seung-Heon Hong,* Yunha Kim,[§] Channy Park,[¶] Sung-Joong Lee,[‖] Shizuo Akira,[#] Raekil Park,*,[2] and Hong-Seob So*,[2]

Dysfunction in immune surveillance during anticancer chemotherapy of patients often causes weakness of the host defense system and a subsequent increase in microbial infections. However, the deterioration of organ-specific function related to microbial challenges in cisplatin-treated patients has not yet been elucidated. In this study, we investigated cisplatin-induced TLR4 expression and its binding to LPS in mouse cochlear tissues and the effect of this interaction on hearing function. Cisplatin increased the transcriptional and translational expression of TLR4 in the cochlear tissues, organ of Corti explants, and HEI-OC1 cells. Furthermore, cisplatin increased the interaction between TLR4 and its microbial ligand LPS, thereby upregulating the production of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, via NF-κB activation. In C57BL/6 mice, the combined injection of cisplatin and LPS caused severe hearing impairment compared with that in the control, cisplatin-alone, or LPS-alone groups, whereas this hearing dysfunction was completely suppressed in both TLR4 mutant and knockout mice. These results suggest that hearing function can be easily damaged by increased TLR expression and microbial infections due to the weakened host defense systems of cancer patients receiving therapy comprising three to six cycles of cisplatin alone or cisplatin combined with other chemotherapeutic agents. Moreover, such damage can occur even though patients may not experience ototoxic levels of cumulative cisplatin concentration. ***The Journal of Immunology*****, 2011, 186: 1140–1150.**

Cis-diamminedichloroplatinum (II) (cisplatin) is one of the most commonly used chemotherapeutic agents in the treatment of a variety of solid tumors, such as tumors of the bladder, head and neck, lung, ovary, and testicles (1). Although cisplatin is effective, serious adverse reactions, such as nausea, vomiting, nephrotoxicity, central and peripheral neuropathies, and ototoxicity, often occur (2). These side effects limit its use in chemotherapeutic agents and affect the dosage, duration, and frequency of cisplatin therapy. Additionally, cisplatin causes myelosuppression, which results in leukopenia, thrombocytopenia, and anemia and thereby directly weakens the host defense response against bacteria, fungi, and viruses and increases the morbidity and mortality of patients through blood-borne microbial infections.

Ototoxic targets of cisplatin are divided into three areas: the hair cells in the organ of Corti (3), the stria vascularis and spiral ligament tissues (4), and the spiral ganglion cells (5). Recent studies have demonstrated that cisplatin ototoxicity is caused by mitochondrial dysfunction and generation of reactive oxygen species (ROS) (6, 7). Furthermore, we demonstrated that proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, play a critical role in cisplatin-induced cochlear injury through activation of ERK and NF-κB (8). In the course of chemotherapy, cisplatin may have an influence on normal cells as well as cancer cells through the modulation of genes and proteins related to cell survival and death responses (9). Tarang et al. (10) reported that cisplatin induces the expression of TLRs in murine peritoneal macrophages in vitro and renders mice more susceptible to subsequent induction of proinflammatory cytokines by TLR ligands. TLRs are a family of mammalian homologues of *Drosophila* Toll protein and play an important role in innate host defense (11). TLRs play key roles as pattern recognition receptors in detecting foreign pathogens and initiating inflammatory responses. A variety of pathogen-associated molecular patterns (PAMPs) from bacteria, fungi, and viruses as well as some host molecules are recognized by TLRs as ligands (12). Activation of intracellular signaling pathways through TLRs results in the recruitment of the cytoplasmic adapter molecule MyD88, activation of serine/threonine kinases of the IL-1R–associated kinase (IRAK) family, and degradation of IκB and translocation of NF-κB to the nucleus (13). Thereafter, various target genes, including those producing inflammatory cytokines,

*Vestibulocochlear Research Center, Wonkwang University School of Medicine, Iksan, Jeonbuk 570-749, Republic of Korea; [†]Department of Microbiology, Wonkwang University School of Medicine, Iksan, Jeonbuk 570-749, Republic of Korea; [‡]Department of Family Medicine, Wonkwang University School of Medicine, Iksan, Jeonbuk 570-749, Republic of Korea; [§]Institute for Innovative Cancer Research, Asan Institute for Life Science, Asan Medical Center, Seoul 138-736, Republic of Korea; [¶]Department of Audiology, Nambu University, Gwangju 506-824, Republic of Korea; [‖]Department of Oral Physiology, College of Dentistry, Seoul National University, Gwanak-gu, Seoul 151-742, Republic of Korea; and [#]Laboratory of Host Defense, World Premier International Research Center, Immunology Frontier Research Center, Osaka University, Osaka 565-0871, Japan

[1]G.-S.O., H.-J.K. contributed equally to this work as first authors.

[2]H.-S.S. and R.P. contributed equally to this work as corresponding authors.

Received for publication June 30, 2010. Accepted for publication November 10, 2010.

This work was supported by the Ministry of Science and Technology/Korea Science and Engineering Foundation through the Vestibulocochlear Research Center at Wonkwang University in 2009 (R13-2002-055-00000-0).

Address correspondence and reprint requests to Hong-Seob So or Raekil Park, Department of Microbiology, Wonkwang University School of Medicine, 344-7 Shinyong-dong, Iksan, Jeonbuk 570-749, Republic of Korea. E-mail addresses: jeanso@wku.ac.kr or rkpark@wku.ac.kr

The online version of this article contains supplemental material.

Abbreviations used in this article: ABR, auditory brain-stem response; AGE, advanced glycation end-product; cisplatin, cis-diamminedichloroplatinum (II); HMGB1, high mobility group box 1; iNOS, inducible NO synthase; IRAK, IL-1R–associated kinase; MEF, mouse embryonic fibroblast; NOX, NADPH oxidase; OHC, outer hair cell; P2, postnatal day 2; PAMP, pathogen-associated molecular pattern; RAGE, receptor for advanced glycation end-products; ROS, reactive oxygen species; siRNA, small interfering RNA; TIR, Toll/IL-1R; TRITC, tetramethylrhodamine isothiocyanate.

are regulated. TLR4 is the most well-characterized TLR: it is localized on the cell surface and acts as the receptor for bacterial endotoxins, such as LPS (14, 15), a cell wall component of Gram-negative bacteria that is known to induce immune activation. LPS is composed of lipid A, O-Ag, and core oligosaccharide. TLR4 recognizes the lipid A component of LPS; this occurs through the TLR4/MD2/CD14 complex. LPS forms a complex with an accessory protein, LPS-binding protein, in serum, but this interaction is dependent on cell type (16–18). Although TLR-mediated signaling pathways play a critical role in the innate immune response (19), they are also involved in a variety of deleterious inflammatory diseases, such as autoimmune disease (20), rheumatoid arthritis (21), and Alzheimer's disease (22). It has been reported that in a mouse model of acute kidney injury, TLR4 signaling mediates cisplatin-induced production of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β, resulting in subsequent renal injury (23, 24). In contrast, Ramesh et al. (25, 26) demonstrated that the combination of cisplatin and LPS, a typical PAMP in bacteria, synergistically induces renal dysfunction through the production of proinflammatory cytokines, including TNF-α, IL-6, and MCP-1: moreover, this renal dysfunction is completely dependent on TLR4 signaling. Therefore, it is reasonable to speculate that cisplatin may induce a systemic decline of immune surveillance in cancer patients, and adverse reactions, including hearing loss, may be aggravated by additional inflammatory responses through TLR signaling pathways elicited by PAMPs of microbial pathogens.

In the current study, we investigated the hearing impairment due to cisplatin alone or cisplatin combined with LPS in a rodent model system. In addition, we estimated cisplatin-induced TLR4 expression and interaction with its specific ligand LPS in vivo and in vitro. Our results suggest the possibility that hearing function may be damaged by increased TLR expression and microbial infections due to the weakened host defense system in cisplatin-treated cancer patients even though the patients may not have an ototoxic level of cumulative cisplatin concentration.

Materials and Methods

Reagents

Cisplatin, LPS (*Escherichia coli* 055:B5 clone), FITC-conjugated LPS (*E. coli* 055:B5 clone), MTT and DMEM media with high glucose (4.5 g/l) were obtained from Sigma Chemical Co. (St. Louis, MO). Abs to TLR4, TNF-α, IL-1β, IL-6, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa Fluor secondary fluorescent Abs were purchased from Molecular Probes (Eugene, OR). Mouse anti–phospho-IκBα and rabbit anti-IκBα Abs were purchased from Cell Signaling Technology (Beverly, MA), and ECL solution was obtained from Thermo Scientific (Pierce Chemical, Rockland, IL). FBS was purchased from Life Technologies (Invitrogen, Grand Island, NY). The plastic culture dishes were bought from Becton Dickinson Labware (Franklin Lakes, NJ), and other tissue culture reagents were obtained from Life Technologies (Gaithersburg, MD).

Culture of HEI-OC1 cells and the organ of Corti explants

The establishment and characterization of the conditionally immortalized HEI-OC1 auditory cells has been described in a our previous report (27). Expression of outer hair cell (OHC)-specific markers, such as Math1 and Myosin 7a, suggests that HEI-OC1 cells represent OHC precursors. HEI-OC1 cells were maintained in high-glucose DMEM containing 10% FBS. For the experiments described later, HEI-OC1 cells were cultured under the following permissive conditions: 33˚C and 5% CO_2 in DMEM supplemented with 10% FBS. HEI-OC1 cells (5×10^4 cells/well on 24-well plates) were incubated with 10 μM cisplatin for 18 h and then further treated with varying concentrations of LPS for 24 h. To determine cell viability, MTT (250 μg/ml) was added to the cells and further incubated for 4 h. After washing the cells with PBS (pH 7.4), the insoluble formazan was dissolved in DMSO. Then, the OD was measured using a microplate reader (Precision Microplate Reader; Molecular Devices, Sunnyvale, CA) at 590 nm. The OD of control cells was taken as 100% viability.

