# Cochlin involves immune response of hair cell degeneration by acoustic trauma

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Directed by Professor Jinsei Jung

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지난 박사 학위과정 동안 여러모로 부족한 저에게 큰 관심과 격려를 아끼지 않으셨던 많은 분들께 깊은 감사의 말씀을 드립니다. 먼저 학위과정 동안 저를 정성껏 이끌어 주시고 열정이 있는 의과학도로 성장할 수 있도록 지도해주신 정진세 교수님께 진심으로 고개 숙여 감사를 드립니다. 바쁘신 와중에도 학위논문을 꼼꼼히 검토해 주시고 격려와 축하를 보내주신 복진웅 교수님, 현영민 교수님, 지헌영 교수님, 그리고 이전미 교수님께 감사 드립니다. 그리고 박사학위 연구에 전념할 수 있도록 강사 후 기초연수과정의 기회를 주신 최재영 교수님과 김세헌 교수님께 감사 드립니다. 항상 가르침을 주시는 김성헌, 문인석 교수님을 비롯한 이비인후과학 교실의 모든 교수님들께 감사 드리고 싶습니다. 또, 아침 일찍 출근하면 항상 먼저 와계셔서 조언해주셨던 윤주헌 교수님께 특별히 감사 드립니다.

지난 2 년 동안 많은 시간 실험실에서 함께했던 최재영교수님 랩 학생 및 연구원선생님에게 감사드립니다. 임해월, 노병화, 김규민, 홍진주, 강민진, 송가배 선생님 앞으로도 잘 부탁드립니다. 특히 이번 연구에 많은 도움을 주신 유지은 선생님께 감사 드립니다. 떠나가신 최혜지 홍한솔 선생님도 건승하기 바랍니다. 또한 지금은 성빈센트병원에 가있는 실험실메이트 곽상현과 강남세브란스에서 열심히 환자를 보고있는 남기성에게 감사합니다.

현영민교수님 실험실의 여러 선생님들, 특히 이미징에 많은 도움을 주신 최영호선생님께 감사 하고, 트랜스웰 어세이에 도움을 주신 강경이, 정소이 선생님께도 감사드립니다. 저의 기초연수과정 지도교수님이기도 하시며, 면역학이라는 제 기억 저편의 학문에 새로운 빛을 비

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# TABLE OF CONTENTS

# LIST OF FIGURES

**ABSTRACT**

**Cochlin involves immune response of hair cell degeneration by acoustic trauma**

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(Directed by Professor Jinsei Jung)

**Introduction:** Recently, the immunologic function of the LCCL peptide cleaved from the cochlin protein was revealed in infectious condition. However, cochlea have distinctly specific immune response in the inflammation induced by acoustic trauma. The present study investigates the cochlin function in sterile inflammation induced by acoustic trauma.

**Methods:** The wild type and *COCH* knock-out C57BL/6 mice were used. The change of LCCL concentration in the perilymph were evaluated by Western Blot. The hearing function, outer hair cell loss, expression of inflammatory molecules and macrophage distribution pattern were measured after acoustic trauma.

**Results:** The LCCL concentration was increased in the perilymph after acoustic trauma. The COCH knock-out mice showed preserved lower

frequency hearing function and outer hair cells after acoustic trauma. The expression of inflammatory molecules were not different between the two genotypes. The recruited macrophages were significantly less in the apical 10% of *COCH* knock-out mouse cochlea, and monocytes showed chemo-attraction by LCCL peptide in transwell assay.

**Conclusion:** The LCCL is cleaved in the sterile inflammation induced by acoustic trauma as well as bacterial infection, but the quantity seems to be limited. The *COCH* knock-out mice showed preserved lower frequency hearing after acoustic trauma. The recruited macrophages were less in the cochlear apex in the *COCH* knock-out mice. Based on the result of the transwell assay, the absence of LCCL seems to be responsible for it.

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Key words: cochlin, LCCL, noise induced hearing loss, monocyte

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## I. INTRODUCTION

The cochlin is a most abundant protein in the inner ear, encoded by COCH gene.[1] Traditionally, the cochlin has been thought to be a structural protein associated with the collagen fibers.[2] In recent studies, the LCCL peptide that is cleaved from the cochlin protein has been revealed an important role in innate immunity associated with a bacterial infection.[3,4] The LCCL showed a protective effect on hearing function and seems to improve the efficacy of innate immunity with the regulation of cytokines, aggregation of the pathogen, and guiding phagocytosis. Totally, the LCCL roles a significant function in inflammation response induced by a pathogen.

