Cochlear supporting cells function as macrophage-like cells and protect audiosensory receptor hair cells from pathogens.

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To protect the audiosensory organ from tissue damage from the immune system, the inner ear is separated from the circulating immune system by the blood-labyrinth barrier, which was previously considered an immune-privileged site. Recent studies have shown that macrophages are distributed in the cochlea, especially in the spiral ligament, spiral ganglion, and stria vascularis; however, the direct pathogen defence mechanism used by audiosensory receptor hair cells (Hcs) has remained obscure. Here, we show that Hcs are protected from pathogens by surrounding accessory supporting cells (Scs) and greater epithelial ridge (GeR or Kölliker’s organ) cells (GeRcs). In isolated murine cochlear sensory epithelium, we established Theiler’s murine encephalomyelitis virus, which infected the Scs and GeRcs, but very few Hcs. The virus-infected Scs produced interferon-α/β, and the viruses efficiently infected the Hcs in the ifn-α/β receptor-null sensory epithelium. Interestingly, the virus-infected Scs and GeRcs expressed macrophage marker proteins and were eliminated from the cell layer by cell detachment. Moreover, lipopolysaccharide induced phagocytosis of the Scs without cell detachment, and the Scs phagocytosed the bacteria. These results reveal that Scs function as macrophage-like cells, protect adjacent Hcs from pathogens, and provide a novel anti-infection inner ear immune system.
Hensen's cells and Claudius' cells. IHCs are surrounded by greater epithelial ridge (GER or Kölliker's organ) cells (GERCs) in the immature neonatal inner ear, which develop later on into mature SCs and are important for HC development. SCs are linked to each other and to HCs via tight and adherent junctions; they form rigid cytoskeletons and maintain the structural integrity of the audiosensory organs during sound stimulation and head movement. Although SCs have several functions in the cochlea related to gap junctions containing connexin proteins including K⁺-recycling, nutrient and energy supply, and generation and maintenance of the unique electrochemical environments of the endolymph and perilymph, their roles in the immune system have not been elucidated.

Macrophages and leukocytes are present in the cochlea of the avian inner ear, especially within the hyaline/ cuboidal cell region, which is located just below the sensory epithelium and near the basilar membrane. A recent study reported that ionized calcium-binding adapter molecule 1 (Iba1)-positive resident macrophages were present in high numbers in the spiral ligament, spiral ganglion, and stria vascula ris of the postnatal cochlea in mice. Moreover, another study using mice with macrophages expressing green fluorescent protein (GFP) showed that very few macrophages (0–4/100 μm) were present in sensory epithelium, and that selective HC lesions increased the number of macrophages, which might have migrated from under the basilar membrane. This suggests that macrophages migrate from outside the sensory epithelium in response to HC damage. In addition, macrophages accumulated around the nerve fibres at the habenula perforata, where nerves enter and exit the organ of Corti, suggesting macrophages might enter from the habenular opening. In the stria vascularis, which supplies oxygen to the HCs, vascular endothelial cells, pericytes and perivascular-resident macrophage-like melanocytes (PVM/Ms) form a blood-labyrinthine barrier. For PVM/Ms, recent findings suggested that perivascular macrophages require more careful analysis to have melanocyte characteristics, but these cells are critical regulatory cells in the fluid-blood barrier and may form the first line of immunological defence. These cells were reported to inhibit pathogen entry via the stria vascularis by regulating the fluid-blood barrier, similar to what occurs in the CNS, and to function as macrophages that protect against pathogens. A recent study using bone marrow chimeric mice showed that macrophages are present in the spiral ligament and spiral ganglion, but not in the sensory epithelium. This suggests that there is a mechanism to prevent infectious agents such as viruses entering the organ of Corti. However, the defence mechanism around HCs when pathogens invade beyond these lines of defence awaits elucidation.

One of the earliest innate antiviral defence mechanisms is the type I interferon (IFN) system. We previously found that Theilier's murine encephalomyelitis virus (TMEV) infection in murine newborn cochlear sensory epithelium results in IFN-α/β production. TMEV is a small RNA picornavirus commonly used as an experimental model system for blood–brain barrier disruption. In the present study, we isolated murine newborn cochlear sensory epithelium and investigated the effects of viral infection on epithelial cells, because these cells can only be isolated from the relatively soft newborn temporal bone. Using this experimental system, we sought to identify whether a protection system against infection in the sensory epithelium of the organ of Corti exists.