The organ culture procedure was similar to that of a previous report (28). Sprague Dawley rats were sacrificed on postnatal day 2 (P2), and the cochlea was carefully dissected out. The stria vascularis and spiral ligament were dissected away, leaving the organ of Corti. The organ of Corti explants were treated with cisplatin and/or LPS for the indicated times. At the end of the experiments, the cultured organ was subjected to histological analysis. The specimen was fixed in 10% formalin solution (Sigma-Aldrich, St. Louis, MO), incubated in 0.2% Triton X-100/TBS, and further treated with first and appropriate secondary fluorescent Ab or tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin (Sigma Chemical Co.; 1:3000). After staining, the specimen was examined under a fluorescence microscope.

In vivo administration of cisplatin and LPS

Wild-type C57BL/6 and TLR4 mutant C3H/HeJ mice were purchased from Japan SLC (Laboratory Animal Medicine, Japan). The homozygous $TLR4^{-/-}$ mice on a C57BL/6 background were kindly provided by Dr. S. Akira (Osaka University, Osaka, Japan). The C3H/HeJ and $TLR4^{-/-}$ mice did not show any developmental abnormalities. Experiments were performed in 8-wk-old mice weighing between 20 and 25 g, and all mice were age matched to within 3 d. Mice were fed a standard commercial diet while housed at an ambient temperature of 20–22˚C with a relative humidity of 50 ± 5% under a 12/12 h light/dark cycle in a specific pathogen-free facility. Each experimental group was composed of five mice. The control group received an i.p. injection of PBS. The cisplatin group was administered cisplatin (5 mg/kg body weight) by an i.p. injection. As shown in Fig. 5*A*, in the groups for LPS-only or cisplatin–LPS combined, LPS doses were divided into three different concentrations (1.25, 2.5, and 5.0 mg/kg body weight) and administered twice at 6 and 24 h after cisplatin injection. On day 7, this experimental regimen was repeated. For the measurement of hearing function, the auditory brain-stem response (ABR) was measured at day 3 and day 10 of the experimental schedule. The changes in ABR threshold between pretreatment and posttreatment were then compared. TDT System-3 hardware and software were used to obtain ABRs, with 1000 stimulus repetitions per record. Mice were anesthetized using a mixture of ketamine (40 mg/kg) and xylazine (10 mg/kg) and kept warm with a heating pad during ABR recording. A subdermal (active) needle electrode was inserted at the vertex, and ground and reference electrodes were inserted subdermally in the loose skin beneath the pinnae of opposite ears. Tone bursts with a duration of 4 ms and a rise-fall time of 1 ms at frequencies of 4, 8, 16, and 32 kHz were then presented to the right ear through an inset speculum in the external auditory meatus. The sound intensity was varied at 10-dB intervals near the threshold. Judgment of the threshold was made off-line by two independent, experimentally blinded observers based on the ABR records. The animals were sacrificed at 5 d after the second ABR measurement. The entire temporal bone of the right ear was removed for immunohistochemical analysis, and the temporal bone of the left ear was also removed to extract total RNA for RT-PCR analysis. The experimental protocol was approved by the Animal Care and Use Committee at the Wonkwang University School of Medicine (Iksan, Jeonbuk, Republic of Korea).

Assay for mRNA expressions by RT-PCR

After extracting total RNA using TRIzol (Invitrogen, Carlsbad, CA) from the experimental specimens, cDNA was synthesized using reverse transcriptase from Roche (Indianapolis, IN). PCR was then performed with *Taq* polymerase (SolGent Co., Daejeon, Korea) for 25 cycles using the following protocol: 94˚C for 30 s, 55˚C for 30 s, and 72˚C for 30 s. The sequence of primers used for PCR amplification was as follows: TLR4 (forward, 5′-AGT GGG TCA AGG AAC AGA AGC A-3′; reverse, 5′-CTT TAC CAG CTC ATT TCT CAC C-3′), GAPDH (forward, 5′-CCT GCA GTG TCT GAT ATT GTT G-3′; reverse, 5′-AAC ACA CCA TTG CGA TGA A-3′), and β-actin (forward, 5′-CCT CTA TGC CAA CAC AGT-3′; reverse, 5′-AGC CAC CAA TCC ACA CAG-3′).

Western blot analysis

Western blot analysis was performed as follows. Cells were harvested and washed twice with ice-cold PBS. Cell pellets were lysed with buffer (10 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM EDTA, 50 mM β-glycerophosphate, 1 mM DTT, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, and 1× protease inhibitor mixture). After centrifugation at the maximum speed, the collected lysates were subjected to electrophoresis on 10% SDS-PAGE and then transferred onto a nitrocellulose membrane. The proteins were visualized by immunoblotting using Abs/Ags and a chemiluminescent solution according to the manufacturer's instructions (Supersignal Pico Substrate; Thermo Scientific, Pierce Chemical).

Flow cytometric analysis

To assess the presence of cell surface molecules and interactions between receptor and ligand by cisplatin and LPS on HEI-OC1 cells, flow cytometric analysis was performed. A total 5×10^5 cells/condition was analyzed using a FACSCalibur System (BD, San Jose, CA) and evaluated with the CellQuest software.

Immunohistochemical study

We performed immunohistochemical staining to examine the expression of TLR proteins and proinflammatory cytokines on the cochlear tissue. The previously removed temporal bone was fixed in 4% paraformaldehyde for 16 h and then decalcified with 10% EDTA in PBS for 2 wk, after which it was dehydrated and embedded in paraffin wax. Sections 5 μm thick were deparaffinized in xylene and rehydrated through graded concentrations of ethanol. For immunohistochemical staining, the LSAB+System-HRP kit (K0679; DAKO, Carpinteria, CA) was used, and all procedures were carried out according to the manufacturer's instructions.

Measurement of proinflammatory cytokines

To quantify the secretion of proinflammatory cytokines from cisplatin- and LPS-treated HEI-OC1 cells, culture supernatant was collected at each condition and stored in a −80°C freezer until analyzed. To measure the serum level of proinflammatory cytokines, whole blood was isolated from mice before sacrifice, incubated at 4°C for 16 h, and centrifuged at 4000 rpm for 20 min. Thereafter, the level of proinflammatory cytokines was determined by ELISA (ELISA, Quantikine Kit; R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Statistical analysis

Each experiment was performed at least three times, and all values are represented as means ± SD of three observations. One-way ANOVA was used to analyze the statistical significance of the results, and p values <0.05 were considered statistically significant.

Results

Cisplatin increased the expression of TLR4 mRNA and protein in HEI-OC1 auditory cells

Cenedeze et al. (24) demonstrated that cisplatin-induced renal toxicity is significantly alleviated in C3H/HeJ mice producing a mutant form of TLR4 compared with that in C3H/HePas control mice. Furthermore, Tarang et al. (10) reported that cisplatin induced the expression of TLRs in peritoneal macrophages. In addition, pretreatment of macrophages with cisplatin and subsequent incubation with TLR ligands significantly enhanced the production of proinflammatory cytokines including TNF-α (10). These results suggest that treatment of macrophages with cisplatin renders them more susceptible to subsequent induction of proinflammatory cytokines by TLR ligands. Therefore, to examine whether cisplatin also induces expression of TLRs in HEI-OC1 cells and mouse cochleae, HEI-OC1 cells were stimulated with 10 μM cisplatin for varying times. As shown in Fig. 1*A*, cisplatin markedly increased the mRNA expression of TLR4 in a time-dependent manner. An increase in the RT-PCR products of TLR4 mRNA was apparent after 18 h of cisplatin treatment and reached the maximum level at 24 or 30 h. Next, to examine whether cisplatin increases TLR4 expression in cochleae, C57BL/6 mice were i.p. administered 10 mg/kg body weight cisplatin and sacrificed at the indicated time points (Fig. 1*B*). We examined TLR4 mRNA levels in whole cochleae using RT-PCR on the total RNA of whole left cochleae from the cisplatin-injected mice. As shown in Fig. 1*B*, TLR4 mRNA levels were markedly increased after cisplatin injection compared with those of PBS-control mice. Injection of cisplatin induced the transcriptional activation of TLR4 from 12 h to 48 h in a time-dependent manner. Next, to confirm the expression of TLR4 in cisplatin-treated HEI-OC1 cells, immunocytochemical analysis was carried out with FITC-labeled Ab against TLR4 protein and DAPI for nuclei. As shown

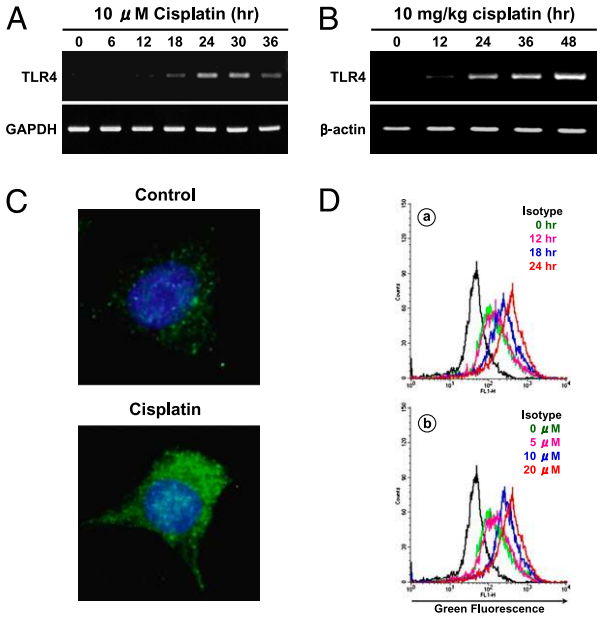

FIGURE 1. Cisplatin increased the mRNA and protein expression of TLR4 in HEI-OC1 auditory cells. *A*, HEI-OC1 cells were treated with 10 μM cisplatin for the indicated times. *B*, C57BL/6 mice were also i.p. administered cisplatin (10 mg cisplatin/kg body weight/one injection). Then, total RNA was isolated from HEI-OC1 cells and cochleae by TRIzol, and cDNA was synthesized by reverse transcription. TLR4, GAPDH, and β-actin cDNAs were amplified using specific primer sets. The results shown are representative of three independent experiments with similar results. *C*, HEI-OC1 cells were incubated with media alone or 10 μM cisplatin for 18 h. The location of the TLR4 protein was determined by immunofluorescent staining. After stimulation, cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100/PBS and stained with anti-TLR4 Ab and FITC-conjugated secondary Ab. Then cells were observed under a fluorescence microscope (original magnification ×200). The results shown are representative of three randomly photographed fields that showed similar results. *D*, HEI-OC1 cells were incubated with cisplatin for indicated times (*a*) and doses (*b*). The expression of TLR4 by cisplatin stimulation was determined by flow cytometric analysis as described in *Materials and Methods*.

in Fig. 1*C*, TLR4 protein expression on the cell membrane was markedly increased by cisplatin exposure, whereas this expression was nearly undetectable in control HEI-OC1 auditory cells. The increase in TLR4 protein expression due to cisplatin was further confirmed by flow cytometric analysis. A rightward shift of the fluorescence signals in cisplatin-treated cells corresponding with the increase of TLR4 expression was obviously induced in a time- (Fig. 1*Da*) and dose-dependent manner (Fig. 1*Db*).