The acoustic trauma, on the other hand, induces sterile inflammation by damage-associated molecular pathway (DAMP).[5] The inflammation induced by acoustic trauma differs from infectious inflammation, although the exact mechanism and characteristics are not fully elucidated. It is known to be regulated mainly by resident macrophages and recruited monocytes, which are recruited 1 ~ 7 days after acoustic trauma.[6] In addition, no neutrophils were found during the inflammation induced by acoustic trauma.[7,8] Interestingly, restriction of inflammation is reported having a protective effect on the hearing function after acoustic trauma. It includes administration of corticosteroid,[9] depletion of monocytes/macrophages,[10] and blocking inflammatory cytokines.[11]

The LCCL has ambivalent functions in inflammation. It increases cytokine level and leukocytes migration, however, it also regulates the distribution of neutrophils and prevents collateral damage.[3] The present study hypothesized that the cochlin involves immune response induced by acoustic trauma and also regulates the inflammation. Thus, *in vivo* experiments using acoustic trauma with loud noise were performed, hearing function and inflammatory characteristics were analyzed in both of the wild type mice and COCH knock-out (KO) mice.

## II. MATERIALS AND METHODS

### 1. Animal

C57BL/6 mice aged 4 ~ 8 weeks were used in the animal experiments. COCH knock-out mice were purchased from the Jackson Laboratory (ME, USA) via Orient Bio (Sungnam, Republic of Korea). Temperature, humidity and light/dark cycle were properly controlled, food and water could be access anytime when the animal need. Less than 5 animals were maintained in a single cage. All animals were carefully maintained not to be exposed to noise in specific pathogen-free environment in the animal facility at Avison Biomedical Research Center in

Yonsei University. The animal experiments were approved by the Institutional Animal Care and Use Committees at the Yonsei University College of Medicine (Protocol number 2019-0182).

### 2. Acoustic trauma

The white noise (300-10,000 Hz) was generated by a personal computer and an amplifier (R-399, Inter M, Seoul, Korea) and delivered through speakers (290-8L, Altec Lansing, Oklahoma City, OK, USA) in a noise booth. Mice were continuously exposed to 115 dB peak equivalent SPL for one hour in awaken status. Before every experiment, noise level was confirmed with a decibel meter.

### 3. Western Blot

For perilymph western blotting, micro needle aspiration from round window was performed in the isolated temporal bone to prevent CSF contamination. Right after the decapitation of mouse head, temporal bone was isolated as soon as possible. Using micro needle, we can obtain 0.2 μl of perilymph from round window. For tissue western blotting, cochleae were ground, and lysed in a sodium dodecyl sulfate (SDS) lysis buffer. Lysed samples or perilymph were mixed with sample buffer and separated by SDS-polyacrylamide gel electrophoresis. For digestion of glycosylated pendrin by N-Glycosidase F (PNGase F; New England Biolabs), protein samples were incubated with PNGase F in solutions containing triton X-100 (1%) for 2 h. The separated proteins were transferred to a nitrocellulose membrane and blotted with appropriate primary and secondary antibodies. Protein bands were detected by enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA). The density of bands were quantified by ImageJ.

### 4. Auditory Brainstem Response

Tests were conducted using an ABR workstation manufactured by Tucker

Davis Technology (TDT) (Alachua, FL, USA). Properly anesthetized mice were placed on a heating pad (37°C) in a soundproofed chamber. After electrode insertion, acoustic stimuli were applied through a receiver probe. The stimuli included 500-repeated click sounds or tone bursts with a 1 ms rise/fall time and a 5-ms plateau at frequencies of 4, 6, 8, 12, 18, 24 and 30 kHz. The sound intensity started from a 90 dB SPL with 5 dB decreasing steps to the auditory threshold. The ABR threshold was collected in both ear at baseline and 2 weeks after noise exposure. The ear that showed threshold more than 40 dB SPL at baseline was excluded from analysis.

### 5. Outer hair cell count

After sacrificing the mouse in a CO2 chamber, the bilateral temporal bone was dissected and fixed in 4% paraformaldehyde for 24 h at 4°C after local perfusion with a fixative through the oval window and the round window. After fixation, the samples were incubated in 1:3 EDTA solution for 24 h at 4°C for decalcification. For investigation of outer hair cells in the cochlea, we applied silicone bond to position the cochlea sample and directly visualized it using 20x objective lens of two-photon microscopy (LSM7MP, Carl-Zeiss, Germany). Mai-Tai HP Ti:Sa Deep See laser system (Spectra-Physics) was used to generate excitation laser (wavelengths of 810 nm). The images were acquired at a resolution of 512 × 512 pixels using band-pass filters with 420–480 nm (blue), 500–550 nm (green), and 575–610 nm (red).