Results

IFN-α/β produced by SCs and GERCs protects HCs against virus infection. As shown in Fig. 1a, the IFN (alpha and beta) receptor 1 (Ifnar1) and IFN (alpha and beta) receptor 2 (Ifnar2) subunits of IFN-α/β receptors were expressed in OHCs and IHCs. Infections with TMEV were mainly established in SCs, and infections in HCs were rarely observed from 9 h post-infection (Fig. 1b, left). Conversely, virus-infected HC numbers were seen to increase significantly in the Ifnar1-null sensory epithelium (Fig. 1b, right), demonstrating that the type I IFN system was present and functional in the studied sensory epithelium. The isolated sensory epithelium contained only HCs, SCs and GERCs, with no lateral wall containing stria vascularis, pericytes and PVM/Ms present. Interestingly, expression of IFN-α/β and macrophage-associated marker mRNA was induced, suggesting the existence of macrophage-like cells in epithelial tissue (Fig. 1c). The macrophage markers F4/80, macrophage-1 antigen (Mac-1), Iba1 and IFN regulatory factor 5 (Ir5) from M1 macrophages, and jumonji domain containing-3 (Jmjd3) and others from M2 macrophages, were also induced by the viral infection (Fig. 1d,e).

Virus-infected SCs and GERCs become detached from their cell layers. To identify which cells express macrophage-associated genes, we analysed the virus-infected sensory epithelium. The virus-infected SCs and GERCs became detached from their cell layers (Supplementary Videos 1 and 2). At 9 h post-infection with TMEV, several SCs left their cell layer and entered the HC layer (Fig. 2a; TMEV 9 h). Subsequently, the virus-infected GERCs also detached from their cell layer (Fig. 2a; TMEV 16 h and 24 h). The virus-infected sensory epithelia were then analysed by electron microscopy (Fig. 2b,c and Fig. S1a–c). Similar SC migration was observed following infection with HSV-1, a major cause of virus-induced sudden sensorineural hearing loss.

Viruses infection and accumulation in high numbers in the spiral ligament, spiral ganglion, and stria vascularis of the postnatal cochlea in mice. A recent study reported that ionized calcium-binding adapter molecule 1 (Iba1)-positive resident macrophages were present in high numbers in the spiral ligament, spiral ganglion, and stria vascularis of the postnatal cochlea in mice. Moreover, another study using mice with macrophages expressing green fluorescent protein (GFP) showed that very few macrophages (0–4/100 μm) were present in sensory epithelium, and that selective HC lesions increased the number of macrophages, which might have migrated from under the basilar membrane. This suggests that macrophages migrate from outside the sensory epithelium in response to HC damage. In addition, macrophages accumulated around the nerve fibres at the habenula perforata, where nerves enter and exit the organ of Corti, suggesting macrophages might enter from the habenular opening. In the stria vascularis, which supplies oxygen to the HCs, vascular endothelial cells, pericytes and perivascular-resident macrophage-like melanocytes (PVM/Ms) form a blood-labyrinthine barrier. For PVM/Ms, recent findings suggested that perivascular macrophages require more careful analysis to have melanocyte characteristics, but these cells are critical regulatory cells in the fluid-blood barrier and may form the first line of immunological defence. These cells were reported to inhibit pathogen entry via the stria vascularis by regulating the fluid-blood barrier, similar to what occurs in the CNS, and to function as macrophages that protect against pathogens. A recent study using bone marrow chimeric mice showed that macrophages are present in the spiral ligament and spiral ganglion, but not in the sensory epithelium. This suggests that there is a mechanism to prevent infectious agents such as viruses entering the organ of Corti. However, the defence mechanism around HCs when pathogens invade beyond these lines of defence awaits elucidation.

