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Differential fates of tissue macrophages in the cochlea during postnatal development

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Abstract

The cochlea contains macrophages. These cells participate in inflammatory responses to cochlear pathogenesis. However, it is not clear how and when these cells populate the cochlea during postnatal development. The current study aims to determine the postnatal development of cochlear macrophages with the focus on macrophage development in the organ of Corti and the basilar membrane. Cochleae were collected from C57BL/6J mice at ages of postnatal day (P) 1 to P21, as well as from mature mice (1–4 months). Macrophages were identified based on their expression of F4/80 and Iba1, as well as their unique morphologies. Two sets of macrophages were identified in the regions of the organ of Corti and the basilar membrane. One set resides on the scala tympani side of the basilar membrane. These cells have a round shape at P1 and start to undergo site-specific differentiation at P4. Apical macrophages adopt a dendritic shape. Middle and basal macrophages take on an irregular shape with short projections. Basal macrophages further differentiate into an amoeboid shape. The other set of macrophages resides above the basilar membrane, either beneath the cells of the organ of Corti or along the spiral vessel of the basilar membrane. As the sensory epithelium matures, these cells undergo developmental death with the phenotypes of apoptosis. Macrophages are also identified in the spiral ligament, spiral limbus and neural regions. Their numbers decrease during postnatal development. Together, these results suggest a dynamic rearrangement of the macrophage population during postnatal cochlear development.

Keywords

Development; Immune cells; Basilar membrane; Cochlea; Macrophage; Immunity

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1. Introduction

The immune capacity of the cochlea is supported by both professional immune cells and resident cells that have immune properties. The professional immune cells consist primarily of macrophages (Hirose et al., 2005; Okano et al., 2008; Yang et al., 2015). Resident cells that have immune properties include fibrocytes (Adams, 2002; Ichimiya et al., 2000; Moon et al., 2006) and cells in the organ of Corti, particularly supporting cells (Cai et al., 2014). Macrophages reside in multiple anatomic regions of the cochlea, including the spiral ligament, spiral limbus, spiral ganglion region, osseous spiral lamina, basilar membrane, as well as around the blood vessels of the stria vascularis (Hirose et al., 2005; Lang et al., 2006; Okano et al., 2008; Sato et al., 2008; Shi, 2010). While the precise functions of these cells are not clear, the widespread distribution of cochlear macrophages suggests their involvement in the maintenance of tissue homeostasis and formation of disease conditions in the cochlea.

The sensory epithelium contains sensory cells that are responsible for converting mechanical stimuli to neural signals. These cells are vulnerable to pathological insults. In this region, the basilar membrane contains macrophages that survey the homeostasis of the sensory epithelium. Under steady-state conditions, these macrophages reside on the scala tympani side of the basilar membrane and display site-specific morphologies (Frye et al., 2017; Yang et al., 2015). In the apical portion of the basilar membrane, macrophages have a dendritic shape. By contrast, macrophages have a branched or amoeboid shape in the middle and basal portions of the basilar membrane. Macrophages in different regions of the basilar membrane also exhibit different expression patterns of immune molecules (Yang et al., 2015) and have different response patterns to cochlear stress (Frye et al., 2017).

The origin of cochlear macrophages is not completely clear. Under acute stress conditions, circulating monocytes infiltrate the cochlea (Fredelius et al., 1990; Hirose et al., 2005; Tornabene et al., 2006; Wakabayashi et al., 2010; Yang et al., 2015) and differentiate locally into macrophages (Yang et al., 2015). Studies using irradiated mice have shown that cochlear macrophages of adult mice are maintained by bone marrow-derived immune cells (Okano et al., 2008; Shi, 2010). These observations suggest that circulating monocytes contribute to the macrophage population in the cochlea. At present, it is not known when and how macrophages populate the basilar membrane during postnatal development. The identification of the origin of tissue macrophages is important for understanding their functional roles in cochlear homeostasis and disease formation.

Under steady-state conditions, the organ of Corti of the mature cochleae is known to lack macrophages (Du et al., 2011; Hirose et al., 2005; Okano et al., 2008; Yang et al., 2015). However, it is not clear whether developmental macrophages are present during postnatal cochlear maturation. Given the role of macrophages in tissue maturation, we hypothesize that developmental macrophages are present in the organ of Corti during postnatal development of the sensory epithelium.

The current study was designed to determine: (1) when macrophages populate the basilar membrane and acquire their site-specific morphology and (2) whether macrophage

populations are present in the organ of Corti during postnatal development. We demonstrate that distinct macrophage populations are present in the basilar membrane and the organ of Corti during the postnatal developmental period. One population resides on the scala tympani side of the basilar membrane. These cells have a monocyte-like morphology and are present at birth. They differentiate into mature macrophages in a site-dependent fashion. The other population is found on the organ of Corti side of the basilar membrane. These cells are short-lived and undergo developmental death, which coincides with the maturation of the sensory epithelium. In addition, macrophages are present in the spiral ligament, spiral limbus and neural regions. Their numbers decrease with the maturation of the cochlea. Together, these results implicate developmental macrophages in the postnatal remodeling of the sensory epithelium.

2. Materials and Methods

2.1. Subjects

C57BL/6J mice (both male and female, the Jackson Laboratory, Bar Harbor, ME, USA and Japan SLC Incorporated, Shizuoka, Japan) were utilized in this investigation. Breeding mice were housed at the University at Buffalo's Lab Animal Facility and were protected from excessive noise exposure for the duration of the investigation. Procedures involving the use and care of the animal subjects were approved by the Institutional Animal Care and Use Committee of the State University of New York at Buffalo and by the Animal Care and Use Committee of Kagawa University.

2.2. Cochlear tissue collection

Cochlear tissues were harvested at multiple periods during postnatal development, including postnatal day 1 (P1)-4, P7-10, P17-21, as well as from mature mice at the age of 1–4 months. At least 12 mice were utilized for each period. Animals were euthanized by CO₂ asphyxiation and subsequently decapitated. The cochleae were quickly removed from the skull and fixed with 10% buffered formalin for 1 day. The cochleae were decalcified with 10% Ethylenediaminetetraacetic acid at 4 °C for 1 day and then were dissected in 10 mM phosphate-buffered saline (PBS) to collect the whole-mounts for subsequent analyses.

2.3. Immunolabeling of immune cells

Immunolabeling of CD45 protein, a pan-leukocyte marker, was used to visualize immune cells. Macrophages were identified based on their expression of F4/80 and Iba1, macrophage-specific marker proteins that have been used in previous studies for identifying macrophages in the cochlea (Fujioka et al., 2006; Hirose et al., 2005; Okano et al., 2008; Tornabene et al., 2006). Macrophage identity was further determined by morphological analysis. These cells are large and have an irregular or branched shape. Macrophage precursors were identified based on their expression of Ly6C, a monocyte marker protein. These cells express F4/80, but the level is lower than that in macrophages (Austyn et al., 1981).

