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IL-10/HMOX1 Signaling Modulates Cochlear Inflammation via Negative Regulation of MCP-1/CCL2 Expression in Cochlear Fibrocytes

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Cochlear inflammatory diseases, such as tympanicogenic labyrinthitis, are associated with acquired sensorineural hearing loss. Although otitis media is extremely frequent in children, tympanicogenic labyrinthitis is not commonly observed, which suggests the existence of a potent anti-inflammatory mechanism modulating cochlear inflammation. In this study, we aimed to determine the molecular mechanism involved in cochlear protection from inflammation-mediated tissue damage, focusing on IL-10 and hemoxynagenase-1 (HMOX1) signaling. We demonstrated that IL-10Rs are expressed in the cochlear lateral wall of mice and rats, particularly in the spiral ligament fibrocytes (SLFs). The rat SLF cell line was found to inhibit nontypeable Haemophilus influenzae (NTHi)-induced upregulation of monocyte chemotactic protein-1 (MCP-1; CCL2) in response to IL-10. This inhibition was suppressed by silencing IL-10R1 and was mimicked by cobalt Protoporphyrin IX and CO-releasing molecule-2. In addition, IL-10 appeared to suppress monocyte recruitment through reduction of NTHi-induced rat SLF cell line-derived chemoattractants. Silencing of HMOX1 was found to attenuate the inhibitory effect of IL-10 on NTHi-induced MCP-1/CCL2 upregulation. Chromatin immunoprecipitation assays showed that IL-10 inhibits NTHi-induced binding of p65 NF-κB to the distal motif in the promoter region of MCP-1/CCL2, resulting in suppression of NTHi-induced NF-κB activation. Furthermore, IL-10 deficiency appeared to significantly affect cochlear inflammation induced by intratympanic injections of NTHi. Taken together, our results suggest that IL-10/HMOX1 signaling is involved in modulation of cochlear inflammation through inhibition of MCP-1/CCL2 regulation in SLFs, implying a therapeutic potential for a CO-based approach for inflammation-associated cochlear diseases. The Journal of Immunology, 2015, 194: 3953–3961.

More than 48 million Americans, 12 years and older, are known to have hearing loss (1). Among the various types of hearing loss, acquired sensorineural hearing loss (SNHL) is clinically important because it is preventable and manageable (2, 3). Because acquired SNHL is frequently associated with inflammation, as observed in acoustic trauma and cisplatin ototoxicity (4, 5), understanding the molecular mechanism involved in cochlear inflammation may provide us with a novel therapeutic approach.

Middle ear infection, one of the most common pediatric diseases, induces inner ear inflammation (6), which may lead to SNHL (7) and vertigo (8). Consistently, we demonstrated that the spiral ligament fibrocytes (SLFs), which are specialized cochlear fibrocytes (9), play a pivotal role in tympanicogenic cochlear inflammation through upregulation of chemokines, such as monocyte chemotactic protein-1 (MCP-1; CCL2) and CXCL2, in response to 

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Abbreviations used in this article: ChIP, chromatin immunoprecipitation; CL, cochlear lateral wall; CoPP, protoporphyrin IX cobalt chloride; CORM, CO-releasing molecule; HMOX1, hemoxynagenase-1; MCP-1, monocyte chemotactic protein-1; NTHi, nontypeable Haemophilus influenzae; rHL-10, recombinant human IL-10; RSL, rat SLF cell line; siRNA, small interfering RNA; SLF, spiral ligament fibrocyte; SNHL, sensorineural hearing loss.

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ates cisplatin ototoxicity through downregulation of proinflammatory cytokines (22), we aimed to further determine the involvement of HMOX1 signaling in IL-10–mediated modulation of cochlear inflammation.

In this article, we show that IL-10Rs are expressed in the cochlear lateral wall (CL) and that SLFs attenuate NTHI-induced MCP-1/CCL2 upregulation in response to IL-10 via HMOX1 signaling. Exogenous and endogenous CO modulated MCP-1/CCL2 regulation through inhibition of p65 NF-kB binding to the promoter region of MCP-1/CCL2. IL-10 deficiency appeared to significantly affect tympanicocochlear inflammation in the animal model. Taken together, this study suggests that IL-10 signaling plays a critical role in protecting the cochlea from inflammation-mediated tissue damage.