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Virus-infected SCs and GERCs each show an activated macrophage phenotype. In the absence of the TMEV infection, SCs, but not GERCs, expressed low levels of F4/80, but upon infection these levels were significantly enhanced or induced the expression of F4/80 in the SCs or GERCs, respectively (Fig. 3a). In response to the virus infection, enhanced expression of Ir5 and Jmjd3 was seen in the SCs (Fig. 3b,c), indicative of both proinflammatory M1 and anti-inflammatory M2 macrophage types. Conversely, the GERCs expressed Jmjd3, but not Ir5, during the virus infection (Fig. 3d), indicative of an anti-inflammatory M2 macrophage phenotype. Moreover, the expression of other macrophage markers was induced by the virus infection (Fig. S2), and both virus-infected SCs and GERCs alike exhibited phagocytosis (Fig. 3e), a hallmark of functional macrophages.
This suggests that SCs and GERCs function similarly to the tissue-resident macrophages previously reported to regulate tissue homeostasis by acting as sentinels responding to physiological changes and pathogenic microorganisms. Conversely, the SCs and GERCs were anchored by tight junctions in the absence of the TMEV infection. Similar to the inner ear, the blood-brain barrier prevents access of the peripheral immune system to the CNS, where microglial cells (resident macrophages), which arise from yolk sac macrophages during foetal development, regulate brain development, maintenance of neuronal networks, and injury repair. Moreover, microglia function as macrophages by eliminating dead cells, microbes and other pathogens that may damage the CNS.

**The sensory epithelium’s immune system contains macrophage-like cells.** Under steady-state conditions, microglial cell receptors, including CD200 receptor 1 (Cd200r1), interact with neural cell-surface ligands, resulting in the inhibition of microglial cell activity. As shown in Fig. 4a, Cd200r1 was expressed in SCs and GERCs, and its ligand, cluster of differentiation 200 (Cd200), was mainly expressed in HCs, as well as in SCs and GERCs, suggesting that SCs and GERCs are inhibited by HCs and by each other. In response to virus infection, Cd200r1 expression was suppressed (Fig. 4a, lower right), suggesting the CD200-Cd200R-mediated suppression of macrophage-like function was inhibited by virus-infection similar to the microglia system. SCs and GERCs differ from the typical microglia that patrol the brain microenvironment in that SCs and GERCs tightly adhere to each other and to HCs. SCs are thought to primarily separate the endolymph and perilymph via...
the tight junction architecture, thereby maintaining the integrity of the sensory epithelium against mechanical stress from the vibrations transmitted by sound waves. As potential macrophage-like cells, this architecture may suppress the shape and function of the SCs and GERCs (Fig. S1a). However, upon virus infection, the SCs and GERCs altered their shapes to become more macrophage-like and were able to migrate (Fig. S1b,c). To conduct a faint sound wave, only a minimal structure is required in the sensory epithelium. Therefore, SCs and GERCs might play diverse roles in sound conduction, homeostasis and immunity. In the present study, we analysed viral infection in cochlear sensory epithelia isolated from newborn mice. In the SHIELD (Shared Harvard Inner-ear Laboratory Database) database, we found that genes associated with macrophages and inflammation that target pathogens were expressed in the SC fractions during development (embryonic day E16, postnatal days P0, P4 and P7; Fig. S3), suggesting that macrophage-like SCs also exist in adult mouse cochleae.

Interestingly, we identified a row of cells that expressed the common microglial marker Iba1 under Deiters’ cells (DCs) and pillar cells (PCs) (Fig. 4b). However, these Iba1+ cells disappeared after viral infection (Fig. 4c), suggesting that they migrate in response to infection and function as typical microglial cells.