### 6. Quantitative real time PCR

To quantify mRNA of inflammatory molecules and ADAMTS 4 in the cochlea, gene-specific primer pairs with GAPDH control primer pairs were used in conjunction with a TaqMan probe (Applied Biosystems, Foster City, CA, USA). After collecting temporal bone from the sacrificed mouse, cochlea was separated from the temporal bone and submerged in the TRIzol (Thermo Fisher). Purified RNA samples from the cochlea were reversetranscribed using an iScript Select

cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Amplification was performed using an ABI 7500 real-time PCR system (Applied Biosystems).

### 7. Immunohistochemistry

Isolated cochleas were obtained by microdissection. Tissues were fixed by submersion in 4% formaldehyde at 4°C overnight. After washing twice with PBS, the fixed temporal bones were decalcified for 24 h in 10% ethylenediaminetetraacetic acid (EDTA)/PBS. The whole-mounted tissues were blocked with 10% donkey serum and incubated with target-specific primary and secondary antibodies at 4°C overnight. The samples were then mounted with mounting solution (Sigma-Aldrich) and viewed under an LSM780 confocal microscope (Zeiss, Jena, Germany).

### 8. Statistical analysis

The results of multiple experiments are presented as means ± SEM. Statistical analysis was performed using Student's *t*-tests or analysis of variance followed by Newman-Keuls multiple comparison test as appropriate. $P < 0.05$ was considered statistically significant.

## III. RESULTS

### 1. The cleaved LCCL increases in perilymph space after acoustic trauma.

After acoustic trauma, perilymph was serially collected from scala tympani of 4 mice temporal bone in each time point. In western blot analysis, LCCL peptide (lower bands, < 18 kDa) increases and peaks at 1 day after acoustic trauma (Figure 1A and 1B). The mRNA expression of Adamts4, which encodes aggrecanase 1 that cleaves LCCL from full length cochlin also increased after acoustic trauma in qPCR (Figure 1C).[3,4] However, the lysate of whole cochlea does not show significant changes in full length cochlin (upper bands, ~ 60 kDa) at any time points after

acoustic trauma (Figure 1A). Taken together, a limited amount of LCCL is cleaved and secreted to perilymph in the inflammation induced by acoustic trauma.