Materials and Methods

Reagents

Recombinant human IL-10 (rhIL-10) and protoporphyrin IX cobalt chloride ([Co2Ru(CO)3Cl2]; CO-releasing molecule [CORM]-2) was purchased from Synthasomes (Nacalai Tesque, Kyoto, Japan). Recombinant human IL-10 (rhIL-10) and protoporphyrin IX cobalt chloride ([Co2Ru(CO)3Cl2]; CO-releasing molecule [CORM]-2) was purchased from Emergent BioSolutions (West Haven, CT). IL-10 was produced from transfected CHO cells and purified by a method similar to that used for the production of recombinant human IL-2. The final product contains less than 0.1% human IL-2 and has a purity of at least 96% as determined by HPLC analysis.

Animal experiments

Male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME), IL-10–deficient mice (http://jaxmice.jax.org/strain/002251.html), and C57BL/6 mice (Charles River Laboratory, Wilmington, MA) were used. All animal experiments were approved by the Institutional Animal Care and Use Committee of University of California, Los Angeles. A total of 105 CFU live NTHI suspended in 10 μl saline was transynaptically inoculated into the middle ear of 8–10-wk-old mice using a 30-G needle and syringe under a surgical microscope. As a control, normal saline was injected into the middle ear of 8–10-wk-old mice using a 30-G needle and syringe under a surgical microscope. All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, Bethesda, MD). Anesthesia was induced using a mixture of 1:1:1 isoflurane, oxygen, and air delivered via a face mask. Mice were allowed to recover for 1 week before experiments.

Cell culture and migration assays

Rat SLF cell line (RSL) (23), primary rat CL, and primary rat splenocytes were maintained in DMEM supplemented with 10% FBS, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) (Life Technologies). Primary SLFs were cultured from explants of the rat CL, as described (10). In brief, rat pups (P3–P6) were euthanatized in a CO2 chamber and then decapitated. The cochlea was isolated, with preservation of its normal structure after dissection of the inner ear from the skull base. After removal of the bony otic capsule, the CL was dissected away from the surrounding tissue (organ of Corti and Reissner’s membrane) using fine forceps. Primary SLFs proliferated from the explants of CL and primary cells of passage 5 or less were used in this study. THP-1 cells (human acute monocytic leukemia cell line) were purchased from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium with 2 mM l-glutamine and 10% FBS. All cells were maintained at 37°C in a humidified atmosphere of 5% CO2/95% air. For migration assays (12), RSL cells were exposed to the NTHI lysate (1 μg/ml), with or without rhIL-10 (50 ng/ml), for 24 h, and the conditioned medium was collected. A 24-well plate with poly-carbonate membrane inserts (5-μm pores; Millipore, Billerica, MA), THP-1 cells and isolated mouse splenocytes were added onto each insert at a density of 5 × 105 cells and conditioned medium was added to the lower chamber. Cells were allowed to migrate for 16–18 h. Migrated cells were counted with a hemocytometer.

Bacterial culture and preparation of bacterial lysate

NTHI strain 12, originally a clinical isolate from the middle ear fluid of a child with acute otitis media, was used in this study (24). NTHI lysates were prepared as described previously (25). In brief, a single colony of NTHI was harvested from a chocolate agar plate, inoculated into 3 ml brain heart infusion broth supplemented with NAD and hemin (both at 10 μg/ml), and placed in a 37°C CO2 incubator overnight. After the addition of 50 ml fresh brain heart infusion broth, bacteria were grown for 4 h to a mid-log phase (A500 = 0.4–0.6). The supernatant was discarded after centrifugation at 6000 × g for 30 min. The bacterial pellet was resuspended in 5 ml PBS and sonicated to lyse the bacteria. The lysate was centrifuged at 12,000 × g for 10 min, and the supernatant was collected. Protein concentrations of the NTHI lysates were determined using a BCA protein assay kit (Pierce Biotechnologies, Rockford, IL).