After dissection, whole-mount preparations were treated with 0.5% Triton X-100 to permeabilize the cells for 30 min at room temperature, and then with 10% donkey or goat

serum albumin in PBS (pH 7.4) for 1 h at room temperature. The tissues were subsequently incubated overnight at 4 °C with a primary antibody or two primary antibodies (goat CD45 polyclonal antibody, 1:100, AF114, RD Inc., Minneapolis, MN, USA; rat anti-F4/80 monoclonal antibody, 1:150, ab6640, Abcam Inc., Cambridge, MA, USA; rabbit anti-Iba1 monoclonal antibody, 1:200, ab178846, Abcam Inc., Cambridge, MA, USA; Anti-Ly6C monoclonal antibody, 1:100, sc-271811, Santa Cruz Biotechnology Inc., TX, USA). After incubation with the primary antibody, the tissues were rinsed 3 times with PBS and incubated in the dark with a secondary antibody or two secondary antibodies (Alexa Fluor® 488 or 568 donkey anti-goat, rat or rabbit IgG, 1:100 in PBS, Invitrogen, Carlsbad, CA, USA) for 2 h at room temperature.

The specificity of CD45, Iba1 and F4/80 primary antibodies used in this study was confirmed in our previous studies. Briefly, Western blotting of lysates from spleen and lymph node tissues was used to confirm the molecular weights of the proteins targeted by the CD45 antibody (Yang et al., 2015). Iba1 antibody has been used for identification of macrophages in oral tissues in Dr. Sharma's laboratory (unpublished observations) and for identification of macrophages/microglia reported in a recent publication (Wang et al., 2017). The specificity of the Ly6C antibody was examined by immunostaining of bone marrow monocytes. To prevent false positive identifications due to non-specific labeling of the secondary antibodies, certain samples were incubated with only the secondary antibodies, and no clear fluorescence was observed.

2.4. Immunolabeling of the basement membrane and the cell membrane

To determine macrophage locations, we used the basement membrane as a landmark to separate the organ of Corti and mesothelial cells. The basement membrane was visualized using the immunoreactivity of Itga3, the alpha subunit of a transmembrane receptor for collagens. We also used E-cadherin to illustrate the membrane of Hensen and Claudius cells (Cai et al., 2012). After dissection, whole-mount preparations were treated with 0.5% Triton X-100 for 30 min at room temperature to permeabilize the cells. The tissues were subsequently incubated overnight at 4°C with an anti-Itga3 primary antibody (Integrin α 3 monoclonal antibody, 1:100, sc-7019, Santa Cruz Biotechnology Inc. Dallas, TX, USA) or an anti-E-cadherin antibody (rat monoclonal anti-E-cadherin antibody, 1:100, sc-59778, Santa Cruz Biotechnology Inc.). After incubation with the primary antibody, the tissues were rinsed 3 times with PBS and incubated in the dark with a secondary antibody (Alexa Fluor® 568 Goat anti-rat or anti-mouse IgG, 1:100 in PBS, Invitrogen, Carlsbad, CA, USA) for 2 h at room temperature. Both the Itga3-stained and the E-cadherin-stained tissues were stained again for CD45, as described above.

2.5. Immunolabeling of ganglion neurons and their fibers

To determine the location of macrophages, we used spiral ganglion neurons and their peripheral and central processes as a landmark for identifying macrophages in neural regions. Ganglion cells and their fibers were observed using the immunoreactivity of tubulin, the dimeric structural protein of microtubules, using a method that has been described in our previous publications (Ding et al., 2011; Ding et al., 2013). After dissection, whole-mount preparations were incubated overnight at 4°C with an anti-Tubulin antibody (Rabbit anti-

Tubulin, ab59680, Abcam Inc., Cambridge, MA, USA) diluted in 1% Triton X-100 and 5% donkey serum in 0.1 M PBS (1:100). The tissues were counterstained with the CD45 or F4/80 primary antibody as described above. After incubation with the primary antibodies, the tissues were rinsed 3 times with distilled water and incubated for 2 hours at room temperature with secondary antibodies (Alexa Fluor® donkey anti-rabbit IgG and Alexa Fluor® donkey anti-goat IgG, 1:100, A10042, Life Technologies, Grand Island, NY, USA) in 1% Triton X-100 and 5% donkey serum in 0.1 M PBS. The specificity of the primary antibody was confirmed in our previous publications (Ding et al., 2011; Ding et al., 2013).

2.6. Nuclear staining

Propidium iodide (PI, P3566, ThermoFisher Scientific) or 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI, D1306, ThermoFisher Scientific) was used to label the nuclei of cells to illustrate the orientation of tissue structures and to define cell death. After immunostaining, the tissues were rinsed in PBS and counterstained with PI (5 µg/ml in PBS) or DAPI (1 µg/ml in PBS) for 10 min.

2.7. Assessment of caspase activity

To confirm the developmental death of macrophages within the organ of Corti, we examined the activity of caspase-3, a primary executioner of apoptosis that has been used in our previous studies to identify apoptotic cells (Hu et al., 2002; Zhang et al., 2017). Caspase activity was assessed with a caspase-3 assay kit (CaspGLOW Fluorescein Active Caspase-3, K183-25, BioVision, Milpitas, California, USA). The staining was performed in *ex vivo*. The animals (n=3; two at P10 and one at P17) were sacrificed and the cochleae were collected. The bony shell of the cochlea facing the middle ear cavity was removed to expose the sensory epithelium. The tissue was placed in the caspase-3 staining solution (1:300 dilution with 10 mM PBS) and was incubated at 37 °C for 40 min. After staining, the cochleae were fixed with 10% formalin overnight and then dissected to collect the whole-mount preparations. The tissues were counterstained with PI as described above.

2.8. Tissue observation and image acquisition

The entire length of the cochlear whole-mount preparation was examined using an epifluorescence illumination microscope (Z6 APO apochromatic zoom system, Leica Microsystems, Buffalo Grove, IL, USA) equipped with a Leica digital camera (DFC3000 G microscope camera) and controlled by Leica Application Suite V4 PC-based software. To visualize detailed structures, the tissues were further examined and photographed using a confocal microscope (LSM510 multichannel laser scanning confocal image system, Zeiss, Thornwood, NY, USA). At each site of interest, a series of confocal images covering the entire thickness of the tissues was collected.

The collected images were processed using ZEN Blue 2012 image processing software (Zeiss, Thornwood, NY, USA) utilizing a previously reported methodology (Yang et al., 2015). Some collected images were further processed to improve the contrast and clarity of cells using the “Levels Adjustment” and “Despeckle” functions offered in Adobe Photoshop CS6 (version 13.0.1, Adobe Systems, San Jose, CA, USA) to correct the tonal range and reduce image noise. These image-processing steps did not create any analytical bias, because

the focus of this study was to examine macrophage morphologies, not expression levels of macrophage marker proteins.

2.9. Scanning electron microscopy

Scanning electron microscopy was used to reveal the morphology of the macrophages on the surface of the basilar membrane in adult mice (1-4 months). Animals were sacrificed and the cochleae were first fixed with 2% glutaraldehyde in 0.1 M phosphate buffer at room temperature for 1 h and then at 4 °C for overnight. The cochleae were decalcified with 10% Ethylenediaminetetraacetic acid at 4 °C for 5 days and dissected to expose the scala tympani side of the basilar membrane. The tissues were dehydrated through a graded series of ethanol (30%, 50%, 70%, 85%, 95%, and 100%) and then with 100% hexamethyldisilazane. The tissues were examined and photographed using a scanning electron microscope (SU-70, Hitachi, Japan).

2.10. Quantitative analysis of cochlear macrophages

We examined cells of interest that had been positively stained with known specific protein markers (either pan-leukocyte marker, CD45 or murine macrophage marker, F4/80 or Iba1). Positive cells were identified and distinguished from surrounding tissue by their ability to exhibit strong immunoreactivity.