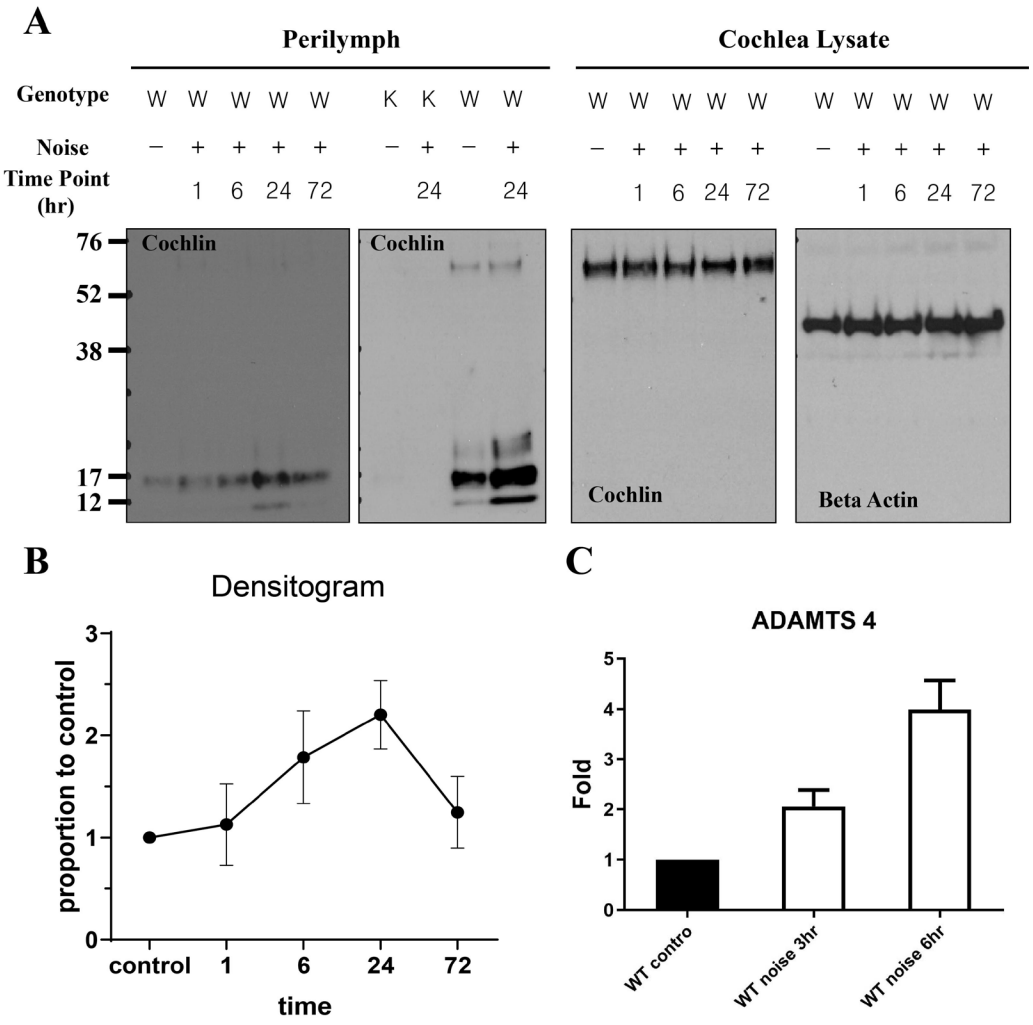


**Figure 1. The LCCL is cleaved and secreted to the perilymph space of cochlea after acoustic trauma**. (A) Western blot analysis of cochlin N-term from perilymph and cochlear lysate. In the cochlin analysis, lower bands demonstrate cleaved LCCL and upper bands demonstrate full length cochlin. The perilymph of COCH knock-out mice show absence of cochlin and LCCL. (B) The densitogram of cochlin in perilymph according to the time point of sample collection. (C) The mRNA

expression of ADAMTS4 from cochlear lysate according to the time point of sample collection using qPCR analysis. *W*, wild type. *K*, COCH knock-out.

### 2. *COCH* KO mice showed preserved low tone hearing after acoustic trauma.

To investigate the function of COCH gene in the acoustic trauma, noise exposure for inducing permanent threshold shift (PTS) was conducted to wild type mouse and COCH KO mouse. The ABR threshold was measured in the frequencies of 4, 6, 8, 12, 18, 24, and 30 kHz at pre-noise and 2 weeks after noise exposure (Figure 2A and 2B). In 2 weeks after acoustic trauma, ABR threshold of higher than 12 kHz almost scaled out in both of the two genotypes. However, ABR threshold of 4k ($p = 0.002$) and 6k ($p < 0.001$) showed statistically significant differences between COCK KO (22 ears) and wild type mice (19 ears).

The outer hair cell (OHC) loss was counted 2 weeks after acoustic trauma. To minimize bias due to tissue preparation, we investigated intact cochlea after decalcification using two-photon microscopy.[12] Because the difference of ABR threshold is limited in low frequencies, OHCs in apical 50 % of the cochlea (represent lower than 20k frequency) were investigated. The OHC loss was significantly more at 30 ~ 50 % distance from cochlea apex in the wild type than in the COCH KO (Figure 2C ~ E).