Quantitative RT-PCR

Quantitative RT-PCR was performed as described previously (10). In brief, SLFs were exposed to the NTHI lysate (1 μg/ml), with and without rhIL-10 (50 ng/ml), and total RNA was extracted using TRIzol reagent (Life Technologies). After cDNA was synthesized using the TaqMan reverse transcription kit (Life Technologies), multiplex PCR was performed using the ABI 7500 Real-Time PCR system (Applied Biosystems) with gene-specific primers (FAM-conjugated probes for MCP-1, IL-10, and HMOX1) and control primers (VIC-conjugated probe for GAPDH). The cycle threshold (Ct) values were determined according to the manufacturer’s instructions. The relative quantity of mRNA was determined using the 2ΔCt method (26). Ct values were normalized to the internal control (GAPDH), and the results were expressed as a fold change in mRNA, with the mRNA levels in the nontreated group set as 1. For conventional PCR, primers were used as follows: rat MCP-1 (537 bp), 5’-TGCTGTTCTCAGCCGAGTCGCAATGA-3’ and 5’-AAAGATGTCTGTTGGTCTGGGA-3’; rat IL-10 (330 bp), 5’-GGGAAAAGACATGTGAACCTTG-3’ and 5’-GTCTTGCTGCAGACCCATT-3’; and 5’-AATCTCGTGAGACAGGGTCTCTC-3’ and 5’-AAATCTGGAGATGGGAGCTTGT-3’. The results were analyzed by electrophoresis on 1.5% agarose gels, stained with GelRed Nucleic Acid Stain (Biotium, Hayward, CA), PCR products and photographed using ChemiDoc (Bio-Rad, Hercules, CA).

Plasmid, small interfering RNA, transfection, and luciferase assay

The luciferase-expressing vector with 5’ flanking regions of rat MCP-1/CCL2 was kindly provided by Dr. D. L. Eizirik (Brussels University, Brussels, Belgium) (27). Luciferase assays were performed as described previously (28). In brief, cells were seeded into 12-well plates at a density of 1.0 × 105 cells/well and transfected at 60% confluence using the Transit-LTI transfection reagent (Mirus, Madison, WI), according to the manufacturer’s instructions. The pRL-TK vector (Promega, Madison, WI) was cotransfected to normalize for transfection efficiency. Transfected cells were starved overnight in serum-free DMEM, followed by exposure to the NTHI (1 μg/ml) lysate, with and without rhIL-10 (50 ng/ml), for 8 h before harvesting. All transfections were carried out in triplicate. After washing with PBS, cells were dissolved in lysis reagent (Promega). Luciferase activity was measured using a luminesometer (BD Monolight, 3010) after adding the necessary luciferase substrate (Promega). Results were expressed as a fold change in luciferase activity, taking the value of the nontreated group as 1. For silencing of IL-10R1 expression, cells were transfected with IL-10R1–specific small interfering RNA (siRNA) (s138796: 5’-CCGUGGAGUGCUAAUCCCAAAT-3’, Life Technologies) using siPORT NeoFX transfection agent (Life Technologies), according to the manufacturer’s instructions. The negative control siRNA (AM4635; Life Technologies) was transfected in parallel. Silencing of IL-10R1 expression was determined with quantitative RT-PCR analysis.

Immunoblotting and ELISA

After overnight starvation with a basal medium, RSL cells were treated with the NTHI lysate (1 μg/ml) for 8 h, with and without rhIL-10 (50 ng/ml). Cells were lysed with cell lysis buffer (Cell Signaling Technology, Danvers, MA) supplemented with a protease inhibitor mixture and 1 mM PMSF (Calbiochem). The lysates were centrifuged at 12,000 × g for 15 min, and the supernatants were collected. An equivalent amount of 20 μg protein was loaded onto 10% Tricine gels (Life Technologies). After electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad) and washed three times for 5 min each in TBST. Membranes were blocked using 5% nonfat dry milk in TBST for

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1 h at room temperature and incubated overnight at 4°C in the presence of a 1:200 dilution of a polyclonal Ab against MCP-1/CCL2 (sc-28879) and α-tubulin (sc-53646; Santa Cruz Biotechnology). After washing, membranes were incubated with an HRP-conjugated secondary Ab in a blocking buffer. Membranes were incubated with a SuperSignal substrate (Pierce Biotechs) for 1 min at room temperature, and chemiluminescence signals were detected by exposure to x-ray films. Protein levels of MCP-1/CCL2 and IL-10 were measured using a rat MCP-1 ELISA Kit (BD Biosciences, San Diego, CA) and a mouse IL-10 ELISA Kit (R&D Systems, Minneapolis, MN), following the manufacturers’ instructions. For silencing of HMOX1 expression, cells were transfected with HMOX1-specific siRNA (s127884; Life Technologies) using siPORT NeoFX transfection agent.