Cisplatin increased the expression of TLR4 protein on the primary organ of Corti explants

Next, we examined cisplatin-induced TLR4 protein expression on the primary organ of Corti explants. The half middle turn of the organs of Corti from neonatal (P2) Sprague Dawley rats was isolated and treated with 5, 10, and 20 μM cisplatin for 24 h. After stimulation with cisplatin, the organ of Corti explants were washed and stained with DAPI, Alexa Fluor 488-labeled TLR4, and TRITC-labeled phalloidin. As shown in Fig. 2, three rows of OHCs and a single row of inner hair cells were clearly observed in phalloidin staining (Fig. 2*C*, 2*F*, 2*I*, and 2L) and were not damaged by the specified ranges of cisplatin concentration. TLR4 staining was undetectable in the hair cell layers in the organs of Corti treated with media alone (Fig. 2*B*) or 5 μM cisplatin (Fig. 2*E*), whereas immunopositive cells for TRL4 were clearly ob-

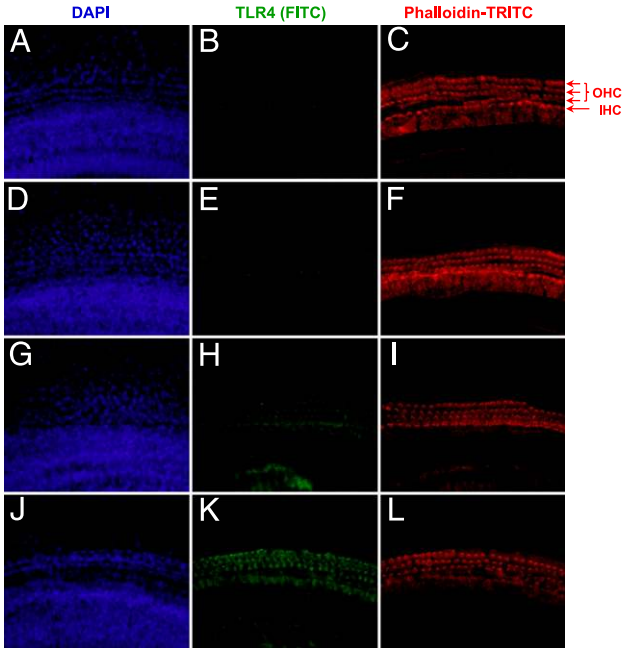

FIGURE 2. Cisplatin increased the expression of TLR4 protein on the primary organ of Corti explants. The organ of Corti explant was treated with cisplatin in a dose-dependent manner for 24 h (*D–F*, 5 μM cisplatin; *G–I*, 10 μM cisplatin; *J–L*, 20 μM cisplatin). The organ of Corti explants were stained with TRITC-conjugated phalloidin (*C*, *F*, *I*, *L*), DAPI (*A*, *D*, *G*, *J*), anti-TLR4 Ab and FITC-conjugated anti-goat secondary Ab (*B*, *E*, *H*, *K*) and then observed under a fluorescence microscope (original magnification ×200). IHC; inner hair cell; OHC, outer hair cell.

served in hair cell layers after exposure to 10 and 20 μM cisplatin (Fig. 2*H* and 2*K*, respectively).

In vitro interaction between cisplatin-induced TLR4 and its ligand LPS

To date, 11 human TLRs and 13 mouse TLRs have been identified, and each TLR has been shown to recognize distinct molecular patterns derived from various microorganisms, including bacteria, viruses, fungi, and protozoa. TLRs are classified into several groups according to the types of PAMPs that they recognize. TLR4 specifically recognizes LPS from Gram-negative bacteria, which causes septic shock (29). To examine whether cisplatin-induced TLR4 interacts with exogenous LPS, HEI-OC1 cells were pre-incubated with cisplatin in time- and dose-dependent manners and then treated with 1 μg/ml FITC-conjugated LPS for 15 min. As shown in Fig. 3*A*, flow cytometric analysis showed that the rightward shift of fluorescence signals was increased by cisplatin in a time- (Fig. 3*Aa*) and dose-dependent manner (Fig. 3*Ab*), which suggests a direct interaction between TLR4 and LPS. We also observed a similar phenomenon in the organ of Corti explant cultures (Fig. 3*B*). Both control and cisplatin-treated groups showed clear nuclei and hair cell layers by DAPI and phalloidin–TRITC staining, respectively, but green fluorescence of LPS appeared only in the cisplatin-treated group. These results suggest that cisplatin causes a marked induction of TLR4 in the hair cells of the organs of Corti, which further directly interacts with exogenous LPS as a ligand. Next, we examined whether the interaction between cisplatin and TLR4 ligand LPS had a direct effect on viability of HEI-OC1 cells. HEI-OC1 cells were pretreated with 10 μM cisplatin for 18 h and then further stimulated with LPS at the indicated concentrations. As shown in Fig. 3*C*, we could not find any significant cytotoxic effect of LPS alone even at

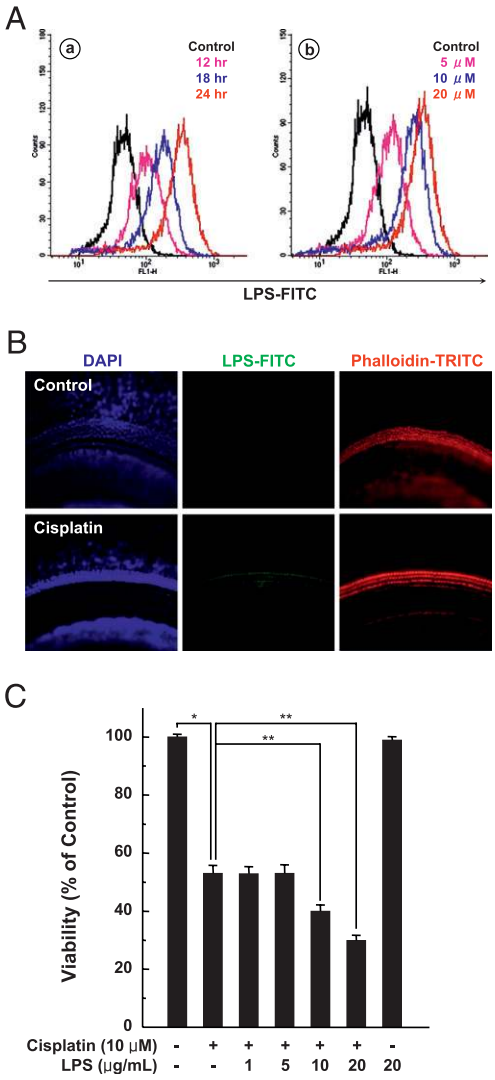

FIGURE 3. In vitro interaction between cisplatin-induced TLR4 and its ligand LPS. *A*, HEI-OC1 cells were incubated with cisplatin for the indicated times (*a*) and doses (*b*) and then treated with FITC-conjugated LPS. The binding interaction between cisplatin-induced TLR4 and LPS was determined by flow cytometric analysis as described in *Materials and Methods*. *B*, The organ of Corti explants were incubated with medium alone and 20 μM cisplatin for 18 h and then treated with FITC-conjugated LPS for 15 min. The organ of Corti explants were stained with TRITC-conjugated phalloidin and DAPI and then observed under a fluorescence microscope (original magnification ×100). *C*, HEI-OC1 cells were pretreated with 10 μM cisplatin for 18 h and further incubated with LPS at the indicated concentrations for 24 h. Cell viability was measured by MTT assay. Values are means ± SD from three independent experiments. $*p < 0.01$; $**p < 0.05$ by one-way ANOVA compared with the cisplatin-only group.

20 μg/ml concentration. However, cell viability was significantly decreased by LPS in a dose-dependent manner after preexposure to cisplatin. Especially, 10 μg/ml or much higher dose of LPS caused a synergistic viability decrease in cisplatin-treated HEI-OC1 cells. Therefore, we applied the 10 μg/ml LPS in our experiment.