Figure 2. SCs and GERCs embedded and fixed in the cochlear sensory epithelium start to migrate to HCs upon virus infection. (a–c) Immunostaining (whole mounts and sections; a) and electron microscope (b,c) analysis of SCs/GERCs [white (a) and black (c) arrowheads] during TMEV infection. Red arrowheads in (b) indicate stereocilia survival in the HCs. Under steady-state conditions, all cells in the sensory epithelium were fixed and arranged as cobblestones. At 9–12 h after TMEV incubation, virus-infected SCs migrated to the epithelial surface and to HCs. At 16–21 h after TMEV infection, virus-infected GERCs also moved to the epithelium and migrated to the HC layer. At 24 h after TMEV infection, most of the HC layer was covered by TMEV-infected SCs/GERCs. (d,e) Immunostaining of SCs during GFP-HSV-1 infection. GFP-HSV-1-infected SCs moved to the epithelial surface (d) and expressed F4/80 (e), as observed during RNA virus infection. Immunostaining and TEM scale bars, 20 µm. SEM scale bars, 5 µm.
Figure 3. Macrophage marker upregulation in SCs and GERCs during TMEV infection. (a) Under steady-state conditions, F4/80 (green) was weakly expressed in SCs, but not in GERCs. Conversely, the SCs and GERCs migrating to the HCs during the virus infection exhibited strong F4/80 expression (green), indicating the virus-induced activation of SCs and GERCs as macrophages. (b–d) Under steady-state conditions, both Irf5 (green) (M1 marker) and Jmjd3 (green) (M2 marker) were expressed in SCs, but not in GERCs (b). Virus incubation increased the expression of Irf5 (green) and Jmjd3 (green) in the SCs, which was confirmed by double-positive (Jmjd3, green; Irf5, red) expression in SCs (c). The GERCs expressed Jmjd3 (green), but not Irf5 (green) during the virus infection (d). These findings indicate that virus-infected SCs exhibit both M1 and M2 characteristics, whereas virus-infected GERCs exhibit only M2 characteristics. (e) Virus-activated SCs and GERCs phagocytosed *Saccharomyces cerevisiae* conjugated with Alexa Fluor 488 (white arrowheads), demonstrating the macrophage-like ability of these cell types. Scale bars, 20 µm.
Scs and GERCs phagocytose bacteria without cell migration. To gain further insight into the mechanisms involved in the macrophage activation of SCs, we stimulated SCs with various pathogen-associated molecules known to activate macrophages. The induction of macrophage-associated markers and proinflammatory cytokines was observed following stimulation with lipopolysaccharide (LPS) and double-stranded (ds)RNA, both of which are recognised by Toll-like receptors (TLRs) (Fig. 5a, b and Fig. S4a–d). Moreover, the LPS-induced phagocytosis of SCs was observed (Fig. 5c), suggesting a protective role for SCs against pathogens other than viruses. Indeed, the SCs were able to phagocytose *Escherichia coli* in the absence of cell migration (Fig. 5d, e). Although *Cdn200* expression was suppressed by LPS and dsRNA stimuli (Fig. S4e, f), the SCs and GERCs did not migrate (Fig. S4g, h). In fact, our cDNA microarray analysis revealed the suppression of many cell adhesion proteins following virus infection, but not after LPS stimulation (Fig. S4i).

**Role played by Irf5 in changing SCs into macrophage-like cells.** In the sensory epithelium of *Irf5*−/− mice where M1 macrophage polarization is suppressed, induction of the genes encoding F4/80, Mac-1 and *Iba1* macrophage markers, suppression of *Cdn200* (Fig. 6b), and migration of the SCs in response to virus infection (Fig. 6a, c) were all impaired. This indicates that virus-induced macrophage activation and SC migration are partly regulated by Irf5 (Fig. 6d). Conversely, the virus-induced migration of GERCs was still observed in the *Irf5*−/− sensory epithelium (Fig. 6a, c). Moreover, *Irf5* knockout accelerated TMEV infection in cochlear sensory epithelia, suggesting that Irf5-mediated macrophage polarization is required for efficient protection against virus-infection (Fig. 6e–g). These results indicate that SCs and GERCs are distinct types of macrophage-like cells, and that different signalling mechanisms are required for macrophage activation and the migration of these cells.