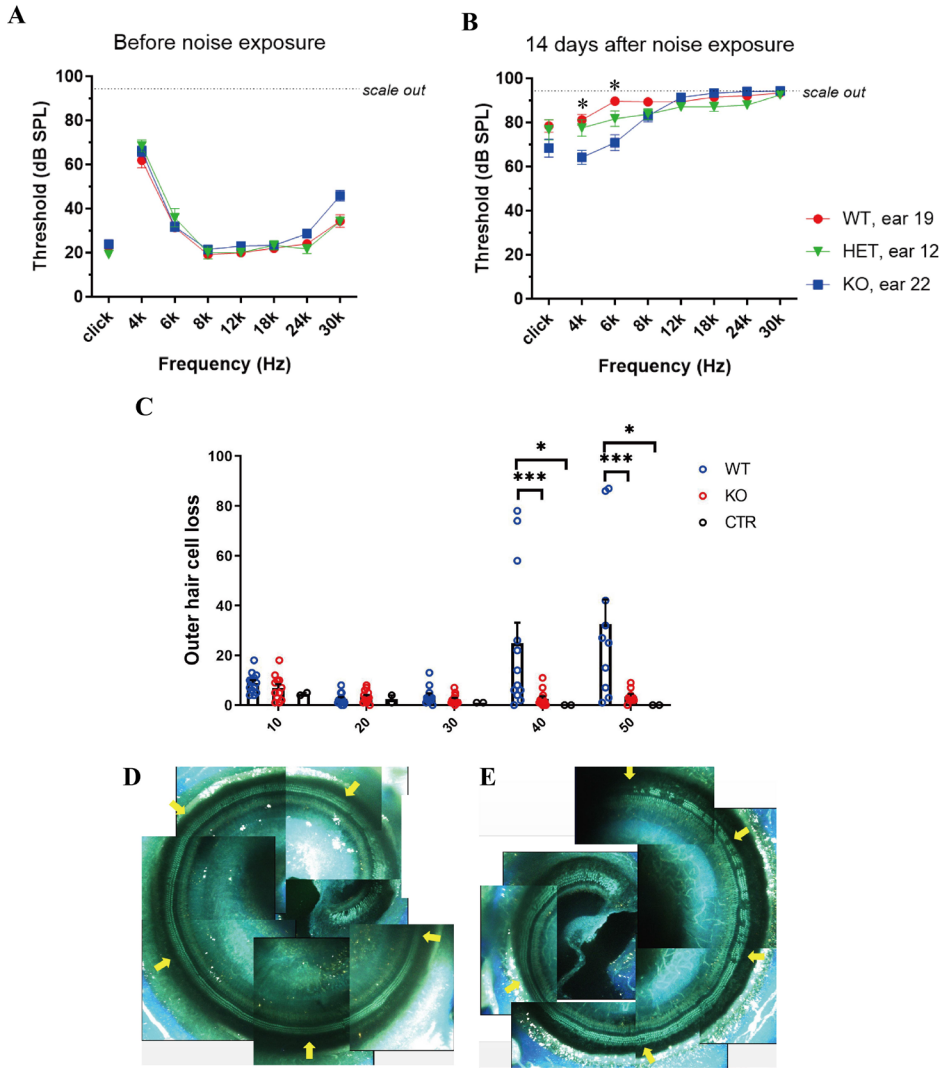


**Figure 2. Lower frequency hearing was preserved after acoustic trauma in COCH knock-out mice.** (A) The ABR thresholds of wild type (WT), COCH+/- (HET), and COCH-/- (KO) showed no difference in all frequencies. (B) Two weeks after acoustic trauma, COCH knock-out mice showed significantly lower ABR threshold in 4, 6 kHz compare to wild type mice. Asterisks denote statistical significance (p-value < 0.05) between wild type and COCH knock-out mice. (C) The outer hair cell loss was significantly greater in the 30~50 % of distance from

apex of cochlea in wilt type than COCH knock-out mice. The x-axis denotes distance from cochlear apex. (D) Representative image of COCH knock-out mouse apical turn of cochlea. Outer hair cells are well visualized by auto-fluorescence in two-photon microscopy. (E) Representative image of wild type mouse apical turn of cochlea. Outer hair cells are well visualized by auto-fluorescence in two-photon microscopy. The degenerated outer hair cells lack of auto-fluorescence. The yellow arrow denotes each 10% of distance from apex of cochlea in (D) and (E).

### 3. The mRNA expression of inflammatory molecules from cochlear resident macrophage in COCH KO mice was not different from that in WT mice.

Based on the previous studies that investigated inflammatory molecule expression after acoustic trauma, the expression of interleukin 1 beta (IL-1β), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α), and monocyte chemoattractant protein 2 (CCL-2) were quantified at 3 hours, 1day, and 1 week post-noise (Figure 3). Because monocytes/macrophage infiltration to the cochlea is known to begin at least 1 day after noise exposure, the inflammatory response at 3 hours post-noise should be affected mainly by the cochlear resident macrophages. All examined molecules showed increased expression after noise in both genotypes, however there were no significant differences between wild type and COCH KO mice. This result implies that COCH gene less likely affects the inflammatory activation of cochlear resident macrophages. In addition, there were no statistically significant differences in mRNA expression at any time points, although KO mice tend to express lesser mRNA of inflammatory molecules than wild type.