NF-κB activation was enhanced by the interaction of TLR4 with LPS

The engagement of TLRs by ligands triggers the activation of signaling cascades, leading to the induction of a variety of genes,

including proinflammatory cytokines. After binding to ligand, TLRs dimerize and undergo conformational changes required for the recruitment of adapter molecules to the Toll/IL-1R (TIR) domain of the TLR. Adapter molecules, such as MyD88 and TRIF, are known to be responsible for the activation of TLR signaling pathways, leading to the production of proinflammatory cytokines and type I IFNs, respectively. Upon association with the adapter molecule MyD88 on the TIR domain of TLRs, signaling cascades involving NF-κB, JNK, and p38 are known to be activated. In this experiment, to confirm that the engagement of TLR4 by LPS also triggers the activation of NF-κB signaling cascades in auditory cells, HEI-OC1 cells were pretreated with 10 μM cisplatin for 18 h and stimulated with LPS for the indicated time periods. Cell lysates were then analyzed by Western blotting for phosphorylation of IκBα, which is critically required for NF-κB activation (30). As shown in Fig. 4*A*, phosphorylation of IκBα was not obviously distinctive in experimental groups of control and single treatment, either cisplatin (10 μM) or LPS (10 μg/ml). However, the combination of cisplatin and LPS markedly increased IκBα phosphorylation, which was clearly detectable at 30 min after exposure to LPS and sustained up to 120 min.

Next, to confirm that LPS-augmented phosphorylation of IκBα in cisplatin-pretreated HEI-OC1 cells is mediated through TLR4, cells were transfected with a TLR4-specific small interfering RNA (siRNA) construct or a scrambled control siRNA. As shown in Fig. 4*B*, LPS-augmented phosphorylation of IκBα in cisplatin-pretreated cells was completely inhibited by the siRNA transfection of TLR4 but not by a scrambled control siRNA. In addition, LPS-augmented phosphorylation of IκBα in cisplatin-pretreated cells was completely blocked in mouse embryonic fibroblasts (MEFs) generated from $TLR4^{-/-}$ mice but not in wild-type MEFs (Fig. 4*C*). We then examined whether the activation of NF-κB signaling pathway exerted by the TLR4–LPS interaction induces proinflammatory cytokine production in HEI-OC1 auditory cells. To address this question, HEI-OC1 cells were preincubated with 10 μM cisplatin for 18 h, washed with fresh media, and then treated with 10 μg/ml LPS for 24 h. Culture supernatant was harvested at 24 h after exposure to LPS to measure the secretion of TNF-α, IL-1β, and IL-6. As shown in Fig. 4*D*, the level of secreted cytokines, such as TNF-α, IL-1β, and IL-6, was significantly elevated in the cisplatin–LPS combined groups compared with that of cisplatin- or LPS-alone groups. In addition, to evaluate whether the production of proinflammatory cytokines by cisplatin–LPS through TLR4 signaling cascade affects viability, cisplatin-pretreated HEI-OC1 cells were further incubated with 10 μg/ml LPS for 24 h in the presence of isotype-matched control IgG, anti–TNF-α, anti–IL-1β, and anti–IL-6 neutralizing Abs, respectively. As shown in Fig. 4*E*, neutralization of TNF-α with anti–TNF-α Ab significantly attenuated the cisplatin–LPS–mediated cytotoxicity in HEI-OC1 cells. However, treatment with control IgG, anti–IL-1β, or anti–IL-6 did not exert any notable cytoprotective effects. We also examined whether the enhanced TNF-α production in cisplatin–LPS treatment caused direct damage of hair cells in cochlear tissues. The half middle turn of the organ of Corti from neonatal (P2) mice was isolated and treated with 10 μM cisplatin for 18 h and then further stimulated with 10 μg/ml LPS for 24 h in the presence of normal IgG or neutralizing anti–TNF-α Ab (20 ng/ml). As shown in Supplemental Fig. 1, media control, cisplatin alone, and LPS alone did not cause any noticeable damage of stereocilia bundles, in which F-actin was intensely labeled with TRITC-conjugated phalloidin. However, loss of F-actin positive-staining cells and the disarray of stereocilia bundles of hair cells were obviously observed in cisplatin–LPS cotreatment with normal IgG Ab. However, the destruction of hair cell arrays of the primary organ of Corti explants in cisplatin–LPS cotreatment was prevented in the presence of anti–TNF-α neutralizing Ab.

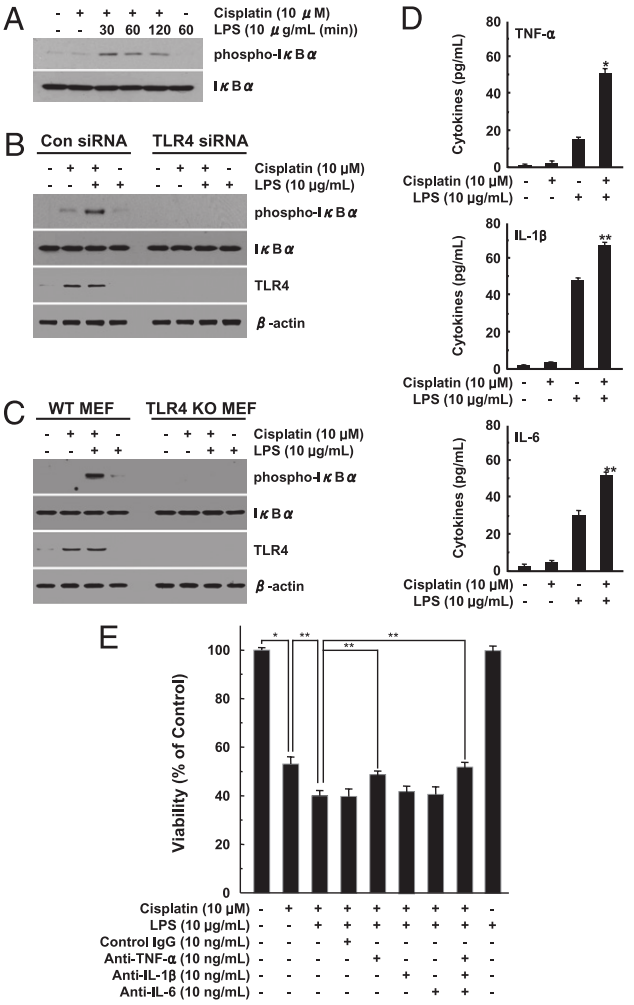

FIGURE 4. NF-κB activation was enhanced by the interaction of cisplatin-induced TLR4 and its ligand LPS. *A*, HEI-OC1 cells were preincubated with 10 μM cisplatin for 18 h and then stimulated with 10 μg/ml LPS at the indicated time points. Phosphorylation of IκBα was confirmed by Western blot analysis. *B*, HEI-OC1 cells were transfected with control scrambled siRNA or TLR4 siRNA and incubated with 10 μM cisplatin for 18 h, followed by 10 μg/ml LPS for 30 min. Cells were harvested, lysed, and then analyzed by Western blot analysis for identifying TLR4-mediated phosphorylation of IκBα. *C*, Wild-type and $TLR4^{-/-}$ MEF cells were used for confirming the TLR4-mediated activation of IκBα. MEF cells were treated with 10 μM cisplatin for 18 h followed by 10 μg/ml LPS for 30 min. Cells were harvested, lysed, and then analyzed by Western blot analysis as described in *Materials and Methods*. Blots shown are representative of three independent experiments. *D*, HEI-OC1 cells were preincubated with 10 μM cisplatin for 18 h and washed with fresh medium. Then, the cells were stimulated with 10 μg/ml LPS for 24 h, and culture supernatants were assessed for cytokine assay as described in *Materials and Methods*. *E*, To evaluate whether the production of proinflammatory cytokines by cisplatin–LPS through TLR4 signaling cascade affects viability, HEI-OC1 cells were treated with 10 μM cisplatin for 18 h and further incubated with 10 μg/ml LPS for 24 h in the presence of isotype-matched control IgG (10 ng/ml), anti–TNF-α (10 ng/ml), anti–IL-1β (10 ng/ml), and anti–IL-6 (10 ng/ml) neutralizing Abs, respectively. Cell viability was measured by MTT assay. Values are means ± SD from three independent experiments. *$p < 0.01$; **$p < 0.05$ by one-way ANOVA compared with the cisplatin-only group.

Combination of cisplatin and LPS caused severe hearing impairment in vivo

Cisplatin combined with other chemotherapeutic agents, administered every 21 d for four to five cycles, has frequently been used as an effective chemotherapy for cancer (31). In vivo model systems for cisplatin-induced hearing loss are well established by several studies (2). In our previous study using rodents, we used four injection schemes composed of 4 mg cisplatin/kg body weight/d for four consecutive days (8). However, to investigate whether cisplatin-induced hearing loss deteriorated further after additional administration of LPS, we used a modification of methods described by Ramesh and colleagues (25). As shown in Fig. 5*A*, two cycles of relatively low-dose cisplatin (5 mg/kg body weight/d) were administered i.p. and followed by two LPS injections at 6 and 24 h after cisplatin exposure. Then, we evaluated the hearing function with the ABR test. ABR threshold shifts were measured at 4, 8, 16, and 32 kHz frequencies before and after injections. As shown in Fig. 5*B*, the ABR threshold shift after the first cycle of cisplatin and/or LPS injection showed that control and LPS alone did not affect normal hearing function, and cisplatin alone caused a small hearing loss at frequencies of 4 and 32 kHz. However, the combination of cisplatin and LPS caused moderate hearing loss in parallel with the LPS dose. After a second cycle of treatment, hearing function in the control or LPS-alone groups was almost the same as that after the first cycle. However, the cisplatin group showed elevated hearing loss compared with that of the first cycle. Of note, the combination of cisplatin and LPS caused severe and significant hearing loss according to the LPS dose (Fig. 5*C*). Furthermore, we also examined whether in vivo injection of cisplatin–LPS induced apoptotic cell death of cochlear tissues by TUNEL staining. As shown in Supplemental Fig. 2, TUNEL-positive cells were not observed in cochleae from PBS control, cisplatin-alone, or LPS-alone injected mice. However, cochlear tissues from the cisplatin–LPS–co-injected groups exhibited many TUNEL-positive cells in the stria vascularis, spiral limbus, and organ of Corti.