The numbers and sizes of macrophages were analyzed in the following anatomic sites of the cochlea: the basilar membrane, the organ of Corti, spiral ligament, and spiral limbus, as well as the neural region in the osseous spiral lamina, ganglion neurons and modiolus. Each subset of the cochlear macrophage population was distinguished from neighboring macrophages using bright-field illumination when observing cells under epifluorescence illumination microscopy or using differential interference contrast view when confocal microscopy was used. These techniques provided clear visual delineation between distinct cochlear partitions (i.e., spiral limbus, spiral ligament and lateral wall). For the neural region, tubulin staining was used to illustrate ganglion cell bodies and their peripheral and central processes. Only macrophages residing within a particular cochlear partition were included in the macrophage distribution analysis for that delimited tissue region. Specifically, basilar membrane macrophages (BM-macrophages) are defined as the macrophages that reside on the scala tympani side of the basilar membrane. Organ of Corti macrophages (OC-macrophages) are defined as the macrophages that reside between the cells of the organ of Corti and the basement membrane. Osseous spiral lamina macrophages are defined as the macrophages that are located in the region around the peripheral nerve bundles of ganglion neurons.

Macrophage number in each anatomic site was quantified. To quantify the macrophage number in the regions of ganglion cell bodies and modiolus, we merged confocal images covering a tissue thickness of 4 µm in these anatomic sites. To compare the distribution patterns of macrophages along the length of the cochlear turns, we divided the whole-mount preparation into the apical, middle and basal regions (approximately, 10–30%, 40–60%, and 80–100% from the apex, respectively). The number and the diameter of macrophages were quantified using images collected with confocal microscopy or epifluorescence microscopy.

The number of macrophages in each region was counted and the mean for these counts was then computed to produce an average value per unit length (1 mm) or area (0.1 mm²) for each pre-delimited cochlear partition.

Diameter measurements were performed for macrophage precursor cells at P1 and in adult mice. If a cell was not in a perfect round shape, the diameter was measured by taking the average of the height and width of the cell. For each region, 5-10 cells were measured and the values were averaged to provide a single representative number for each individual sample. The numbers were averaged within groups to generate the group means and standard deviations.

2.11. Data analyses

Group means were acquired by averaging measurements per unit length or area across cochleae for each experimental condition. Group means were statistically compared with a two-tailed Student's *t* test, one-way or two-way ANOVA (see the Results section for details) using SigmaStat (Version 3.5) (San Jose, CA, USA) or GraphPad Prism (Version 5) (La Jolla, CA, USA). An α -level of 0.05 was selected for significance for all statistical tests.

3. Results

Immune cells were identified using immunolabeling of F4/80 (a macrophage marker protein), Iba1 (a macrophage marker protein), Ly6C (a monocyte marker protein) and CD45 (a pan-leukocyte marker). The tissues were stained with either one or two antibodies to identify immune cells. Because the immunoreactivity of CD45 was much stronger than that of other marker proteins, we used CD45 staining to illustrate macrophage morphology in the current study.

3.1. The basilar membrane and the organ of Corti contain immune cell populations

The cochlear whole-mount sensory epithelium preparation contains CD45-positive cells in multiple tissue partitions (Fig. 1A). We first examined the CD45-positive cells in the organ of Corti and the basilar membrane. To determine the precise location of CD45-positive cells, we double-stained the tissues with PI or DAPI to illustrate nuclei or with an antibody against Itga3 to illustrate the basement membrane. Using confocal microscopy, we found that CD45-positive cells were present in two locations relevant to the mesothelial cells and the basement membrane. One was on the scala tympani side of mesothelial cells (Fig. 1B). The other was on the organ of Corti side of the mesothelial cells (Fig. 1B and C). Some of these cells were found between the cells of the organ of Corti and the basement membrane (Figs. 1C and 1D) and others were found along the spiral vessels (Figs. 1B and 1E). Noticeably, the cells in different locations underwent distinct developmental changes and had different fates that will be described in the following sections.

3.2. Differentiation of basilar membrane immune cells

CD45-positive cells were found on the surface of the scala tympani side of the mesothelial cells at P1. These cells were distributed across the entire length of the basilar membrane. To define their developmental changes, we quantified the numbers at three basilar membrane

sites (the apex, middle and base) and at multiple developmental periods. The average number of cells per 1 mm length of the basilar membrane decreased from 35.4 ± 6.4 during the P1-4 to 26.2 ± 6.5 at P10 and to 16.8 ± 2.4 during P17-21 (Fig. 2A). This reduction is statistically significant (two-way ANOVA; $F = 22.3$; $df = 2,36$; $P < 0.001$; Tukey test: P1-4 vs. P10, $P = 0.006$; P10 vs. P17-21, $P = 0.005$). The analysis also revealed that the average number of CD45-positive cells in the apical region (31.9 ± 11.7) was slightly, but statistically, higher than those in the middle and basal regions (24.1 ± 8.2 and 22.4 ± 8.3 ; $F = 6.5$, $df = 2,36$; $P = 0.004$; Tukey test: apical region vs middle region: $P = 0.005$; apical region vs. basal region: $P = 0.023$; Fig. 2B). To determine the identity of CD45-positive cells, we double-stained the tissues with CD45 and F4/80 or with CD45 and Iba1. The immunoreactivity of F4/80 and Iba1 was detectable at P1 and their immunoreactivity became stronger at later time points (Fig. 3). The majority of CD45-positive cells ($80\% \pm 9$) display strong F4/80 and Iba1 immunoreactivity, suggesting that these cells are macrophages.

CD45-positive cells displayed a round shape with limited cytoplasm and a round nucleus at P1 (Fig. 4A). The average diameter of these cells is $14.5 \pm 1.5 \mu\text{m}^2$. To provide a context for assessing cell size, we measured the size of macrophage precursor cells in mature cochleae following acoustic overstimulation in our previous investigation (Yang et al., 2015). Postnatal cells are significantly larger than macrophage precursor cells (14.48 ± 1.54 vs. $8.71 \pm 0.54 \mu\text{m}^2$; two-tailed Student's *t*-test, $t(17) = 15.11$; $P < 0.0001$; Fig. 4B). Noticeably, CD45-positive cells exhibited heterogeneous CD45 immunoreactivity around the nuclei, forming a patch of immunoreactivity in one side of the cells. At P4, some CD45-positive cells became elongated or acquired an irregular shape (Fig. 4C).

By P10, the morphologies of CD45-positive cells started to diverge in a site-specific fashion. In the apical region, macrophages displayed multiple projections (Fig. 5A), whereas in the basal region, they showed an irregular shape with fewer projections (Fig. 5B). The difference between the apical and basal CD45-positive cells became more distinctive at P17 (Figs. 5C and 5D). The apical cells appeared dendritic in shape with multiple long processes. In contrast, the basal cells had short, thin projections. In mature cochleae (5 weeks), the apical-basal distinction became even more evident. The long processes of the apical cells remained (Fig. 5E), while the processes of basal cells were shorter and the amoeboid shape was common in the basal extreme (approximately 80-100% distance from the apex; Fig. 5F). These site-specific differences were also evident in the scanning electron micrographs of mature macrophages (7 weeks; Figs. 5G and 5H). Together, these observations suggest that apical and basal macrophages differentiate locally into their mature phenotypes.