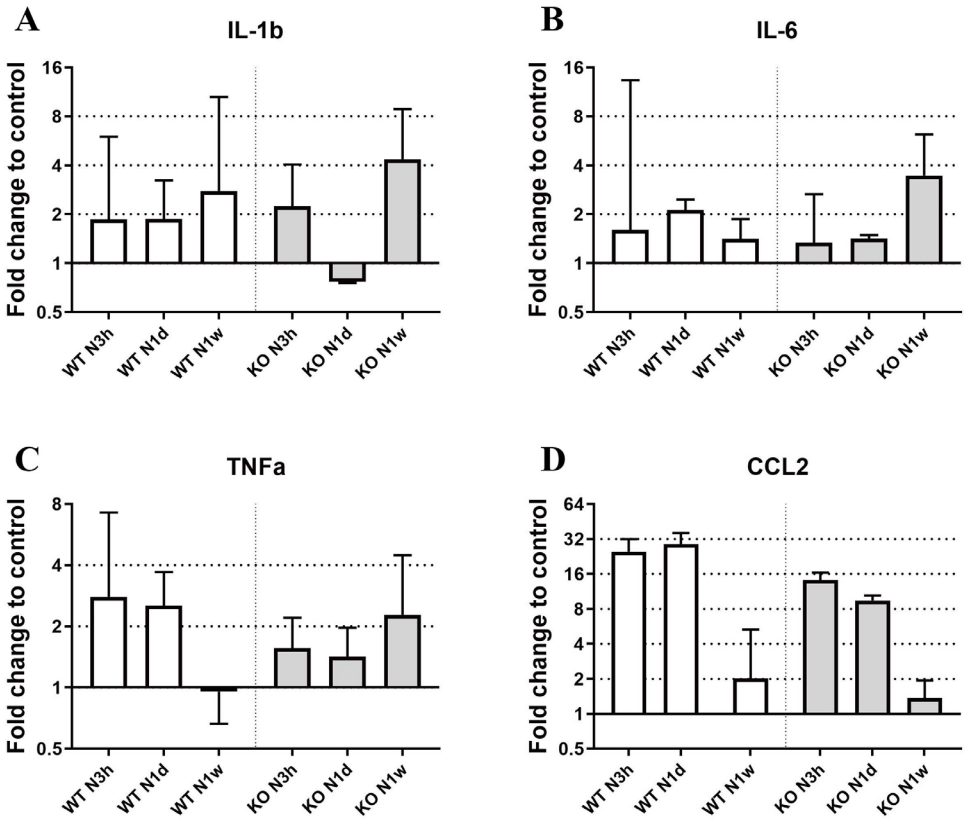


**Figure 3. mRNA expression of inflammatory molecules are not different between wild type and COCH knock-out mice.** Interleukin 1 beta (IL-1b), interleukin 6 (IL-6), tumor necrosis factor alpha (TNFa), and Monocyte chemoattractant protein 2 (CCL2) showed no significant differences between wild type and COCH knock-out mice at baseline and after the acoustic trauma. The y-axis denotes relative fold change of mRNA expression to control (log2). Box denotes median value and error bar denotes interquartile range. (n = 4, each time point)

**4. Recruited monocytes were significantly less in the cochlear apex of**

**COCH KO mice.**

Based on the previous studies that reported recruited monocytes play significant role in the inflammation induced by acoustic trauma, we performed in vivo experiments to investigate the distribution pattern of the monocytes/macrophages. The ovoid shaped macrophages without any dendrite were defined as recruited macrophages and counted in the spiral ganglion ~ organ of Corti in the scala tympani side (Figure 4A ~ D). Interestingly, the macrophage count of apical 10 % (represent lower than 7k frequency) was significantly less in COCH KO mouse compare to wild type mouse after acoustic trauma (Figure 4E). However, macrophages count of apical 20 % (represent lower than 9k frequency) showed no significant difference (Figure 4F).

The LCCL is known to have a chemo-attractive effect on neutrophils and increase the recruitment of leukocytes in the previous study, however, there is no evidence so far that monocyte/macrophage also have a chemo-attractive property to the LCCL. Thus, we investigated the chemo-attractive effect of the LCCL on monocytes which have a critical role in the inflammation induced by acoustic trauma. Bone marrow derived monocytes/macrophage cells showed increased migration with the LCCL in the trans-well assay (Figure 4G ~ I). Taken together, the COCH gene involves in immune cell distribution in the inflammation induce by acoustic trauma.