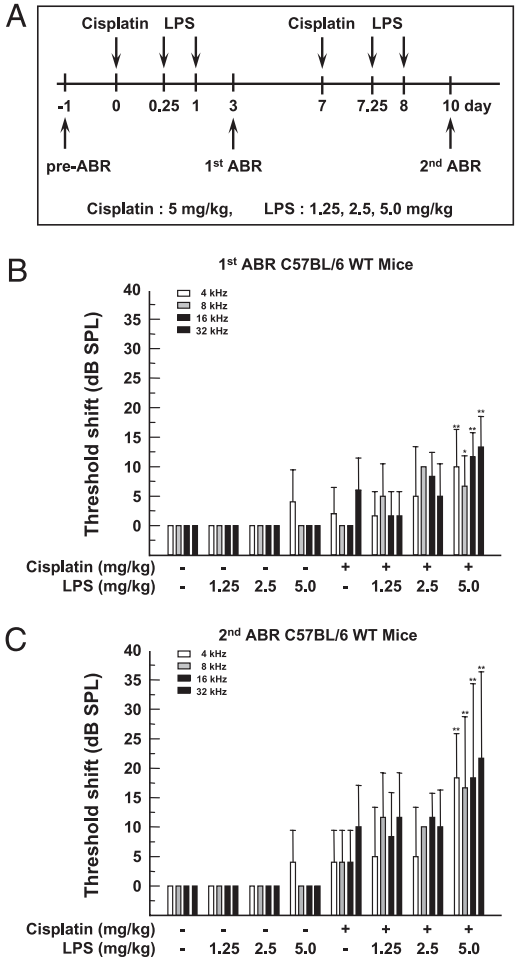

FIGURE 5. In vivo combined injection of cisplatin and LPS caused severe hearing impairment compared with injection of cisplatin or LPS alone. *A*, Experimental scheme for the in vivo ototoxic mouse model with cisplatin and/or LPS. Mice were administered PBS, cisplatin, LPS, or cisplatin–LPS by i.p. injection at the indicated times. ABR was measured at three time points as indicated: before injection (pre-ABR), (*B*) after first injection of cisplatin and/or LPS (1st ABR), and (*C*) after second injection (2nd ABR). ABR threshold shifts are represented as mean ± SD at each frequency tested in C57BL/6 mice. $*p < 0.05$; $**p < 0.01$ by one-way ANOVA compared with the cisplatin-only group at each frequency.

In vivo combined injection of cisplatin and LPS increased the expression of TLR4 and proinflammatory cytokines in the cochleae of mice

Many previous studies have demonstrated that the proinflammatory cytokines TNF-α, IL-1β, and IL-6 are important mediators for pathological consequences in a variety of clinical situations, such as sepsis, ischemia, and acute renal failure (32). We also reported that cisplatin-induced ototoxicity is closely related to the production and release of proinflammatory cytokines (8, 27). In the current study, we demonstrated that cisplatin induces transcriptional and translational upregulation of TLR4 in HEI-OC1 cells in vitro and in ex vivo primary organ of Corti explants. In addition, the production of proinflammatory cytokines is significantly enhanced by the interaction of TLR4 and its exogenously introduced ligand LPS. We therefore investigated by immunohistochemical staining whether in vivo injection of cisplatin and/or LPS affects the expression of TLR4 and proinflammatory cytokines in the cochlear tissue after two cycles of treatment. As shown in Fig. 6, the cisplatin- or LPS-alone groups showed very slight expression of TLR4 protein similar to that of the PBS control. However, the expression of TLR4 protein after cisplatin treatment was markedly

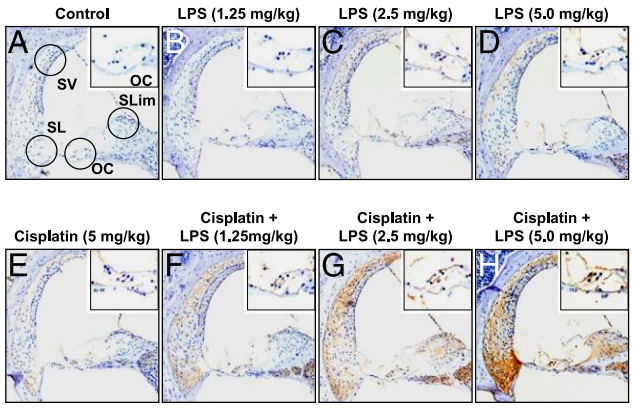

FIGURE 6. In vivo effect of cisplatin and/or LPS on TLR4 expression in the cochleae. *A–H*, Cochleae from C57BL/6 mice injected with PBS, cisplatin, LPS, or cisplatin plus LPS were removed, decalcified, and embedded in paraffin, and 5-μm sections were prepared. In an immunohistochemical study, an immunohistochemistry kit (DAKO LSAB+ System-HRP, K0679) was used, and all procedures were carried out according to the manufacturer's instructions. Goat polyclonal Ab was used as the primary TLR4 Ab. This figure is representative of three individual animals from each group. An enlarged image of the organ of Corti is presented in the inset of each panel. The results were observed under a microscope (original magnification ×100 and ×200 in OC part). OC, organ of Corti; SL, spiral ligament; SLim, spiral limbus; SV, stria vascularis.

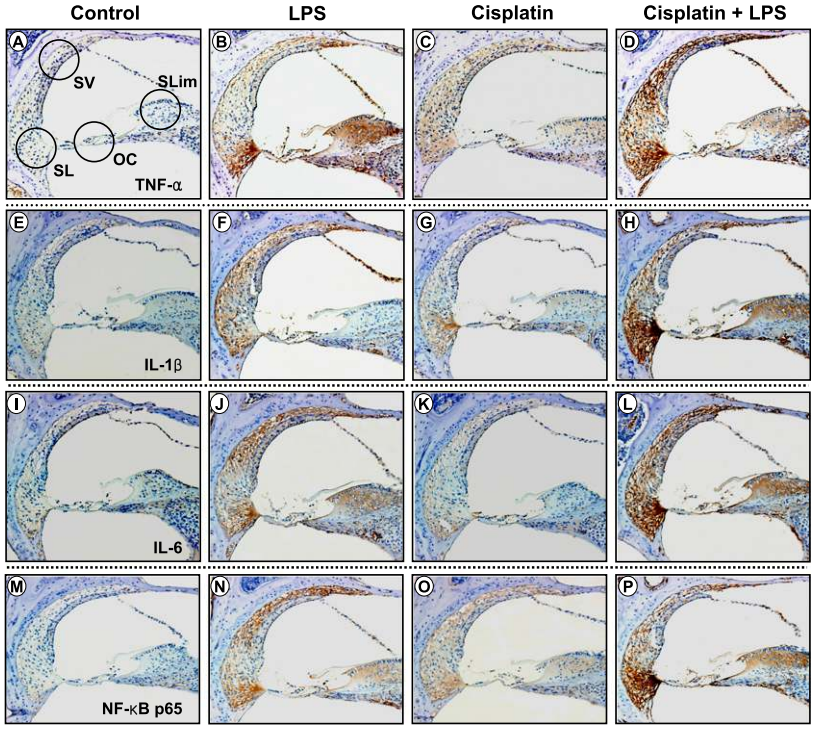

FIGURE 7. In vivo combined injection of cisplatin and LPS increased the expression of proinflammatory cytokines in the cochleae of mice. The cochleae were embedded in paraffin and prepared in 5-μm sections for immunohistochemical study of proinflammatory cytokine expression. An immunohistochemistry kit (DAKO LSAB+ System-HRP, K0679) was used, and all procedures were carried out according to the manufacturer's instructions. The following primary Abs were used: *A–D*, anti–TNF-α (rabbit polyclonal Ab); *E–H*, anti–IL-1β (rabbit polyclonal Ab); *I–L*, anti–IL-6 (rabbit polyclonal Ab); *M–P*, anti–NF-κB p65 (rabbit polyclonal Ab). The results were observed under a microscope (original magnification ×100). OC, organ of Corti; SL, spiral ligament; SLim, spiral limbus; SV, stria vascularis.

enhanced by LPS in a dose-dependent manner in all regions of the cochlear tissue. In addition, as shown in Fig. 7, cisplatin or LPS alone slightly increased the expressions of proinflammatory cytokines and NF-κB p65 compared with that of the control group. However, combined treatment with cisplatin and LPS markedly enhanced the expression of proinflammatory cytokines and NF-κB p65 in the entire cochlear tissue, including the stria vascularis, spiral ligament, and spiral ganglion neurons as well as in the sensory hair cell layers in the organ of Corti. In parallel with the enhanced expression of proinflammatory cytokines in cochlear tissue from mice injected with cisplatin alone, LPS alone, or in cisplatin–LPS–co-injected mice, the serum levels of TNF-α and IL-1β were increased in the cisplatin- or LPS-injected mice compared with that of PBS-control mice (Fig. 8). Notably, the increased serum level of TNF-α due to cisplatin was further highly enhanced by LPS in a dose-dependent manner in the cisplatin–LPS–co-injected mice compared with that of mice injected with cisplatin or LPS alone. However, the increased serum level of IL-1β after injection with cisplatin was not further enhanced by LPS injection.

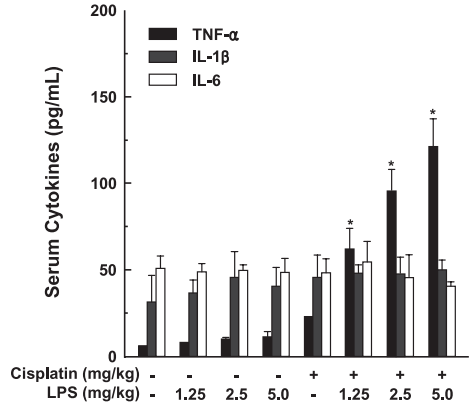

FIGURE 8. In vivo combined injection of cisplatin and LPS increased the serum level of proinflammatory cytokines. After performing the in vivo experimental schedule, mice were sacrificed for bleeding and removal of the cochleae. The levels of proinflammatory cytokines (TNF-α, IL-1β, and IL-6) in sera were analyzed with an ELISA kit, and all procedures were carried out according to the manufacturer's instructions. $*p < 0.05$.