Immunolabeling
To determine the translocation of NRF2, cells were cultured on a four-chamber microscope slide and treated with rhIL-10 (50 ng/ml) for 2 h. After fixation with 4% paraformaldehyde and permeabilization of cell membranes with 0.15% Triton X-100 (Sigma-Aldrich), cells were blocked using 10% goat serum and subsequently incubated in the presence of rabbit anti-NFκB Ab (1:200; Santa Cruz Biotechnology) overnight at 4°C. Alexa Fluor 488 goat anti-rabbit IgG (1:500; Life Technologies) was used as the secondary Ab. For paraffin sections, paraffin was removed with a series of washes with xylene, ethanol, and PBS. Sections were blocked with 10% goat serum, incubated with rat anti–IL-10R1 Ab (1:200; Thermo Scientific) overnight at 4°C, and incubated with rhodamine-conjugated goat anti-rat IgG (1:200; Life Technologies). Sections were mounted with antifade mounting media (Life Technologies). Samples were viewed and photographed using a TCS SP5 confocal microscope (Leica, Buffalo Grove, IL).

Chromatin immunoprecipitation
Binding of p65 NF-κB to the enhancer region of the MCP-1/CCL2 gene was determined as described (11). Briefly, RSL cells were treated for 2 h with the NTHi lysate in the presence or absence of rhIL-10 and fixed with 1% formaldehyde for 10 min. After lysis of cells with 1 ml ice-cold lysis buffer supplemented with a protease inhibitor mixture and PMSF, the nuclear fraction was collected using a Nuclear Extract Kit (Active Motif, Carlsbad, CA). After resuspending the nuclear extract, enzymatic shearing of chromatin was conducted. The sheared DNA samples were centrifuged at 18,000 x g for 10 min at 4°C, and the supernatant was collected (input DNA). Input DNA samples were incubated with 2 μg/ml the polyclonal rabbit anti–NFκB p65 Ab (Abcam, Cambridge, MA) and 25 μl protein G magnetic beads overnight at 4°C. The samples were placed on the magnetic stand to pellet the beads, and the supernatant was discarded carefully. After washing, the pelleted beads were resuspended and named chromatin immunoprecipitation (ChIP) DNA. To reverse cross-links, ChIP DNA samples and input DNA samples were incubated at 95°C for 10 min. Mixed with 1 μg/μl proteinase K, and incubated at 37°C for 1 h. Conventional PCR was performed on input DNA and ChIP DNA samples using the following primer pairs: distal NFκB motif (223 bp), 5′-AGCATCTG-GAGGTATATCTCCAGC-3′ and 5′-CAGTTGATATCCGATGCAACA-CAGT-3′ and proximal NFκB motif (200 bp), 5′-GCAAGCTCTATT-GCTCCAGTATG-3′ and 5′-TTATGGTTAAGGCAAGGGTGGAGT-CAGG-3′ (29).

Statistics
All experiments were carried out in triplicate and repeated twice independently. For quantitative RT-PCR analysis, luciferase assays, and ELISA, results were analyzed with the Student’s t test and ANOVA followed by the Tukey post hoc test using R2.14.0 software for Windows (The R Foundation for Statistical Computing). A p value < 0.05 was considered significant. For histological analysis of cochlear inflammation, we performed mixed-effects model analysis and the Fisher exact test using SPSS 12.0 (IBM, Armonk, NY). A Bonferroni corrected p value < 0.025 was considered significant.

Results
IL-10Rs are expressed in the CL
SLFs, which reside in the CL (Fig. 1A), play an important role in cochlear inflammation through upregulation of chemokines (10–12), but it is unclear whether SLFs are able to respond to a potent anti-inflammatory cytokine, IL-10. To determine whether IL-10Rs are expressed in SLFs, we performed RT-PCR analysis using the isolated rat CL tissues and RSL cells. The CL tissues and RSL cells expressed both IL-10R1 and IL-10R2 (Fig. 1B). Notably, IL-10R1, but not IL-10R2, was found to be upregulated by treatment with the lysate of NTHi, a middle ear pathogen. Consistently, immunolabeling showed the expression of IL-10R1 in RSL cells (Fig. 1C). Next, we sought to determine IL-10R1–expressing cochlear cells in the murine temporal bone sections. IL-10R1 was expressed in the spiral ligament area and stria vascularis (Fig. 1D). Particularly, type II SLFs were found to markedly express IL-10R1 after NTHi injection. In addition to the CL, IL-10R1 was expressed in the organ of Corti and the Reissner’s membrane (data not shown), suggesting that the cochlea largely consists of IL-10–responding cells.