3.3. Developmental death of OC-macrophages

It has been reported that the mature organ of Corti lacks tissue macrophages (Du et al., 2011; Hirose et al., 2005; Okano et al., 2008; Yang et al., 2015). Here, we present evidence that the postnatal organ of Corti contains macrophages. CD45-positive cells were found at P1. However, their morphology was difficult to detect at this time point due to weak CD45 immunoreactivity. At P4, CD45 immunoreactivity increased and the cell morphology became clearly visible (Fig. 6A). All these cells displayed F4/80 and Iba1 immunoreactivity

(Fig. 6B–D and 6E–G). In both age groups, their number was significantly less than that of basilar membrane macrophages (two-way ANOVA; $F = 10.173$, $df = 1,63$; $P < 0.001$; Tukey test: BM-macrophages vs. OC-macrophages at P1-4, $P < 0.001$; BM-macrophages vs. OC-macrophages at P7-10, $P < 0.001$; Fig. 6H).

OC-macrophages were arranged in one row along the long axis of the organ of Corti. Their abundance was relatively homogeneous from the apex to the base as their numbers were similar among the apical, middle and basal regions (Fig. 6I; one-way ANOVA on Ranks, $H(2) = 2.065$, $P = 0.356$). Moreover, there was no significant difference in the numbers of these cells between the P1-4 and the P7-10 group, suggesting that the macrophage number remains consistent during the early phases of postnatal development. However, by P17-21, there is a significant decrease in number of OC-macrophages (one-way ANOVA; $F = 635.23$, $df = 14$; $P < 0.001$; Tukey test: OC-macrophages at P1-4 vs. P17-21, $P < 0.001$; OC-macrophages at P7-10 vs. P17-21, $P < 0.001$). $n = 5$ cochleae for each bar.

At the early stages of postnatal development, OC-macrophages and their nuclei are irregular in shape (Figs. 7A and 7B). Starting at P7, the cell bodies began to shrink. The shrinkage was first observed at the basal end of the cochlea and then progressed toward the apical portion of the cochlea. Double-labeling of the tissues with PI, a nuclear dye, showed that the cells with shrunken cell bodies also displayed fragmented or shrunken nuclei (Figs. 7C and 7D). Nuclear condensation and fragmentation are morphological signs of apoptotic cell death. To provide further evidence for occurrence of apoptosis, we stained three cochleae for the activity of caspase-3, a protease for degradation of apoptotic cells (Boatright et al., 2003; Springer et al., 2001). We used sensory cell apoptosis as a positive control that was described in our recent publication (Zhang et al., 2017) and used the intact macrophages residing apical to dying macrophages as a negative control. As shown in Figure. 7E, a macrophage that displays nuclear fragments exhibited strong caspase-3 fluorescence. Both nuclear fragmentation and caspase activation are the phenotypes of apoptosis. In contrast, cells without nuclear changes displayed very weak caspase fluorescence. At P17-21, the developmental death of OC-macrophages reached the apical region of the cochlea. At this age, the condensed nuclei of degrading macrophages in the basal region of the cochlea disappeared, leaving anucleated residual bodies (Fig. 7F). This basal to apical gradient of macrophage degradation is consistent with the basal to apical maturation of the sensory epithelium (Kraus et al., 1981; Pujol et al., 1998; Souter et al., 1997).

3.4. CD45-positive cells along the spiral vessel

The cochlea has a spiral vessel that runs parallel to the edge of the osseous spiral lamina. In the mouse cochlea, this vessel was observed at P1 (Fig. 8A), consistent with a previous report that this vessel is formed embryonically after gestational day 17 (Iwagaki et al., 2000). We found CD45-positive cells along this vessel (Fig. 8B). These CD45-positive cells also displayed F4/80 and Iba1 immunoreactivity (Figs. 8C and 8D). They had a long, slim cell body with a long nucleus that ran parallel to the long axis of the vessel (Figs. 8E and 8F). Noticeably, these cells were identified in only a portion of examined regions and cochleae. Like OC-macrophages, these cells started to shrink several days after birth and this change was accompanied by nuclear fragmentation (Figs. 8G and 8H). The time frame of

the cell degradation was similar to that seen for the degradation of OC-macrophages. At P21, these cells became anucleated remnants (Figs. 8I and 8J).

3.5. Presence of macrophage precursors in the basilar membrane

We wanted to determine whether macrophage precursors continue to contribute to the BM-macrophage population during postnatal development. As shown in Figure 4A, CD45-positive cells with a round shape were first observed at P1. To prevent the misidentification of immune cells that infiltrated the region postnatally as these embryonically distributed cells, we examined cochleae at P4 or later when embryonically distributed cells started to transform into mature macrophages. We used Ly6C which marks monocytes/macrophages ($n=3$ cochleae). The tissues were double-stained with an antibody against F4/80 because F4/80 expression is weaker in blood monocytes compared to tissue macrophages (Austyn and Gordon, 1981). We found CD45-positive cells that were round or oval with fine, short projections at P10 (Fig. 9A). These cells were about 6-8 μm , which is much smaller than macrophages. Tissues that were stained for Ly6C and F4/80 displayed small, round cells with Ly6C immunoreactivity, but lacked F4/80 immunoreactivity (Figs. 9B, 9C and 9D). This immunophenotype is a typical phenotype of blood monocytes (Austyn et al., 1981). We did not perform statistical analysis for infiltrating monocytes because they were low in number (approximately 2-4 per cochlea). These observations suggest that macrophage precursors continue to contribute to the postnatal macrophage population in the basilar membrane.

3.6. CD45-positive cells in the spiral ligament

In order to gain a more comprehensive understanding of the changes in cochlear immune cells during postnatal development, we examined CD45-positive cells in three additional cochlear partitions that display an abundance of macrophages in mature cochlea (Hirose et al., 2005; Okano et al., 2008; Sato et al., 2008; Shi, 2010): the spiral ligament of the lateral wall, the spiral limbus, and the neural regions of the cochlea at ages P4, P10 and P17 ($n=4$ cochleae for each group).

CD45 staining revealed diverse shapes of immune cells in the spiral ligament. At P4, most cells displayed an irregular, distended, globular morphology (Fig. 10A). At P10 and P17, the CD45-positive cells became more dendritic in morphology (Figs. 10B and 10C). Most CD45-positive cells with irregular shapes had F4/80 and Iba1 immunoreactivity (Figs. 10D-10G). However, a few CD45-positive cells that exhibited a round-to-ovoid phenotype were F4/80 negative (arrows, Fig. 10E). When compared to cells with both CD45 and F4/80 colocalization, cells positive for only CD45 constitute 3.1% of the total spiral ligament immune cell population at age P4 and 3.3% of the population at ages P10 and P17. Overall, a greater number of spiral ligament macrophages was found in the earlier stage of postnatal development compared to later developmental stages. A systematic survey of the number of CD45-positive cells in this tissue revealed a mean of 58.3 ± 2.7 cells per 0.1 mm^2 of spiral ligament tissue at age P4. A reduction in the average number of CD45-positive cells in the spiral ligament was seen at P10 (50.6 ± 1.6) and P17 (50.3 ± 2.6). This reduction in the number of cells, while moderate, is statistically significant (Fig. 10H; one-way ANOVA; $F=14.7$; $\text{df} = 11$; $P < 0.001$; Tukey test: P4 vs. P10, $P = 0.003$; P4 vs. P17, $P = 0.003$).