Synergistic deterioration of hearing function by LPS after exposure to cisplatin was suppressed in TLR4 mutant C3H/HeJ or TLR4$^{-/-}$ mice

In the current study, we demonstrated that cisplatin increased TLR4 expression and that its interaction with LPS resulted in hearing impairment through proinflammatory cytokine production and NF-κB activation. Therefore, to confirm the involvement of TLR signaling in the deteriorated hearing function of cisplatin-injected mice after exogenous LPS injection, TLR4 mutant C3H/HeJ or TLR4$^{-/-}$ mice were injected with cisplatin and/or LPS using the same schedule shown in Fig. 5*A*. We then examined the hearing threshold using the ABR test. As shown in Fig. 9*A* and 9*B*, although cisplatin alone or high-dose LPS (5 mg/kg body weight) induced a very slight increase in ABR threshold compared with that of the PBS-control group, the significant changes in the ABR threshold at 4, 8, 16, and 32 kHz were not detected in the cisplatin- and/or LPS-injected TLR4 mutant C3H/HeJ (Fig. 9*A*) and TLR4$^{-/-}$ mice (Fig. 9*B*) after either the first or second cycle of treatment. In addition, the serum levels of proinflammatory cytokines were not elevated in cisplatin- and/or LPS-injected TLR4$^{-/-}$ mice compared with those of the PBS-control group (Fig. 9*B*, inset). In addition, the expression of the proteins TLR4 and TNF-α was not observed in cisplatin- and/or LPS-treated TLR4$^{-/-}$ mice (Fig. 10). These results suggest that TLR4 signaling cascade is critically required for cisplatin–LPS–mediated ototoxicity through production of proinflammatory cytokines.

Discussion

Many drugs in clinical use are ototoxic and have the potential to cause inner ear damage as a side effect despite their therapeutic

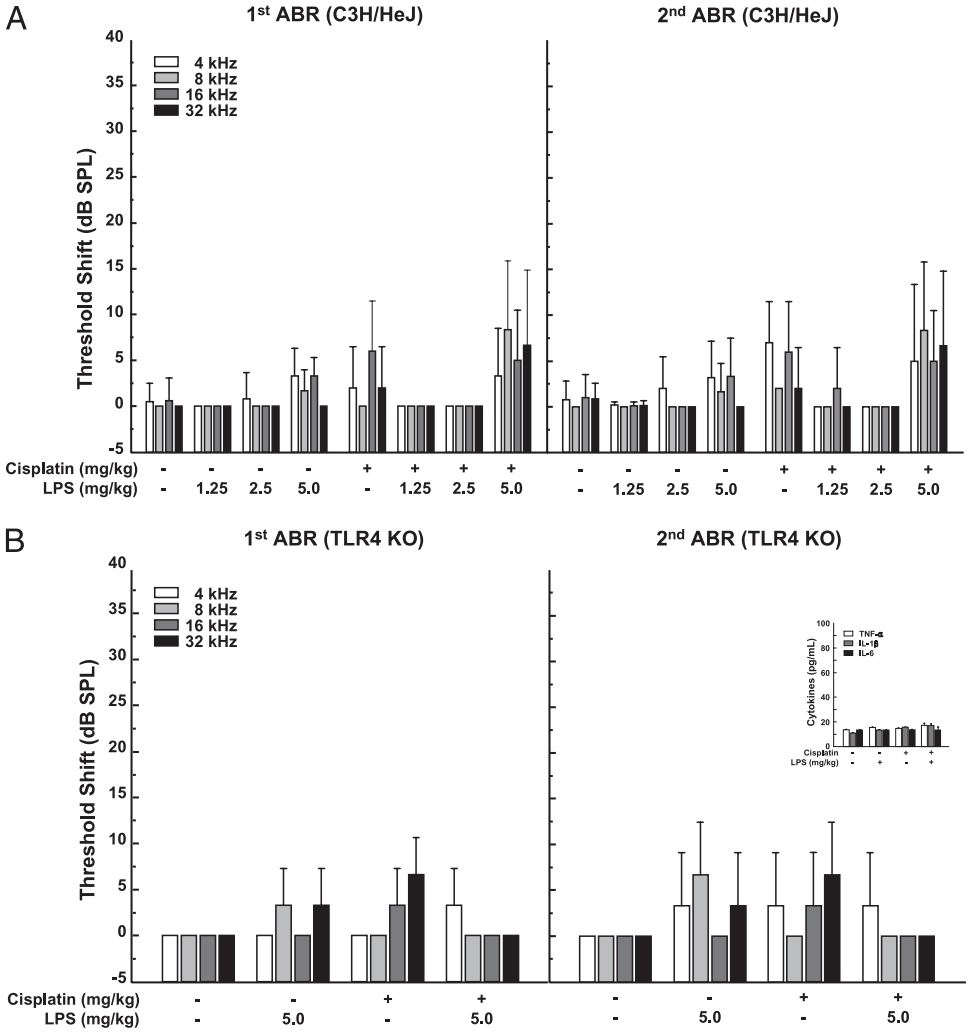

FIGURE 9. Synergistic deterioration of hearing function by LPS after exposure to cisplatin was blocked in TLR4 mutant C3H/HeJ or TLR4$^{-/-}$ mice. TLR4 mutant C3H/HeJ (*A*) and TLR4$^{-/-}$ (*B*) mice were administered PBS, cisplatin, LPS, or cisplatin–LPS by i.p. injection as described for Fig. 5*A*. ABR was measured at three time points as indicated: before injection (pre-ABR), after first injection of cisplatin and/or LPS (1st ABR), and after second injection (2nd ABR). ABR threshold shifts were represented as mean ± SD at each frequency tested in these mice. In addition, the inset of Fig. 10*B* represents the serum levels of proinflammatory cytokines in cisplatin- and/or LPS-injected TLR4$^{-/-}$ mice.

value. These drugs can either be vestibulotoxic (i.e., they disturb the balance organs in the vestibular system) or cochleotoxic (i.e., they cause hearing loss by affecting the sensory cells of the cochlea) (33). Cisplatin is a widely used chemotherapeutic agent for the treatment of various solid tumors, but its usefulness is limited by side effects, which include nephrotoxicity, neurotoxicity, and ototoxicity. Many cancer patients also experience other side effects, such as nausea and vomiting, anemia, fever, and alopecia during their chemotherapy. Incidence of ototoxicity in the course of clinical chemotherapy is highly variable and depends on dose and duration of drugs as well as on the disease state of the patients. Wang et al. (34) reported that the incidence of hearing loss was increased from 8.9 to 28.8% in 150 patients with nasopharyngeal carcinoma after chemotherapy and radiation. Bokemeyer et al. (14) revealed that the frequency of cisplatin-induced ototoxicity was ~20–40% after a high cumulative dose of 400 mg/m^2 in patients with testicular cancer. De Jongh et al. (15), however, reported that the ototoxicity of weekly high-dose cisplatin (70–85 mg/m^2) in 400 patients with advanced solid tumors was only observed in 2.5% of the patients. In addition, cancer patients

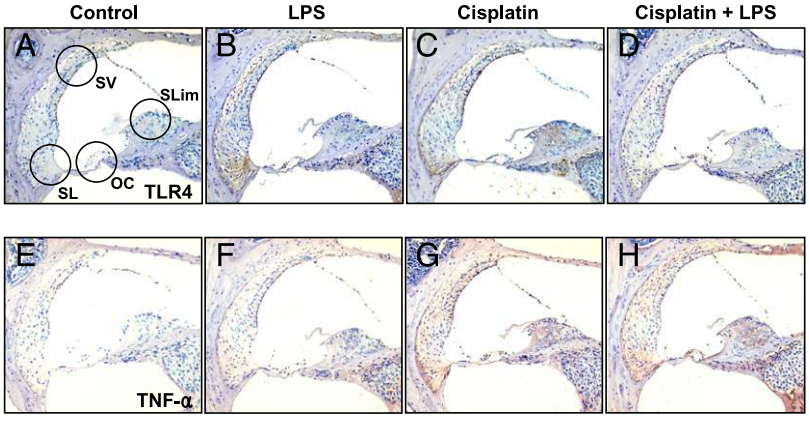

FIGURE 10. The protein expression of TLR4 and TNF-α was not observed in cisplatin- and/or LPS-treated TLR4$^{-/-}$ mice. TLR4$^{-/-}$ mice were administered PBS, cisplatin, LPS, and cisplatin–LPS by i.p. injection as described for Fig. 5*A*. Cochleae were removed, decalcified, and embedded in paraffin. Then, 5-μm sections were prepared. In an immunohistochemical study, an immunohistochemistry kit (DAKO LSAB+ System-HRP, K0679) was used, and all procedures were carried out according to the manufacturer's instructions. The following primary Abs were used: *A–D*, anti-TLR4 (goat polyclonal Ab); *E–H*, anti–TNF-α (rabbit polyclonal Ab). The results were observed under a microscope (original magnification ×100). OC, organ of Corti; SL, spiral ligament; SLim, spiral limbus; SV, stria vascularis.

receiving cisplatin have been found to experience leukopenia, anemia, and reduction of platelets as well as hearing loss. These side effects induce severe toxicity and may result in additional diseases. Infection has a significant influence on the care and outcome of patients with cancer, notably in those patients who become neutropenic during the course of disease (35). Important factors for infection in cancer patients may be therapy-related myelosuppression and immune defects inherent to the underlying disease process. Although cisplatin-induced ototoxic mechanisms have been well defined in various reports, the effects of accidental infection on ototoxicity during the course of chemotherapeutic cycles with cisplatin remain to be elucidated.