IL-10 inhibits NTHi-induced MCP-1/CCL2 upregulation in SLFs
We sought to determine whether IL-10 is expressed in the CL in response to the NTHi lysate. ELISA showed that IL-10 protein was expressed in the isolated CL tissues (42.03 ± 11.27 pg/ml/μg) and splenocytes (85.30 ± 31.66 pg/ml/μg) of WT mice but not IL-10–deficient mice (Fig. 2A). RT-PCR analysis showed that IL-10 was upregulated only in the isolated rat CL tissues, and not in the RSL cells, in response to the NTHi lysate (Fig. 2B); this indicates that IL-10–expressing cells reside in the CL. In contrast, MCP-1/CCL2 was upregulated in both isolated rat CL tissues and RSL cells. Because macrophages are known to reside in the mouse cochlea (30–32), we performed RT-PCR analysis to determine localization of macrophages in the rat CL. As shown in Fig. 2C, CD68 (a marker for monocytes/macrophages) and Aif-1 (a marker for resident macrophages) were found to be expressed in the isolated rat CL tissue, but not in the RSL cells, which suggests that cochlear-resident macrophages are a potential source of IL-10 in the CL. However, further studies are necessary to localize and characterize IL-10–producing cells in the cochlea. Next, we sought to determine whether IL-10 affects MCP-1/CCL2 regulation in SLFs, which may contribute to modulation of cochlear inflammation through upregulation of chemokines (10–12), but it is unclear whether SLFs are able to respond to a potent anti-inflammatory cytokine, IL-10. To determine whether IL-10Rs are expressed in SLFs, we performed RT-PCR analysis using the isolated rat CL tissues and RSL cells. The CL tissues and RSL cells expressed both IL-10R1 and IL-10R2 (Fig. 1B). Notably, IL-10R1, but not IL-10R2, was found to be upregulated by treatment with the lysate of NTHi, a middle ear pathogen. Consistently, immunolabeling showed the expression of IL-10R1 in RSL cells (Fig. 1C). Next, we sought to determine IL-10R1–expressing cochlear cells in the murine temporal bone sections. IL-10R1 was expressed in the spiral ligament area and stria vascularis (Fig. 1D). Particularly, type II SLFs were found to markedly express IL-10R1 after NTHi injection. In addition to the CL, IL-10R1 was expressed in the organ of Corti and the Reissner’s membrane (data not shown), suggesting that the cochlea largely consists of IL-10–responding cells.
inflammation. Quantitative RT-PCR analysis and luciferase assays showed that RSL cells inhibit NTHi-induced transcriptional regulation of MCP-1/CCL2 upregulation upon exposure to rIL-10 (Fig. 2D, 2E). Interestingly, IL-10 appeared to shift a peak in MCP-1/CCL2 upregulation to 12 h later compared with the control not treated with IL-10. Moreover, immunoblot analysis showed that RSL cells inhibit NTHi-induced release of THP-1-attracting molecules upon exposure to IL-10. (F) Migration assays show that RSL cells inhibit NTHi-induced release of THP-1-attracting molecules compared with WT splenocytes. Original magnification ×100. The experiments were performed in triplicate and repeated twice independently. Data are mean ± SD (n = 3). *p < 0.05. Con, conditioned medium from RSL cells not exposed to NTHi; NTHi, conditioned medium from NTHi-exposed RSL cells; pMCP-1–Luc, luciferase-expressing reporter construct of rat MCP-1; S, splenocytes as a positive control; 18S, 18S rRNA.

HMOX1 mediates IL-10–dependent inhibition of NTHi-induced MCP-1/CCL2 regulation in SLFs

The anti-inflammatory effect of IL-10 is mainly mediated by SOCS3 (33, 34) and HMOX1 (35). In our previous studies, HMOX1 was found to protect the cochlea from cisplatin ototoxicity through downregulation of proinflammatory cytokines (22), which led us to further explore the involvement of HMOX1 in the modulation of cochlear inflammation. First, we sought to determine whether SLFs activate NRF2, a basic leucine zipper transcription factor regulating HMOX1 (36), by exposure to NTHi (12), we sought to determine whether IL-10 affects chemotraction of RSL-derived molecules. Migration assays showed that rIL-10 suppresses migration of THP-1 cells by reducing NTHi-induced RSL-derived chemotractionants (Fig. 2H). Moreover, IL-10–deficient splenocytes appeared to migrate more actively in response to NTHi-induced SLF-derived molecules compared with WT splenocytes (Fig. 2I). To further determine the involvement of IL-10R1 in IL-10–mediated inhibition of MCP-1/CCL2 regulation, RSL cells were transfected with an siRNA specific to IL-10R1. Luciferase assays showed that silencing of IL-10R1 significantly suppressed an inhibitory effect of IL-10 on NTHi-induced MCP-1/CCL2 upregulation (Fig. 3A). Quantitative RT-PCR analysis showed siRNA-mediated silencing of IL-10R1 expression (Fig. 3B). Collectively, these results suggest that the IL-10/IL-10R1 axis is involved in modulation of cochlear inflammation through attenuation of MCP-1/CCL2 expression in SLFs.