3.7. Spiral limbus macrophages

In the cochlear scala media, a thickening of the periosteum on the upper plate of the spiral lamina forms the spiral limbus, and a substantial number of CD45-positive cells were found within this cochlear partition early in postnatal development as can be observed at age P4 (Fig. 11A). There was no significant change in the morphology of the cells from P4 to P17 (Fig. 11B). However, a dramatic decline in the number of macrophages residing in this cochlear partition was found with progressing postnatal age, diminishing from 51.3 ± 3.2 cells/ 0.1mm^2 at P4 to 23.8 ± 3.2 at P10 and to 15.6 ± 1.3 at P17 in the cochlear middle turn. This reduction is statistically significant (Fig. 11C; two-way ANOVA; $F = 570.56$; $df = 2$; $P < 0.001$; Tukey test: P4 vs. P10, $P < 0.001$; P10 vs. P17, $P < 0.001$). All CD45-positive cells in this region displayed strong F4/80 and Iba1 immunoreactivity (Figs. 11D-11G).

3.8. Macrophages in cochlear neural tissues

We examined immune cells in three neural regions of the cochlea: the ganglion cell bodies within the Rosenthal's canal, the peripheral processes within the osseous spiral lamina, and the central processes within the modiolus. In these regions, all CD45-positive cells with the macrophage morphology displayed Iba1 immunoreactivity (Fig. 12).

3.8.1. Osseous spiral lamina macrophages—Changes in the macrophages residing amongst the neural tissue of the osseous spiral lamina were evaluated as a function of postnatal development (Figs. 13A and 13B). Tubulin staining confirmed that the cells that were analyzed resided along with the peripheral fibers of ganglion cells (Figs. 13C and 13D). Macrophages in the osseous spiral lamina ran radially toward the edge of the osseous spiral lamina. They began to differentiate and adopted a more mature morphological phenotype earlier in development when compared to macrophages from other nearby cochlear partitions (e.g., BM-macrophages and spiral ligament macrophages). Even as early as age P4, these immune cells are observed with a branched morphology (Fig. 13A) and phenotypically closely resemble the cells at P17 (Fig. 13B).

However, when scrutinizing the number of macrophages extant in this cochlear partition, a clear and significant reduction in the number of the immune cells is observed with advancing age (Fig. 13E; two-way ANOVA; $F = 109.72$; $df = 2$; $P < 0.001$; Tukey test: P4 vs. P10, $P < 0.001$; P4 vs. P17, $P < 0.001$). While the count of osseous spiral lamina macrophages was calculated for each of three anatomical cochlear turns (apical, middle and basal) for each cochlea, the overall the number of macrophages present in this tissue was found to be largely similar amongst these cochlear turns. Representatively, though at P4 42.8 ± 3.1 macrophages resided in 0.1 mm^2 of osseous spiral lamina tissue of the middle cochlear turn, the number of these cells per same unit area significantly reduced to 26.2 ± 2.7 at P10 and 25.6 ± 1.0 at P17.

3.8.2. Macrophages among the spiral ganglia in Rosenthal's canal—We quantified the number of macrophages residing among the spiral ganglia in Rosenthal's canal at three postnatal ages (Figs. 14A, B and C). The macrophage location was confirmed with tubulin staining, which illustrated ganglion cell bodies (Fig. 14D). In this cochlear region, macrophages appeared with an irregular morphology throughout postnatal

development. Yet, when analyzing changes in the number of macrophages present in this cochlear partition, a reduction of cells was observed as age increased. While at P4 macrophages in the spiral ganglion region numbered 41.7 ± 3.2 cells per 0.1mm^2 , fewer macrophages were present at age P10 (35.3 ± 3.3), and a further reduction in the number of these cells was measured at P17 (27.3 ± 1.2). This decrease in the number of macrophages in the spiral ganglion region is significant (Fig. 14E; two-way ANOVA; $F = 19.32$; $df = 2$; $P = 0.001$; Holm-Sidak test: P4 vs. P10, $P < 0.05$; P4 vs. P17, $P < 0.01$; P10 vs. P17, $P < 0.01$).

3.8.3. Macrophages in the modiolar region—A survey of macrophages within the modiolar region was conducted during postnatal development. Alterations in both macrophage morphology and number occurred with advancing postnatal age. Macrophages at P4 presented with a distended globular shape (Fig. 15A) and occurred at a rate of 56.0 ± 2.0 per 0.1mm^2 . By P10, macrophage morphology became increasingly branched with more abundant processes (Fig. 15B). Concurrently, the number of macrophages reduced to 37.3 ± 5.5 per tissue survey area at this age. By P17, macrophages appeared with a somewhat stretched, linear shape (Fig. 15C), and a further decrease in the number of these cells was recorded (28.7 ± 2.6). Again, tubulin staining confirmed the location of macrophages in the modiolus. We also performed a statistical analysis of macrophage numbers. The age-related reduction in macrophage number in the modiolar region is statically significant (Fig. 15E; two-way ANOVA; $F = 36.01$; $df = 2$; $P = 0.001$; Holm-Sidak test: P4 vs. P10, $P < 0.01$; P4 vs. P17, $P < 0.01$; P10 vs. P17, $P < 0.05$).

4. Discussion

We examined the postnatal development of macrophages in the whole-mount preparation of the mouse cochlea and found two distinct macrophage populations in the organ of Corti and the basilar membrane: one on the organ of Corti side of the basilar membrane and the other on the scala tympani side of the basilar membrane. These two macrophage populations exhibit distinct developmental patterns and fates. The macrophages on the organ of Corti side of the basilar membrane are fully differentiated in morphology at birth. These cells are short-lived and undergo a developmental degradation coincident with the reported maturation of the sensory epithelium. These results provide evidence linking developmental macrophages with the postnatal maturation of the sensory epithelium. In contrast, the macrophages on the scala tympani side of the basilar membrane display a round shape at birth. These cells undergo site-specific differentiation into mature phenotypes with postnatal development. Moreover, we found macrophages in the spiral ligament, spiral limbus and the neural region during postnatal development. The numbers of these cells decrease during postnatal development. Finally, we found the presence of macrophage precursors in the basilar membrane during the period of postnatal development. These cells differentiate locally into mature macrophages. Together, this study reveals the dynamic development of postnatal macrophages in the cochlea.

4.1. Origin of BM-macrophages

The basilar membrane contains macrophages. In mature cochleae, these cells reside on the scala tympani side of the basilar membrane. While BM-macrophages have been observed

throughout adulthood in mice (Frye et al., 2017), their origin is not completely clear. Tissue macrophages are known to originate embryonically, from blood monocytes, self-renewal or from multiple origins (Epelman et al., 2014; Gentek et al., 2014). Studies on cochleae exposed to acute stresses have revealed time-dependent monocyte infiltration (Fredelius et al., 1990; Hirose et al., 2005; Tornabene et al., 2006; Wakabayashi et al., 2010). These cells locally differentiate into macrophages (Yang et al., 2015). Similarly, when tissue macrophages are experimentally depleted, circulating monocytes infiltrate the cochlea (Sato et al., 2008; Shi, 2010). These observations suggest that bone marrow-derived macrophages are a source of tissue macrophages in the basilar membrane.