The cytotoxic mechanisms of cisplatin, including DNA adducts, mitochondrial dysfunction, and generation of ROS, have been extensively studied. In addition, we demonstrated another mechanism for cisplatin ototoxicity; that is, proinflammatory cytokines play a critical role in the pathogenesis of cisplatin-induced ototoxicity (8). Cisplatin-induced ototoxicity occurs primarily in the OHCs of the organ of Corti. Cisplatin induces apoptosis of OHCs in the organ of Corti explants and in vivo, respectively (3, 36). Inflammatory cytokines are known to be related to innate and adaptive immunity in the host defense system. Cisplatin activates macrophages in vitro to enhance the tumoricidal state and enhances their Ag-presenting ability (14). It also increases the production of ROS and proinflammatory cytokines both in vitro and in vivo (37–39). In addition, cisplatin increases the expression of TLRs, including TLR4 in murine peritoneal macrophages in vitro, and subsequent stimulation with individual TLR-related ligands induces the production of proinflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, and IL-12 (30). TLR4, as a pattern recognition receptor, is known to recognize its specific ligand LPS in innate immunity. By using TLR4 mutant C3H/HeJ mice, Cenedeze et al. (24) reported that cisplatin-induced renal toxicity is partly mediated through TLR4. Ramesh et al. (25) also demonstrated that the combination of cisplatin and LPS acts synergistically to produce inflammatory cytokines, such as TNF-α, IL-6, MCP-1, KC, and GM-CSF, thereby inducing nephrotoxicity in an acute renal failure model. Leukocytes are involved in defending the body against both infectious disease and foreign materials. It has been well known that cisplatin promotes the infiltration of leukocytes to kidney through TLR4 and cytokine production (23, 40). However, in our previous report, we demonstrated that cisplatin induces secretions of proinflammatory cytokines from various types of cells in the cochlear tissues including hair cells and supporting cells, and these cytokines mediate ototoxicity without leukocyte infiltration (8). Moreover, in this study, proinflammatory cytokines were also considered as one of the major effective molecules for cisplatin–LPS–induced ototoxicity through TLR4 signaling. Therefore, we investigated whether production of proinflammatory cytokines in the cisplatin- and/or LPS-treated cochlear tissues could lead to recruitment of inflammatory leukocytes into cochlear tissues, thereby causing ototoxicity. We examined the recruitment of inflammatory leukocytes in the cochlear tissues using H&E staining and immunohistochemical analysis for CD45R as a leukocyte surface marker. As shown in Supplemental Figs. 3 and 4, the specific infiltration of leukocytes in the cochlear tissues from cisplatin- and/or LPS-treated mice was not detected by H&E staining and immunohistochemical analysis for CD45R, even though it just showed very slight nonspecific expression of CD45R in the prominence region of the spiral ligament. Based on these results, we conclude that the immune response by infiltrated leukocyte is not involved in cisplatin- and/or LPS-induced ototoxicity.

In this study, we clearly demonstrated that the transcriptional and translational expression of TLR4 was increased in mouse cochleae in vivo (Figs. 1*B*, 6), HEI-OC1 auditory cells in vitro (Fig. 1*A*, 1*C*, 1*D*), and organ of Corti explants ex vivo (Fig. 2) after cisplatin exposure. We also showed that cisplatin-induced TLR4 interacts with FITC-conjugated LPS in vitro (Fig. 3*A*, 3*B*). Furthermore, the engagement of LPS with its receptor TLR4 elicited NF-κB activation by IκBα phosphorylation after cisplatin treatment in HEI-OC1 cells (Fig. 4*A*), and this activation was completely inhibited in TLR4 siRNA-transfected HEI-OC1 cells (Fig. 4*B*) or TLR4 knockout MEF cells (Fig. 4*C*). Of note, NF-κB activation is a critical bridge to the expression of inflammatory cytokines and other mediators involved in inflammatory responses (30). After dimerizing of TLR4 through engagement with its ligand, adapter molecules, such as TIRAP and TRAM, are recruited on the cytoplasmic domain of TLR4 and further interact with MyD88 and TRIF, respectively, and then transduce a signal to the nucleus. MyD88 is critical for signaling by all TLRs except TLR3. After stimulation, MyD88 associates with the cytoplasmic portion of the TLR and then recruits IRAK-4 and IRAK-1 through a homophilic interaction of the death domains (13). Subsequently, TNFR-associated factor 6, transforming growth factor-β–activated kinase 1, and NF-κB are activated, and then NF-κB is translocated into the nucleus regulating the genes for proinflammatory cytokines as well as many other genes. In the current study, we also observed that combined treatment with cisplatin and LPS markedly enhanced the expression and secretion of proinflammatory cytokines in the whole cochlear tissue (Fig. 7) and blood sera (Fig. 8). Notably, the increased serum level of TNF-α after treatment with cisplatin was further highly enhanced by LPS, but IL-1β secretion was not affected by LPS injection. In agreement with our previous report (8), these results suggest that TNF-α plays a key role in the cisplatin- or cisplatin–LPS–induced ototoxicity.

In our previous study, we also demonstrated that NADPH oxidases (NOXs) played a critical role in cisplatin-induced cochlear injury, and TNF-α and ERK played a key role in NOX activation (41). These findings suggest that inflammation and ROS generation through NOXs are one of the major mechanisms in cisplatin ototoxicity. Therefore, we further examined the expression of other cellular mediators, such as high mobility group box 1 (HMGB1), receptor for advanced glycation end-product (RAGE), advanced glycation end-product (AGE), inducible NO synthase (iNOS), and NOXs, which are closely related to inflammation and oxidative stress (42, 43). As shown in Supplemental Fig. 5 and Supplemental Table I, combined injection of cisplatin and LPS markedly enhanced the expression of these cellular mediators, including HMGB1, RAGE, AGE, cyclooxygenase 2, iNOS, and NOXs, in the entire cochlear tissue, such as the stria vascularis, spiral ligament, and spiral ganglion neurons as well as the sensory hair cell layers in the organ of Corti. These findings suggest that other cellular mediators, such as HMGB1, RAGE, AGE, cyclooxygenase 2, iNOS, and NOXs, may be also involved in acceleration of cisplatin ototoxicity by TLR4–LPS interaction. However, the effects of these molecules on cisplatin–LPS–mediated ototoxicity should be further extensively studied.

The development of experimental animal models has played a critical role in understanding the mechanisms of ototoxicity by these drugs and in the search for oto-protective materials. Typically, the cochlear effects of ototoxic drugs are studied in the guinea pig, which is the most common animal model used in hearing research (2, 44). The mouse model for studying ototoxicity is of interest because it allows for more flexibility and offers the potential for transgenic studies (45). In our previous studies, 8-wk-old mice received cisplatin i.p. for four consecutive days (4 mg cisplatin/kg

body weight per injection) (8, 41). These mice showed a significant hearing loss by ABR measurement. However, human clinical chemotherapy schedule depends on tumor type, condition of patients, and type of drugs. In the clinical use of cisplatin alone or combined with other drugs, treatment plans generally call for treating several times with 3- or 4-wk intervals (46). Considering the cisplatin-induced TLR4 expression in our in vivo and in vitro systems, we speculate that cisplatin can induce a systemic decline of immune surveillance in cancer patients. In this situation, hearing function may be deteriorated by additional inflammatory responses through the signaling pathways mediated by the interaction of TLR4 and PAMPs of infected microbial organisms. Therefore, to address this question, we used a C57BL/6 mouse model subjected to two cycles of low-dose cisplatin injection and a double treatment of LPS after cisplatin injection to mimic the result of infection during the chemotherapy cycles (Fig. 5*A*). Cisplatin alone with a final cumulative dose of 10 mg cisplatin kg/body weight did not result in any deterioration of hearing function. LPS alone also could not induce hearing impairment. However, the combined injection of cisplatin and LPS caused severe hearing impairment in C57BL/6 mice (Fig. 5*C*), whereas this hearing dysfunction was completely inhibited in both TLR4 mutant (Fig. 9*A*) and TLR4$^{-/-}$ mice (Fig. 9*B*). These results support our hypothesis that hearing function can be synergistically damaged by the increased expression of TLRs and by microbial infections in cancer patients who are in a treatment regimen composed of multiple cycles of cisplatin-based chemotherapy. Moreover, such damage can occur even though patients do not exhibit an ototoxic level of cumulative cisplatin concentration. Taken together, the results of the current study suggest the possibility that anticancer chemotherapy occasionally deteriorates the host defense system against infection, and thus, an increase in chance of infection occurrence may cause additional hearing loss through activation of TLRs signaling pathway.

Disclosures

The authors have no financial conflicts of interest.