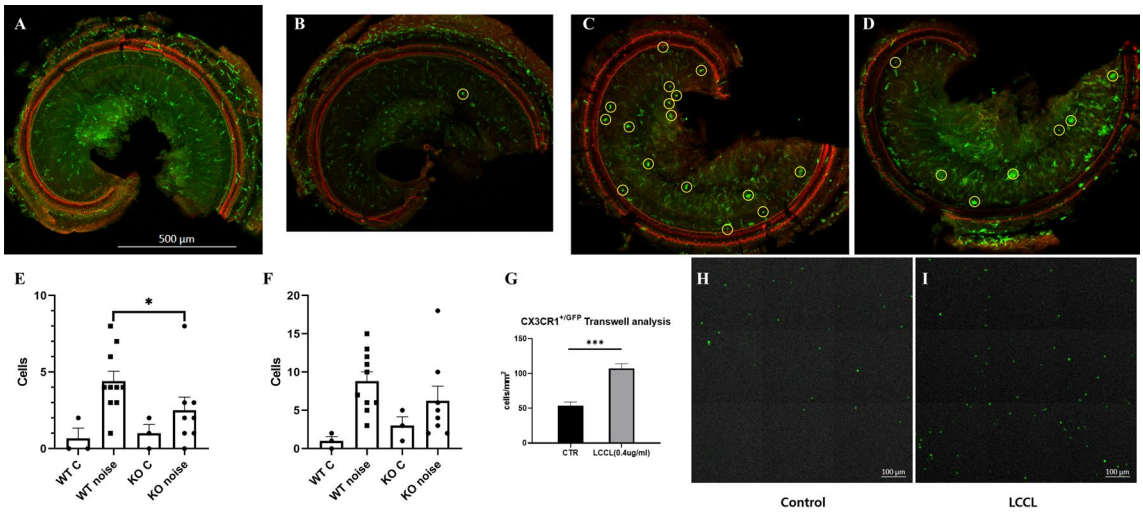


**Figure 4. The LCCL chemo-attracts bone marrow monocytes/ macrophages and COCH KO mice showed less recruited macrophages in the apex of cochlea.** (A) Untreated cochlear apex of wild type mouse, (B) untreated cochlear apex of COCH KO mouse, (C) cochlear apex of wild type mouse at 3 days after acoustic trauma, (D) cochlear apex of COCH KO mouse at 3 days after acoustic trauma. Green, F4/80. Red, Phalloidin. Yellow circle, recruited macrophage. (E) Recruited macrophages on the scala tympani side of the basilar membrane in 0 ~ 10 % distance from cochlear apex, (F) 0 ~ 20 % distance from cochlear apex. Asterisk denotes statistical significance ($p < 0.05$). (G) GFP-positive cell count in the transwell assay. Triple asterisks denotes statistical significance ($p < 0.001$). (H) Lower chamber image after transwell assay of control (PBD treated) well, (I) LCCL well.

## IV. DISCUSSION

The present study demonstrates the increase of LCCL peptide in the

perilymph space after acoustic trauma. This observation implies that LCCL peptide/COCH gene has some role in the inflammatory response induced by noise exposure as well as by bacterial infection. Indeed, knock-out of COCH gene resulted in preservation of low frequency in PTS situation. Furthermore, the distribution pattern of the monocytes/macrophages after acoustic trauma was different between the two genotypes. As the LCCL peptide showed a chemo-attractive effect on monocytes, the changes in the monocytes/ macrophages distribution pattern may be affected by the presence of LCCL peptide. However, mRNA expression failed to show the difference between the two genotypes. Considering the tendency of inflammatory molecule mRNA expression and partially protected hearing function, the function of LCCL in noise-induced inflammation seems not considerable compare to the bacterial infection.

Recently, the importance of LCCL in inflammatory response is revealed not only in the cochlea, but also in other organ.[3,4,13] However, the function and mechanism of this peptide are not fully elucidated in many aspects. Based on the previous studies and the result of the present study, the LCCL seems to enhance the inflammatory response by interacting with leukocytes. Interestingly, the knock-out of COCH gene showed aggravation of organ damage with decreased local inflammation in previous studies. However, the result of the present study seems opposite because wild type mice showed more severe hearing loss in lower frequencies after acoustic trauma compares to COCH knock-out mice. This discrepancy might be resulted from the different immune responses between PAMP and DAMP. In the cochlea, several evidences showed that inhibition of inflammatory response can rescue from hearing loss after acoustic trauma representing the DAMP pathway. Y. Mizushima et. al. reported preserved low frequency hearing function after acoustic trauma in monocyte/macrophage depleted mouse.[10] K. Wakabayashi et. al. reported blockade of IL-6 improved low frequency hearing after acoustic trauma.[11] Knock-out of Toll-like receptor 4 (Tlr4), which is a key protein of DAMP pathway, also showed improved hearing function after

acoustic trauma in the study by R. Vethanayagam et. al.[14] In the ototoxic inflammation model, E. Sato et al reported increased macrophage is positively correlated to hair cell loss.[15] In line with these previous studies, the knock-out of COCH gene also showed improved low-frequency hearing function after acoustic trauma in the present study. Given that the LCCL enhancing inflammatory response in the bacterial infection, the absence of LCCL might reduce the inflammatory burden after acoustic trauma resulting in protective effect on the low-frequency hearing function. The hearing protection by reducing inflammatory burden after noise exposure is previously studied using glucocorticoid treatment in the mouse.[9]