In the cochlea, embryonic macrophages has been found near the otic vesicle at embryonic day 10 (Hirose et al., 2017), indicating the presence of embryonic origin of cochlear macrophages. Here, we found immune cells with macrophage phenotypes at birth. These cells differentiate locally into mature macrophages. We also observed the presence of macrophage precursors in the basilar membrane after birth. These results indicate that tissue macrophages with different ontological origins coexist in the postnatal cochlea. While these results suggest the multiple sources of cochlear macrophages, it not clear the exact contribution of embryonic and postnatal infiltrating cells to the adult macrophage pool because of the dynamic nature of macrophage balance. Future fate mapping analysis could provide direct evidence for analyzing such contribution.

4.2. Site-specific differentiation of BM-macrophages

Our previous studies demonstrated that BM-macrophages in mature cochleae display distinct site-specific morphologies (Frye et al., 2017; Yang et al., 2015). In the apical portion of the basilar membrane, the macrophages are dendritic, whereas those in the middle and basal portions are branched or amoeboid in shape. These studies also revealed differences in the expression of macrophage proteins under both steady-state and pathological conditions. Here, we demonstrated that the apical-basal difference is not embryonically established, but develops during the postnatal development of the cochlea. We found that, at birth, both apical and basal primitive cells display a round shape, suggesting that these cells are derived from the same precursors. Moreover, we found that these macrophage precursors locally transform into their adult macrophage phenotypes along with the maturation of the sensory epithelium. This observation is consistent with our previous observation in acutely damaged cochleae, where the transformation into mature macrophages from infiltrated monocytes is also related to the location in which they reside (Yang et al., 2015). This apical-basal difference in macrophage morphology suggests the difference in their functional state because changes in macrophage morphology can cause changes in their function (McWhorter et al., 2013).

The biological mechanism for the shape difference is not clear. Macrophages are mobile. They change their shapes as they migrate through tissues (Hirose et al., 2017). Therefore, it is likely that the shape difference is associated with the movement of the cells. However, an intriguing finding reported here as well as in our previous observations of mature cochleae (Frye et al., 2017; Yang et al., 2015) is that no amoeboid cells were found on the apical basilar membrane, nor were cells with long and thin processes found in the basal region of

the basilar membrane. We suspect that the difference is due to the difference in macrophage functional states because these cells display different expression pattern of immune molecules that has been demonstrated in our previous publication (Yang et al., 2015). It is likely that local environmental signals play an essential role in controlling the differentiation of macrophage precursors. It would be interesting to determine how the apical and basal immune environments differ because such differences could affect the immune capacity of the tissue.

4.3. Macrophages on the organ of Corti side of the basilar membrane are short-lived

The mouse cochlea is immature at birth and the maturation process continues until adulthood. During this time, the sensory epithelium undergoes several gross structural modifications, including a reduction in the number of mesothelial cells, regression of the spiral vessel, formation of lymph-filled spaces within the organ of Corti, as well as fine subcellular changes in sensory cells and their neuronal connections (Kraus and Aulbach-Kraus, 1981; Pujol et al., 1998; Souter et al., 1997). These processes start from the base and progress toward the apex of the cochlea, consistent with the progression of functional maturation from high to low frequencies of hearing. Here, we provide evidence that OC-macrophages are implicated in cochlear maturation. First, OC-macrophages are fully differentiated morphologically and therefore are likely functional as well at birth. Second, these cells reside in the immediate vicinity of the structures that undergo developmental changes. Third, these cells maintain their viability until sensory epithelium maturation is complete. Importantly, the degradation of these cells proceeds from the base to the apex, consistent with the maturation pattern of the sensory epithelium. Together, these findings implicate macrophages in the developmental maturation of the sensory epithelium.

In addition to OC-macrophages, we found the presence of short-lived macrophage-like cells around the spiral vessel at early stages of postnatal developmental. The spiral vessel is the only vessel near the sensory epithelium. This vessel has branches projecting toward the external wall and the spiral lamina at birth (Iwagaki et al., 2000), which gradually regress with the maturation of the sensory epithelium. Like the macrophages in the organ of Corti, the macrophage-like cells around the spiral vessel undergo a time-dependent degradation as the vessel regresses. This correlation suggests a role for these cells in vascular pruning.

We also found immune cells with condensed or fragmented nuclei in other cochlear partitions (the lateral wall and the surface of the basal membrane) that displayed developmental reorganization. This observation suggests that local death of macrophages occurs in these regions. Because the presence of dying cells are scarce in these regions, we did not document their number. Lack of detection of dying macrophages could be due to a quick removal of dying cells by survival macrophages in these regions.

The contribution of macrophages to the maturation of the sensory epithelium is not clear. Developmental macrophages have been found to phagocytize degraded cells during tissue remodeling and to interact with the extracellular matrix during general organogenesis and vasculogenesis (Lang et al., 1994; Poche et al., 2015; Pollard, 2009). In the developing ear, molecules that modulate macrophage functions have been found to play a role in the

development of the inner ear (Bank et al., 2012). Future studies are expected to define the functional role of macrophages in sensory epithelium maturation.

4.4. Macrophages in the spiral ligament, spiral limbus and the neural regions of the cochlea

The spiral ligament, spiral limbus, and cochlear neural tissue were selected for immune cell analysis during postnatal development because in mature cochleae, these regions contain abundant macrophages (Hirose et al., 2005; Okano et al., 2008; Sato et al., 2008; Shi, 2010). Our analysis reveals that postnatal development is accompanied by a reduction in the number of macrophages within each of these tissue regions, although there is certainly not a complete loss of these cells. This reduction is consistent with the general trend of macrophage reduction in the region of the basilar membrane. Moreover, our data clearly show that macrophages are present in each of these cochlear partitions from an early stage of postnatal development, though the rate of macrophage maturation was found to vary between these tissue regions. While a more mature, adult-like morphology was displayed by macrophages in the spiral limbus and amongst the neural tissue of the osseous spiral lamina as early as P4, macrophages in the spiral ligament still exhibited an immature, globular phenotype at this age. Mature morphologies of spiral ligament macrophages did not emerge until later in postnatal development. This difference in maturation rate for macrophages in distinct cochlear partitions may be associated with the difference in maturation stages of microenvironments around macrophages.

In summary, macrophages are present in the neonatal basilar membrane and the organ of Corti, and these cells have distinct developmental fates. The presence of short-lived macrophages around the cells of the organ of Corti suggests a developmental role for the immune system in postnatal remodeling of the sensory epithelium.

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Abbreviations

P	Postnatal day
PBS	Phosphate-buffered saline
PI	Propidium iodide
DAPI	4',6-Diamidine-2'-phenylindole dihydrochloride
BM-macrophages	Basilar membrane macrophages
OC-macrophages	Organ of Corti macrophages

Highlights

- Postnatal basilar membrane and organ of Corti contains distinct macrophage populations
- Organ of Corti-macrophages undergo developmental death with the maturation of the sensory epithelium
- Basilar membrane macrophages differentiate in a site-dependent manner
- Macrophage number decreases in multiple cochlear regions with the maturation of the cochlea

Figure 1. Distribution of CD45-positive cells in the basilar membrane and the organ of Corti

A. Cochlear whole-mount sensory epithelium preparation at P4 showing CD45-positive cells in multiple tissue partitions, BM = basilar membrane, OSL = osseous spiral lamina, GN = ganglion neurons. **B.** A side view of a whole-mount surface preparation. The image was projected from a series of confocal images of a whole-mount preparation from a cochlea at P7. Red fluorescence represents the staining of PI, which binds to nuclear DNA and cytoplasm RNA for illustration of both the nuclei and cytoplasm of cells. The green fluorescence represents CD45 immunoreactivity. The arrows point to the CD45-positive cells that reside in the organ of Corti side of mesothelial cells and the double-arrow indicates a CD45-positive cell on the surface of mesothelial cells facing the scala tympani. **C.** A side view of a whole-mount preparation showing the immunostaining of the basement membrane from a cochlea at P17. The basement membrane was illustrated using Itga3 immunoreactivity (red fluorescence pointed by the arrow). The double-arrow points to a CD45-positive cell that resides on the organ of Corti side of the basement membrane. **D.** A side view of the whole-mount preparation showing the spatial relationship between a CD45-positive cell and organ of Corti-cells in a cochlea at P17. E-cadherin immunoreactivity (red fluorescence) is used to illustrate cell membrane. DAPI (blue fluorescence) is used to illustrate nuclei. Notice that CD45 immunoreactivity (green fluorescence pointed by the double-arrow) is adjacent to Hensen and Claudius cells pointed by the arrow. **E.** Schematic drawing showing the locations of CD45-positive cells in the basilar membrane and the organ of Corti.