References

1. Fram, R. J. 1992. Cisplatin and platinum analogues: recent advances. *Curr. Opin. Oncol.* 4: 1073–1079.
2. Rybak, L. P., and C. A. Whitworth. 2005. Ototoxicity: therapeutic opportunities. *Drug Discov. Today* 10: 1313–1321.
3. Alam, S. A., K. Ikeda, T. Oshima, M. Suzuki, T. Kawase, T. Kikuchi, and T. Takasaka. 2000. Cisplatin-induced apoptotic cell death in Mongolian gerbil cochlea. *Hear. Res.* 141: 28–38.
4. Lee, J. E., T. Nakagawa, T. S. Kim, F. Iguchi, T. Endo, Y. Dong, K. Yuki, Y. Naito, S. H. Lee, and J. Ito. 2003. A novel model for rapid induction of apoptosis in spiral ganglions of mice. *Laryngoscope* 113: 994–999.
5. Lee, J. E., T. Nakagawa, T. Kita, T. S. Kim, F. Iguchi, T. Endo, A. Shiga, S. H. Lee, and J. Ito. 2004. Mechanisms of apoptosis induced by cisplatin in marginal cells in mouse stria vascularis. *ORL J. Otorhinolaryngol. Relat. Spec.* 66: 111–118.
6. Rybak, L. P., C. Whitworth, and S. Somani. 1999. Application of antioxidants and other agents to prevent cisplatin ototoxicity. *Laryngoscope* 109: 1740–1744.
7. McAlpine, D., and B. M. Johnstone. 1990. The ototoxic mechanism of cisplatin. *Hear. Res.* 47: 191–203.
8. So, H., H. Kim, J. H. Lee, C. Park, Y. Kim, E. Kim, J. K. Kim, K. J. Yun, K. M. Lee, H. Y. Lee, et al. 2007. Cisplatin cytotoxicity of auditory cells requires secretions of proinflammatory cytokines via activation of ERK and NF-kappaB. *J. Assoc. Res. Otolaryngol.* 8: 338–355.
9. Jamesdaniel, S., D. Ding, M. H. Kermany, B. A. Davidson, P. R. Knight, III, R. Salvi, and D. E. Coling. 2008. Proteomic analysis of the balance between survival and cell death responses in cisplatin-mediated ototoxicity. *J. Proteome Res.* 7: 3516–3524.
10. Tarang, S., A. Sodhi, and P. Chauhan. 2007. Differential expression of Toll-like receptors in murine peritoneal macrophages in vitro on treatment with cisplatin. *Int. Immunol.* 19: 635–643.
11. Hoffmann, J. A., F. C. Kafatos, C. A. Janeway, and R. A. Ezekowitz. 1999. Phylogenetic perspectives in innate immunity. *Science* 284: 1313–1318.
12. Akira, S., and H. Hemmi. 2003. Recognition of pathogen-associated molecular patterns by TLR family. *Immunol. Lett.* 85: 85–95.
13. McGettrick, A. F., and L. A. O'Neill. 2004. The expanding family of MyD88-like adaptors in Toll-like receptor signal transduction. *Mol. Immunol.* 41: 577–582.
14. Bokemeyer, C., C. C. Berger, J. T. Hartmann, C. Kollmannsberger, H. J. Schmoll, M. A. Kuczyk, and L. Kanz. 1998. Analysis of risk factors for cisplatin-induced ototoxicity in patients with testicular cancer. *Br. J. Cancer* 77: 1355–1362.
15. de Jongh, F. E., R. N. van Veen, S. J. Veltman, R. de Wit, M. E. van der Burg, M. J. van den Bent, A. S. Planting, W. J. Graveland, G. Stoter, and J. Verweij. 2003. Weekly high-dose cisplatin is a feasible treatment option: analysis on prognostic factors for toxicity in 400 patients. *Br. J. Cancer* 88: 1199–1206.
16. Haziot, A., E. Ferrero, F. Köntgen, N. Hijiya, S. Yamamoto, J. Silver, C. L. Stewart, and S. M. Goyert. 1996. Resistance to endotoxin shock and reduced dissemination of gram-negative bacteria in CD14-deficient mice. *Immunity* 4: 407–414.
17. Nagai, Y., S. Akashi, M. Nagafuku, M. Ogata, Y. Iwakura, S. Akira, T. Kitamura, A. Kosugi, M. Kimoto, and K. Miyake. 2002. Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat. Immunol.* 3: 667–672.
18. Chauhan, P., A. Sodhi, and A. Shrivastava. 2009. Cisplatin primes murine peritoneal macrophages for enhanced expression of nitric oxide, proinflammatory cytokines, TLRs, transcription factors and activation of MAP kinases upon co-incubation with L929 cells. *Immunobiology* 214: 197–209.
19. Kaisho, T., and S. Akira. 2000. Critical roles of Toll-like receptors in host defense. *Crit. Rev. Immunol.* 20: 393–405.
20. Uematsu, S., and S. Akira. 2006. The role of Toll-like receptors in immune disorders. *Expert Opin. Biol. Ther.* 6: 203–214.
21. Huang, Q. Q., and R. M. Pope. 2009. The role of toll-like receptors in rheumatoid arthritis. *Curr. Rheumatol. Rep.* 11: 357–364.
22. Landreth, G. E., and E. G. Reed-Geaghan. 2009. Toll-like receptors in Alzheimer's disease. *Curr. Top. Microbiol. Immunol.* 336: 137–153.
23. Zhang, B., G. Ramesh, S. Uematsu, S. Akira, and W. B. Reeves. 2008. TLR4 signaling mediates inflammation and tissue injury in nephrotoxicity. *J. Am. Soc. Nephrol.* 19: 923–932.
24. Cenedeze, M. A., G. M. Gonçalves, C. Q. Feitoza, P. M. Wang, M. J. Damião, A. P. Bertocchi, A. Pacheco-Silva, and N. O. Câmara. 2007. The role of toll-like receptor 4 in cisplatin-induced renal injury. *Transplant. Proc.* 39: 409–411.
25. Ramesh, G., B. Zhang, S. Uematsu, S. Akira, and W. B. Reeves. 2007. Endotoxin and cisplatin synergistically induce renal dysfunction and cytokine production in mice. *Am. J. Physiol. Renal Physiol.* 293: F325–F332.
26. Ramesh, G., S. R. Kimball, L. S. Jefferson, and W. B. Reeves. 2007. Endotoxin and cisplatin synergistically stimulate TNF-alpha production by renal epithelial cells. *Am. J. Physiol. Renal Physiol.* 292: F812–F819.
27. So, H., H. Kim, Y. Kim, E. Kim, H. O. Pae, H. T. Chung, H. J. Kim, K. B. Kwon, K. M. Lee, H. Y. Lee, et al. 2008. Evidence that cisplatin-induced auditory damage is attenuated by downregulation of pro-inflammatory cytokines via Nrf2/HO-1. *J. Assoc. Res. Otolaryngol.* 9: 290–306.
28. Zheng, J. L., and W. Q. Gao. 1996. Differential damage to auditory neurons and hair cells by ototoxins and neuroprotection by specific neurotrophins in rat cochlear organotypic cultures. *Eur. J. Neurosci.* 8: 1897–1905.
29. Akira, S., S. Uematsu, and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell* 124: 783–801.
30. Ferreiro, D. U., and E. A. Komives. 2010. Molecular mechanisms of system control of NF-kappaB signaling by IkappaBalpha. *Biochemistry* 49: 1560–1567.
31. de Ruysscher, D. 2010. Treatment of limited disease small cell lung cancer. *Front. Radiat. Ther. Oncol.* 42: 173–179.
32. Levi, M. 2010. The coagulant response in sepsis and inflammation. *Hamostaseologie* 30: 10–12, 14–16.
33. Schacht, J., and J. E. Hawkins. 2006. Sketches of otohistory. Part 11: ototoxicity: drug-induced hearing loss. *Audiol. Neurootol.* 11: 1–6.
34. Wang, L. F., W. R. Kuo, K. Y. Ho, K. W. Lee, and C. S. Lin. 2003. Hearing loss in patients with nasopharyngeal carcinoma after chemotherapy and radiation. *Kaohsiung J. Med. Sci.* 19: 163–169.
35. Morrison, V. A. 2005. An overview of the management of infection and febrile neutropenia in patients with cancer. *Support. Cancer Ther.* 2: 88–94.
36. Kopke, R. D., W. Liu, R. Gabaizadeh, A. Jacono, J. Feghali, D. Spray, P. Garcia, H. Steinman, B. Malgrange, R. J. Ruben, et al. 1997. Use of organotypic cultures of Corti's organ to study the protective effects of antioxidant molecules on cisplatin-induced damage of auditory hair cells. *Am. J. Otol.* 18: 559–571.
37. Singh, R. K., A. Sodhi, and S. M. Singh. 1991. Production of interleukin-1 and tumor necrosis factor by cisplatin-treated murine peritoneal macrophages. *Natural Immunity and Cell Growth Regulation* 10: 105–116.
38. Sodhi, A., and K. Pai. 1992. Increased production of interleukin-1 and tumor necrosis factor by human monocytes treated in vitro with cisplatin or other biological response modifiers. *Immunol. Lett.* 34: 183–188.
39. Sodhi, A., and P. Gupta. 1986. Increased release of hydrogen peroxide (H2O2) and superoxide anion (O-2) by murine macrophages in vitro after cis-platin treatment. *Int. J. Immunopharmacol.* 8: 709–714.
40. Ramesh, G., and W. B. Reeves. 2002. TNF-alpha mediates chemokine and cytokine expression and renal injury in cisplatin nephrotoxicity. *J. Clin. Invest.* 110: 835–842.
41. Kim, H. J., J. H. Lee, S. J. Kim, G. S. Oh, H. D. Moon, K. B. Kwon, C. Park, B. H. Park, H. K. Lee, S. Y. Chung, et al. 2010. Roles of NADPH oxidases in cisplatin-induced reactive oxygen species generation and ototoxicity. *J. Neurosci.* 30: 3933–3946.
42. Bierhaus, A., and P. P. Nawroth. 2009. Multiple levels of regulation determine the role of the receptor for AGE (RAGE) as common soil in inflammation, immune responses and diabetes mellitus and its complications. *Diabetologia* 52: 2251–2263.

43. Castiglioni, A., V. Canti, P. Rovere-Querini, and A. A. Manfredi. 2010. High-mobility group box 1 (HMGB1) as a master regulator of innate immunity. *Cell Tissue Res.* In press.
44. Aran, J. M., J. P. Erre, D. Lima da Costa, I. Debbarh, and D. Dulon. 1999. Acute and chronic effects of aminoglycosides on cochlear hair cells. *Ann. N. Y. Acad. Sci.* 884: 60–68.
45. Brown, S. D., R. E. Hardisty-Hughes, and P. Mburu. 2008. Quiet as a mouse: dissecting the molecular and genetic basis of hearing. *Nat. Rev. Genet.* 9: 277–290.
46. Wagner, A. D., S. Unverzagt, W. Grothe, G. Kleber, A. Grothey, J. Haerting, and W. E. Fleig. 2010. Chemotherapy for advanced gastric cancer. *Cochrane Database Syst. Rev.* (3): CD004064.