Nevertheless, we could not found any significant differences in the expression of inflammatory molecules between two genotypes. As the monocytes infiltration into the cochlea is known to begin from 1 day after acoustic trauma, our result at 3 hours after noise exposure represents the inflammatory response mediated by resident cochlear macrophages.[5,6] Moreover, given that protective effect to acoustic trauma was limited to low frequencies, the significance of LCCL in noise-induced inflammation may not be so critical. Although, LCCL seems to have some role in the noise-induced inflammation because the inflammatory molecule mRNA expression tends to be less in COCH knock-out mice especially at 1 day after noise and the chemo-attract to monocytes of LCCL itself. Consistently, the monocyte recruitment to perilymph surface of spiral lamina showed decreased in COCH knock-out mice. Taken together, the protective effect to the acoustic trauma in COCH knock-out mice seems to be mainly resulted from the alteration of newly recruited monocytes/ macrophages.

The present study has several limitations. First, there may be differences in mechanical properties of cochlea in COCH KO mice as Robertson et al pointed.[2] However there are also no evidences that hearing functions or cochlea histology is different between wild type and COCH KO mice.[16,17] Second, the exact mechanism why macrophages are less dispersed to apical turn in the COCH KO mice after acoustic trauma is not clearly elucidated in this study. Although we found that

LCCL peptide have chemo-attractive effect to macrophages, the receptor of LCCL expressed in macrophages or its intracellular pathway is unrevealed to the best of our knowledge. Moreover, the trajectory of recruited macrophages in cochlea is not clear so far and exact function has controversy.[6,18] These remain to be determined in future work.

## V. CONCLUSION

The LCCL is cleaved in the sterile inflammation induced by acoustic trauma as well as bacterial infection, but the quantity seems to be limited. The COCH knock-out mice showed preserved lower frequency hearing after acoustic trauma. The mRNA expression of inflammatory molecules was not significantly different between the two genotypes at 3 hours and 1 day after acoustic trauma, although knock-out mice tend to express lesser mRNA. Based on the transwell assay that showed the LCCL has chemo-attract effect on monocytes, the LCCL is responsible for the reduced recruitment of macrophage in the cochlear apex.

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**ABSTRACT (in Korean)**

## 소음 노출로 인한 유모세포 손상에서 일어나는 면역반응에서의 코클린 단백질의 영향 규명.

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배성훈

Cochlin 단백질은 내이에서 많이 발현되는 구조단백질로 알려져 있었으나, 최근의 연구에서 Cochlin 단백질에서 잘려 분비되는 LCCL 단백질의 선천면역 기능이 보고되며 주목을 받고 있다. 한편, 소음노출에 의한 달팽이관의 염증은 세균 감염에 의한 면역반응과 여러 가지 다른 점이 보고되고 있다. 따라서, 본 연구에서는 Cochlin 단백질이 소음노출에 의한 달팽이관 염증에서 어떤 역할을 하는지 보고하고자 한다. 이를 위해, Cochlin 유전자를 Knock out 시킨 마우스와 wild type 마우스를 비교 분석하였고, 115dB 의 백색소음에 1시간 동안 노출시켜 소음에 의한 달팽이관 염증을 유도하였다. 그 결과, 소음 노출 후 Cochlin 단백질에서 절단된 LCCL 단백질의 농도가 달팽이관의 외림프액에서 점점 증가하였고, 소음 노출 1일 후에 가장 높은 농도를 보였다. 또한 동일한 소음 노출 후 Cochlin knock out 마우스의 청력이 wild

type 마우스와 비교하였을 때, 저음역대에서 보존되는 것을 확인하였다. 이는 외유모세포의 수를 비교하였을 때에도 동일한 양상을 보였으나, 소음 노출 3시간, 1일, 1주일 후의 염증관련 물질들의 RNA 발현에는 통계적으로 유의한 차이를 보이지 않았다. 소음 노출 후 외림프액 공간으로 유입된 단핵구 수에서는 cochlin knock out 마우스가 달팽이관 첨부에서 유의하게 적었으며 이는 마우스의 골수 단핵구에 대한 LCCL 단백의 화학주성실험에서도 재현되었다. 결론적으로, cochlin 단백질은 소음노출에 의해 유도된 달팽이관 염증에서도 LCCL로 절단되어 분비되었다. Cochlin 단백이 없을 때, 저음역대에서 소음노출에 대한 보호효과가 있었고 이는 아마도 LCCL 단백질의 단핵구에 대한 화학주성과 관련이 있을 것으로 보인다.

핵심되는 말: 코클린, LCCL, 단핵구, 소음성 난청

# PUBLICATION LIST

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