Figure 2. Comparisons of the numbers of CD45-positive cells among different postnatal periods and anatomic sites of the basilar membrane

A. Comparison of the average numbers of CD45-positive cells among three postnatal periods ($n = 5$ cochleae). There is a statistically significant reduction in the number of CD45-positive cells from P1-4, to P10, and to P17-21 (two-way ANOVA; $F = 22.3$; $df = 2,36$; $P < 0.001$; Tukey test: P1-4 vs. P10, $P = 0.006$; P10 vs. P17-21, $P = 0.005$). **B.** Comparison of the average numbers of CD45-positive cells among the apical, middle and basal regions of the basilar membrane ($n = 5$ cochleae). The number of apical cells is slightly, but significantly, higher than those in the middle and basal regions (two-way ANOVA; $F = 6.5$, $df = 2,36$; $P = 0.004$; Tukey test: apical region vs. middle region: $P = 0.005$; apical region vs. basal region: $P = 0.023$).

Figure 3. Most CD45-positive cells on the surface of the basilar membrane express macrophage marker proteins

A, B and C. Double-staining of CD45 and F4/80 in a cochlea at P4. **D, E and F.** Double-staining of CD45 and Iba1 in a cochlea at P10. Notice that the majority of CD45-positive cells exhibit F4/80 and Iba1 immunoreactivity. The scale bar presented in **B** is applicable to all panels.

Figure 4. Differentiation of CD45-positive cells on the surface of the basilar membrane from P1 to P4

A. Image showing the morphology of CD45-positive cells at P1. These cells are round with limited cytoplasm (arrows). The dark area within each cell is the nucleus, which is also round in shape. **B.** Comparison of the diameters of the CD45-positive cells and macrophage precursor cells. The size of macrophage precursor cells was measured from the basilar membranes collected during our previous investigation of cochlear responses to acoustic injury (Yang et al., 2015). Postnatal CD45-positive cells are larger than macrophage precursor cells (two-tailed Student's *t*-test, $t(17) = 15.11$; $P < 0.0001$). $n = 4$ cochleae for the P1-2 group and $n = 5$ cochleae for the mature cochleae group. **C.** Image showing the morphology of CD45-positive cells at P4. Some CD45-positive cells possess an irregular or elongated shape (arrows).

Figure 5. Apical and basal CD45-positive cells on the surface of the basilar membrane acquire different morphologies from P10 to maturation

A and B. Images showing typical morphologies of CD45-positive cells at P10. The apical and basal CD45-positive cells show different morphologies. In the apical portion of the basilar membrane, CD45-positive cells show multiple projections with different lengths (arrows in panel A). In the basal portion, CD45-positive cells display irregular shapes (arrows in panel B). Some have projections, but these projections are usually short. **C and D.** Images showing CD45-positive cell morphologies at P17. In the apical portion of the basilar membrane, the cells display a dendritic shape. In the basal portion, the projects of the cells are short and thin. **E and F.** Images showing typical CD45-positive cell morphology at 5 weeks. The apical cells retain their dendritic shape, whereas the cells in the basal extreme (80-100% distance from the apex) acquire an amoeboid shape. **G and H.** Typical morphologies of macrophages revealed by scanning electron microscopy. Notice that the apical macrophage displays a long and thin body (panel G). The macrophage from the basal region displays a bulky shape (panel H). Scale bars in A to F = 20 μ m.

Figure 6. Developmental changes of OC-macrophages

A. CD45 immunostaining of a whole-mount preparation collected at P4. CD45-positive cells display an irregular shape and are arranged in one row parallel to the long axis of the basilar membrane (arrows). **B, C and D.** Double-staining of tissues for CD45 and F4/80. Notice that CD45-positive cells display strong F4/80 immunoreactivity. **E, F and G.** Double-staining of CD45 and Iba1. Notice that CD45-positive cells also display strong Iba1 immunoreactivity. **H.** Comparison of the average numbers of BM-macrophages and OC-macrophages per 1 mm at two developmental periods (P1-4 and P7-10). The numbers of OC-macrophages are significantly less than those of BM-macrophages at both developmental periods (two-way ANOVA; $F = 10.17$, $df = 1,63$; $P < 0.001$; Tukey test: BM-macrophages vs. OC-macrophages at P1-4, $P < 0.001$; BM-macrophages vs. OC-macrophages at P7-10, $P < 0.001$). $n = 5$ cochleae for each bar. **I.** Comparison of the average numbers of OC-macrophages among the apical, middle and basal regions of the organ of Corti. There is no significant difference in the numbers among the three regions (one-way ANOVA on Ranks, Kruskal-Wallis test, $H(2) = 2.07$, $P = 0.356$), indicating that the cells are evenly distributed from the apex to the base. $n = 5$ cochleae for each bar. **J.** Comparison of the average numbers of OC-macrophages between three postnatal periods (P1-4, P7-10 and P17-21). There is no significant difference in the number of macrophages between P1-4 and P7-10, suggesting the maintenance of OC-macrophages during this developmental period. However, by P17-21, there is a significant decrease in the number of OC-macrophages (one-way ANOVA; $F = 635.23$, $df = 14$; $P < 0.001$; Tukey test: OC-macrophages at P1-4 vs. P17-21, $P < 0.001$; OC-macrophages at P7-10 vs. P17-21, $P < 0.001$). $n = 5$ cochleae for each bar.

Figure 7. Developmental death of OC-macrophages

The tissues were double-stained with a CD45 antibody (green fluorescence) and PI (red fluorescence). **A** and **B**. Images showing a normal macrophage from a cochlea at P10. The cell displays an irregular morphology with a large nucleus (panel A). An enlarged view of this nucleus is seen in panel B (arrow). **C** and **D**. Images showing the shrinkage of an OC-macrophage in the basal region of the same cochlea. Compared to the healthy macrophage (panel A), the macrophage displays a shrunken cell body and a fragmented nucleus (panel C), which are signs of cell degradation. An enlarged view of the fragmented nucleus of the macrophage can be observed in panel D. **E**. Image showing activation of caspase-3 activity in a cochlea collected at P10. The arrow points to a cell with a fragmented nucleus (see the inset for an enlarged view of the nucleus) in the region of OC-macrophages where macrophage death was evident. Notice that this cell displays strong caspase-3 fluorescence, an indication of caspase activation. **F**. Image showing complete loss of macrophage nuclei in the organ of Corti at the basal end of the cochlea. However, residual CD45 immunoreactivity is present (arrows).