**FIGURE 2.** IL-10 inhibits NTHi-induced MCP-1 upregulation in SLFs. (A) ELISA shows the expression of IL-10 protein in the CL and splenocytes (S) of WT mice. IL-10 was not detectable (ND) in the CL of IL-10–deficient mice (IL-10−/−). (B) Rat CL tissue, but not RSL cells, upregulated IL-10 expression in response to NTHi. (C) RT-PCR analysis shows that CD68 and Aif-1 are expressed in isolated rat CL tissue but not in RSL cells. Quantitative RT-PCR analysis (D), luciferase assays (E), and immunoblot analysis (F) show that IL-10 inhibits NTHi-induced MCP-1 upregulation. (G) Luciferase assays show that NTHi-induced MCP-1 upregulation is reduced by IL-10 (50 ng/ml) but is enhanced by IL-1β and TNF-α (both 20 ng/ml). (H) Migration assays show that RSL cells inhibit NTHi-induced release of THP-1-attracting molecules upon exposure to IL-10. (I) Migration assays with Giemsa staining (left panel) and cytometry (right panel) show that IL-10–deficient splenocytes (IL-10−/−) migrate more in response to NTHi-induced SLF-derived molecules compared with WT splenocytes.
determine the involvement of HMOX1 in IL-10 signaling in the cochlea, quantitative RT-PCR analysis was performed using the primers specific to HMOX1. IL-10 was found to upregulate HMOX1 expression in the primary rat SLFs (Fig. 4B). Furthermore, silencing of HMOX1 suppressed an inhibitory effect of IL-10 on NTHi-induced MCP-1/CCL2 regulation in RSL cells (Fig. 4C). ELISA analysis showed that RSL cells reduce NTHi-induced MCP-1/CCL2 production when HMOX1 expression is depleted (Fig. 4D). Taken together, these findings suggest that HMOX1 is required for the IL-10–mediated modulation of cochlear inflammation.

**CO is involved in IL-10/HMOX1 signaling in SLFs**

HMOX1, which catalyzes the initial and rate-limiting step in heme metabolism, degrades heme into CO, free iron, and biliverdin (37). Because CO gas (10–500 ppm) has a potent anti-inflammatory effect (38, 39), we determined whether it serves as a key molecule in IL-10/HMOX1 signaling in SLFs. Quantitative RT-PCR analysis showed that CO, an HMOX1 inducer augmenting endogenous CO generation, markedly inhibits NTHi-induced MCP-1/CCL2 regulation (∼70%), mimicking the inhibitory effect of IL-10 (Fig. 5A). Furthermore, we found that exogenous CO generated by CORM-2, a ruthenium-based CO releaser, inhibits NTHi-induced MCP-1/CCL2 upregulation in a dose-dependent manner (Fig. 5B). Consistently, ELISA analysis showed that RSL cells significantly reduce NTHi-induced MCP-1/CCL2 production (∼50%) upon exposure to CORM-2 (Fig. 5C). Altogether, our data suggest that HMOX1/CO signaling is critically involved in IL-10–mediated modulation of cochlear inflammation.

**IL-10 inhibits NTHi-induced binding of p65 NF-κB to distal NF-κB motifs of the MCP-1/CCL2 gene**

In our previous study, we demonstrated that p65 NF-κB binds to the distal NF-κB–binding motif of the rat MCP-1/CCL2 gene in response to the NTHi lysate (10), but it is unclear whether IL-10 affects NTHi-induced NF-κB activation in SLFs. Luciferase assays using a reporter construct of NF-κB activity showed that RSL cells reduce NTHi-induced NF-κB activation upon exposure to IL-10 (Fig. 6A). Consistently, exogenous CO generated by CORM-2 was found to inhibit NTHi-induced NF-κB activation (Fig. 6B). Analysis of transcription factor binding sites in the 3′-flanking region (−3000 bp) of the MCP-1/CCL2 gene predicted six NF-κB–binding motifs in humans but only three in mice and rats (Fig. 6C). Interestingly, multiple sequence–alignment analysis with Clustal Omega showed that the distal NF-κB–binding motifs (D-κB1 and D-κB2) between −3000 and −2000 bp of the MCP-1/CCL2 gene are highly preserved in humans, mice, and rats (Fig. 6D). This finding led us to further explore whether IL-10 affects NTHi-induced binding of p65 NF-κB to the promoter region of the MCP1/CCL2 gene. ChIP analysis showed that RSL cells inhibit NTHi-induced binding of p65 NF-κB to D-κB1 and D-κB2 of the MCP-1/CCL2 gene (Fig. 6E, 6F). In contrast, IL-10 did not appear to significantly affect NF-κB binding to the proximal NF-κB motif (P-κB). Collectively, it is suggested that IL-10/CO signaling attenuates NTHi-induced MCP-1/CCL2 regulation.