Figure 8. Developmental death of CD45-positive cells along the spiral vessel

A. Differential interference contrast view of the basilar membrane of a cochlea examined at P4. Arrows point to a spiral vessel containing red blood cells. **B.** Immunostaining of the same tissue for CD45. Arrows indicate CD45-positive cells with long cell bodies that are parallel to the long axis of the vessel. **C.** CD45-positive cells along the spiral vessel display strong Iba1 immunoreactivity in a P10 cochlea. Note that CD45 immunoreactivity is not shown. **D.** CD45-positive cells along the vessel also display strong F4/80 immunoreactivity in a P10 cochlea. Again, CD45 immunoreactivity is not shown. **E** and **F.** CD45 immunolabeling (green fluorescence) and PI nuclear staining (red fluorescence) showing a normal CD45-positive cell (arrow) distributed along the spiral vessel (the vessel is not shown). The tissue was collected from a cochlea at P10. Notice that the nucleus is oval and displays a normal morphology (see the enlarged view of the nucleus in panel F). **G** and **H.** CD45 immunolabeling and PI nuclear staining showing a cell with a fragmented nucleus (arrow), a sign of cell degradation, from a cochlea at P10. An enlarged view of the fragmented nucleus can be seen in panel H. **I** and **J.** Double-staining of CD45 and nuclei (with PI) of a whole-mount preparation collected from a cochlea at P21. The arrow points to a CD45-positive residual body where the cell body appears degraded (panel I). Double-

staining of the tissue with PI shows the absence of the nuclear fluorescence (panel J), indicating complete degradation of the nucleus.

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Figure 9. Immune cells with the phenotypes of macrophage precursors on the surface of the basilar membrane

A. Image showing CD45-positive cells on the surface of the basilar membrane at P10. The arrow points to a CD45-positive cell that has a round shape with thin projections. Notice that the size of this cell is much smaller than that of neighboring cells with the macrophage morphology (double-arrows). **B, C and D.** Double-staining for Ly6C (panel A) and F4/80 (panel C) in a cochlea at P10. The arrow points to a Ly6C-positive cell that has a small round shape and lacks F4/80 immunoreactivity, which are phenotypes of the monocyte. Double-arrows indicate the F4/80 positive cells with the macrophage morphology that display only weak Ly6C immunoreactivity.

Figure 10. Developmental changes in spiral ligament macrophages

A. Image showing abundant CD45-positive cells with diverse shapes in the spiral ligament at age P4. While most cells exhibit an irregular, distended, globular morphology, a few cells are small and round-to-ovoid. **B** and **C.** Images showing CD45 immunostaining of the spiral ligament at P10 and P17. More mature dendritic-like cells emerge by P10 (panel B) and this phenotype is maintained at P17 (panel C). **D** and **E.** Double-staining of a spiral ligament tissue at P10 for CD45 and F4/80. Note that irregular-shaped cells possess strong F4/80 immunoreactivity indicating a macrophage phenotype. Note that a few round-to-ovoid CD45-positive cells are F4/80 negative (cells in green pointed by arrows in panel E). **F** and **G.** Double-staining of a spiral ligament tissue at P10 for CD45 and Iba1. Irregular-shaped cells possess strong Iba1 immunoreactivity, which is a macrophage phenotype. **H.** Comparison of the numbers of spiral ligament macrophages across three development ages. $n = 4$ cochleae for each bar.

Figure 11. Developmental changes in spiral limbus macrophages

A and **B**. Images show the immunolabeling of CD45 in the spiral limbus at P4 (panel **A**) and P17 (panel **B**). Positive cells at P17 display more branches and their number is substantially diminished. **C**. Comparison of the numbers of CD45-positive cells among three developmental stages. Each cochlear tissue is divided into the apical, middle and basal sections, representing the regions of approximately 10-30%, 40-60%, and 80-100% from the apex, respectively. A significant reduction in the number of CD45-positive cells within spiral limbus tissue occurs with progressive postnatal development (two-way ANOVA; $F = 570.56$; $df = 2$; $P < 0.001$; Tukey test: P4 vs. P10, $P < 0.001$; P10 vs. P17, $P = 0.001$). $n = 4$ cochleae for each bar. **D** and **E**. Double-staining of a cochlea at P4 for CD45 and F4/80. All CD45-positive cells display F4/80 immunoreactivity as indicated by yellow in panel **E**. **F** and **G**. Double-staining of a cochlea at P10 for CD45 and Iba1. All CD45-positive cells display strong Iba1 immunoreactivity indicated by yellow in panel **G**.

Figure 12. CD45-positive cells in the neural region (the modiolus, osseous spiral lamina and ganglion neuron region) display strong Iba1 immunoreactivity
Images showing immunolabeling of CD45 and Iba1 in a cochlea at P10. Notice that all CD45-positive cells with the macrophage morphology exhibit strong immunoreactivity of Iba1, a macrophage marker protein.

Figure 13. Developmental changes in osseous spiral lamina macrophages
A and **B**. Immunolabeling of macrophages in the neural region of the osseous spiral lamina at P4 (panel A) and P17 (panel B). General branched macrophage morphologies are maintained from P4 to P17, though with a diminished number of cells observed at P17. **C** and **D**. The location of macrophage among the neural fibers is confirmed by tubulin staining. Macrophages in this cochlear region have a branched shape and are oriented in parallel to the peripheral fibers of ganglion neurons. **E**. Comparison of macrophage numbers among three developmental stages. Each cochlear tissue is divided into the apical, middle and basal sections, representing the regions of approximately 10-30%, 40-60%, and 80-100% from the apex, respectively. A significant reduction in the number of osseous spiral lamina macrophages occurs in later stages of postnatal development (two-way ANOVA; $F = 109.72$; $df = 2$; $P < 0.001$; Tukey test: P4 vs. P10, $P < 0.001$; P4 vs. P17, $P < 0.001$). $n = 4$ cochleae for each bar.

Figure 14. Developmental changes in ganglion region macrophages

A, B and C. CD45-positive macrophages among ganglion neurons within Rosenthal's canal at P4, P10 and P17. **D.** Macrophages can be seen juxtaposed with spiral ganglion neurons labeled with tubulin at P4. **E.** Comparison of macrophage numbers among three developmental stages. A significant reduction in the number of macrophages occurs with advancing postnatal development (two-way ANOVA; $F = 19.32$; $df = 2$; $P = 0.001$; Holm-Sidak test: P4 vs. P10, $P < 0.05$; P4 vs. P17, $P < 0.01$; P10 vs. P17, $P < 0.01$). P4 and P17, $n = 3$ cochleae; P10, $n = 4$ cochleae.

Figure 15. Developmental changes in modiolar region macrophages

A, B and C. Macrophages in the modiolar region at P4, P10 and P17. **D.** The image shown a superimposed image of CD45-positive cells and modiolar nerve fibers illustrated by immunolabeling of tubulin at age P4. **E.** Comparison of macrophage numbers among three developmental stages. With progressive postnatal development, fewer macrophages are observed in the modiolar region, and this reduction in macrophage number is significant (two-way ANOVA; $F = 36.01$; $df = 2$; $P = 0.001$; Holm-Sidak test: P4 vs. P10, $P < 0.01$; P4 vs. P17, $P < 0.01$; P10 vs. P17, $P < 0.05$). $n = 3$ cochleae for each bar.