**FIGURE 3.** IL-10R1 is involved in IL-10–mediated inhibition of NTHi-induced MCP-1 upregulation in RSL cells. (A) Luciferase assays show that silencing of IL-10R1 attenuates an inhibitory effect of IL-10 on NTHi-induced MCP-1 upregulation. (B) Quantitative RT-PCR analysis showing siRNA-mediated inhibition of IL-10R1 expression. Results are expressed as a fold induction, taking the value of the nontreated group as 1. Experiments were performed in triplicate and repeated twice independently. Data are mean ± SD (n = 3). *p < 0.05. NC, a nonspecific siRNA.

**FIGURE 4.** HMOX1 is involved in IL-10–mediated inhibition of NTHi-induced MCP-1 upregulation in SLFs. (A) Confocal microscopic images show that NRF-2 is translocated into the nucleus by IL-10 in RSL cells. Original magnification ×630. (B) Quantitative RT-PCR analysis shows that primary rat SLFs upregulate HMOX1 in response to IL-10. Quantitative RT-PCR analysis (C) and ELISA analysis (D) show that silencing of HMOX1 suppresses an inhibitory effect of IL-10 on NTHi-induced MCP-1 upregulation. Results are expressed as a fold induction, taking the value of the nontreated group as 1. Experiments were performed in triplicate and repeated twice independently. Data are mean ± SD (n = 3). *p < 0.05. Con, a negative control without IL-10 treatment; NC, a nonspecific siRNA.
in SLFs through inhibition of p65 NF-κB binding to the distal NF-κB motifs of the MCP-1/CCL2 gene.

**IL-10 deficiency affects cochlear inflammation secondary to middle ear infection**

Because our results showed that IL-10 inhibits NTHi-induced MCP-1/CCL2 regulation in SLFs, we sought to determine whether IL-10 is involved in the modulation of cochlear inflammation in vivo. Live NTHi was injected into the middle ear cavity of IL-10–deficient mice, and histological analysis was conducted. H&E staining of the midmodiolar sections of the temporal bone showed that intratympanic injection of live NTHi leads to accumulation of serous substances and infiltration of inflammatory cells in the scala tympani of the mouse cochlea, particularly in the cochlear basal turn (Fig. 7A). According to the number of infiltrated inflammatory cells, cochlear samples were divided into four groups from 0 to 3+. The Fisher exact test showed that inflammatory cells infiltrate into the cochlea more in IL-10–deficient mice than in WT mice on postinjection day 2 but not on postinjection day 6 (Table I). To further quantify cochlear inflammation,
Cochlear inflammation frequently associated with acquired SNHL, such as cisplatin ototoxicity (5) and acoustic trauma (4). However, like the brain and the retina, the cochlea is protected from immune-mediated tissue damage because the cochlea is an essential organ for survival in mammals. For example, the cochlea is relatively isolated from the immune system by a blood–labyrinthine barrier (18). Cochlear Hensen’s cells were found to release a large amount of annexin A1, a potent inhibitor of leukocyte migration, in response to glucocorticoids (19). Recently, it was reported that CX3CR1-expressing cochlear macrophages play a protective role in aminoglycoside ototoxicity (40). In addition to these cochlear protections, our study introduces the molecular mechanism involved in IL-10–mediated modulation of cochlear inflammation. Interestingly, IL-10 is known to inhibit CD80/CD86 expression in CX3CR1-expressing myeloid cells, resulting in prevention of T cell–dependent colitis (41); however, further studies are needed to determine whether cochlear protection by CX3CR1-expressing cochlear macrophages is mediated by IL-10.

SLFs, the most abundant inner ear cell type, express a variety of ion channels serving as part of the potassium recycle pathway required for normal hearing (42, 43). In our prior studies, we demonstrated that SLFs are involved in cochlear inflammation through TLR2/NF-κB–mediated MCP-1/CCL2 regulation and ERK2/c-Jun–mediated CXCL2 regulation (10, 11). However, our understanding about the negative regulation of chemokines in SLFs is limited. This study provides us with insight into IL-10–mediated modulation of chemokine regulation via HMOX1/CO signaling. Furthermore, we found that IL-10R1 is expressed in the CL, particularly in the area of type II SLFs. Type II SLFs are positive for Na+,K+-ATPase (9) and selectively activate NF-κB to the distal NF-κB activation. The immunological role for each type of SLF remains to be revealed.

Discussion

In our previous studies, we showed that SLFs play a pivotal role in cochlear inflammation through the regulation of chemokines (10–12). In this study, we aimed to determine whether IL-10 modulates cochlear inflammation via downregulation of a model chemokine, MCP-1/CCL2, in SLFs. We found that SLFs downregulate NTHi-induced MCP-1/CCL2 expression in response to IL-10 through IL-10R1/HMOX1–mediated signaling. Exogenous and endogenous CO inhibited NTHi-induced MCP-1/CCL2 upregulation through suppression of NF-κB activation. Interestingly, IL-10 inhibited NTHi-induced binding of p65 NF-κB to the distal NF-κB motifs of the MCP-1/CCL2 gene but not to the proximal motif. Moreover, we found that IL-10 deficiency exaggerates tympanicogenic cochlear inflammation in the murine model, implying a protective role for IL-10 against inflammation-mediated cochlear damage.

Cochlear inflammation induced by intratympanic injection of live NTHi

<table>
<thead>
<tr>
<th>Postinjection</th>
<th>WT</th>
<th>IL-10−/−</th>
<th>Fisher Exact Test Value</th>
<th>Exact Significance (Two-Sided)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 2</td>
<td>13/15b</td>
<td>2</td>
<td>10/10b</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>10</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>2</td>
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<tr>
<td></td>
<td>+++</td>
<td>1</td>
<td>+++</td>
<td>7</td>
</tr>
<tr>
<td>Day 6</td>
<td>1/6b</td>
<td>−</td>
<td>5</td>
<td>4/8b</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>++</td>
<td>0</td>
<td>++</td>
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</tr>
<tr>
<td></td>
<td>+++</td>
<td>1</td>
<td>+++</td>
<td>1</td>
</tr>
</tbody>
</table>

*bCorrected for two Fisher exact tests, p value must be <0.025.

**Ears showing cochlear infiltration of inflammatory cells/total ears.

−, no cochlear infiltration of inflammatory cells; +, cochlear infiltration of 1–30 inflammatory cells; ++, cochlear infiltration of 31–50 inflammatory cells; ++++, cochlear infiltration of ≥51 inflammatory cells.
The authors have no financial conflicts of interest.

IL-10-deficient mice spontaneously develop a chronic inflammatory bowel disease due to uncontrolled cytokine production (47, 48). They are abnormally sensitive to bacterial LPS (49) and inflammatory bowel disease due to uncontrolled cytokine production of cells and a narrow window of IL-10 induction. We plan to investigate the treatment of vascular dysfunction and immune-mediated diseases (58), CO has been emerging as a promising drug target for inflammation-associated cochlear diseases.

cause CORM-2 is not very soluble in water, we will assess further implications of IL-10–MEDIATED MODULATION OF COCHLEAR INFLAMMATION. Therefore, targeting of specific molecules downstream of IL-10 signaling, such as HMOX1 and CO, may represent better “druggable” approaches. Despite a potential risk for prenatal CO exposure (58), CO has been emerging as a promising drug target for the treatment of vascular dysfunction and immune-mediated diseases (38, 39). Ruthenium-based CORMs are able to directly liberate a small quantity of CO in biological systems (59, 60). Our result demonstrated that CORM-2 inhibits MCP-1/CCL2 upregulation by suppression of NTHi-induced NF-κb activation. Because CORM-2 is not very soluble in water, we will assess further the therapeutic potential of a water-soluble CORM-2 derivative, CORM-3 (61), for the management of cochlear inflammation.

In conclusion, we demonstrated that IL-10/HMOX1 signaling is protective for cochlear inflammation through inhibition of MCP-1/CCL2 regulation. This study is expected to provide us with a scientific basis for a novel nonsteroidal approach to manage inflammation-associated cochlear diseases.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental data

Schematic illustration showing a hypothetical negative regulatory mechanism inhibiting cochlear inflammation secondary to NTHi-induced otitis media.