**Discussion**

The present study provides initial evidence supporting the macrophage-like nature of cochlear SCs and their potential importance in anti-viral and anti-bacterial defences in the inner ear. Many tissue-resident macrophages, especially microglia, arise from the embryonic precursors that take residence prior to birth and maintain a local position throughout adulthood. It has been shown that the third DC row and inner PCs adjacent to HCs (Fig. 4b, c) act as HC progenitors following injury, suggesting that their origin is the same as that of HCs. Like HCs, SCs are also considered to be ectodermal cells, but our results suggest the possibility that some SCs, including Hensen’s cells and Claudius’ cells, have a mesodermal cell origin. Indeed, virus- or LPS-stimulated SCs displayed characteristics typical of macrophages, as determined by our analysis of macrophage marker protein expression, transcriptional profiles, and phagocyte activity and morphology, suggesting that SCs are tissue-resident macrophages.
Figure 5. Phagocytosis of SCs and GERCs without migration. (a) qRT-PCR analysis of macrophage markers (F4/80, Mac-1, and Iba1) and M1 markers (Il1b, Il6, and interleukin 12b (Il12b)) after LPS treatment (time point, 9 h; *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, t-test; 0 ng/ml: n = 4, 100 ng/ml: n = 4, 0 ng/ml: n = 6, 1000 ng/ml: n = 4 for Il12b; 0 ng/ml: n = 4, 0 ng/ml: n = 4, 1000 ng/ml: n = 4 for the other genes). LPS-induced macrophage marker expression indicates that LPS stimulates SCs and GERCs as cochlea-resident macrophages. (b) qRT-PCR analysis of macrophage markers (F4/80, Mac-1, and Iba1) and M1 markers (Il1b, Il6, and Il12b) after poly I:C treatment (time point, 9 h; *P < 0.05, **P < 0.01, t-test; 0 µg/ml: n = 4, 20 µg/ml: n = 4, 0 µg/ml: n = 5, 200 µg/ml: n = 4 for F4/80; 0 µg/ml: n = 4, 20 µg/ml: n = 4, 0 µg/ml: n = 3, 200 µg/ml: n = 4 for the other genes). Poly I:C upregulated macrophage marker expression, indicating that poly I:C also activates SCs and GERCs as cochlea-resident macrophages. (c) SC phagocytosis was observed during LPS treatment. (d) Sets of immunostained whole mounts and sections showing EGFP-E. coli in the SCs (white arrowheads) during EGFP-E. coli infection. The lower right image of SCs also shows green signals in these.
cells, indicating SC phagocytosis of the bacteria. (e) The confirmation of SC phagocytosis of EGFP-E. coli using cryosections from the cochlear sensory epithelium after EGFP-E. coli infection. SC cryosections after EGFP-E. coli infection showing EGFP signals inside (arrowheads) and outside (arrows) the SCs. EGFP signals inside the SCs indicate phagocytosis of E. coli by the SCs, while EGFP signals outside the SCs indicate E. coli attachment to the SC surfaces. These findings show that SCs undergo phagocytosis during viral and bacterial infections, which strongly supports a role for SCs in mounting an innate immune response against microbes as macrophages. Scale bars, 20 µm. Error bars, standard errors.

Moreover, under our experimental conditions, isolated sensory epithelium contained rows of Hensen’s cells and 4–6 rows of Claudius’ cells, both of which were infected with the virus and migrated with increased expressions of macrophage markers including F4/80, Irf5, and Jmd3. This suggested that Hensen’s cells and Claudius’ cells function as macrophages. A notable difference between SCs and other resident macrophages, especially microglia, is that uninfected SCs show no typical features of macrophages, particularly in terms of their morphology, and are anchored by tight junctions in their resting state. However, it has been shown that tight junction proteins are expressed in lung epithelium macrophages41.

Here, we analysed viral infections in isolated cochlear sensory epithelium from the soft temporal bones of newborn mice, because it is difficult to obtain viable organs of Corti that have maintained their tissue structures from the temporal bones of adult mice. Therefore, our analyses have some limitations. During development, cochlear extension and coiling continue until approximately E19 or P0, by which time the duct has reached its mature shape. At the tissue level, cellular differentiation within the prosensory domain is observable in the mid-basal region of the cochlea between E14 and E15. Developing OHCs are observable by E15 to E16, and developing SCs become morphologically distinct around the same time. By E17, HCs and SCs along the length of the cochlear duct have become arranged into the characteristic pattern for the organ of Corti7,14. Although full cochlear function takes some time to complete after birth, it is considered that the cellular differentiation and organization of the organ of Corti is completed before birth. This is supported by our findings that show that the genes associated with macrophages and inflammation were expressed in the cochlear SC fractions after birth (P0, P4 and P7; Fig. S3). Therefore, although the neonatal organ of Corti is in a different functional state when compared with the adult mouse, our experimental results were at least obtained from tissues with the same cell composition as the adult mouse. Therefore, in the same way that microglial cells arise from embryonic precursors, it is conceivable that embryonic-stage macrophage-like cells play a role in infection prevention in the adult mouse.

Unlike viral infections, in bacterial infections, the expression of cell adhesion molecules in HCs does not change and bacterial phagocytosis occurs. This phenomenon seems to be a beneficial system to eliminate bacteria while causing minimal tissue damage. However, our current results only showed the phagocytic activity of SCs. Further analyses are required to elucidate the protective role of SCs against bacterial infection, using clinically known bacteria that cause inner ear infection. In addition, we showed that microglia-like Iba1-positive cells were present under the HC layer. These cells disappeared after viral infection, but we have no direct evidence that they migrated to the site of viral infection, at present. Moreover, it is still unclear whether these cells exist in adult mice. However, very few macrophages are present in the sensory epithelium13. Therefore, if an infection occurs near HCs, these pre-existing macrophages might traffic to the infected area to protect HCs from the pathogen in cooperation with the SCs. These double-defence mechanisms may protect audiosensory organs from viruses and other pathogens.

Methods

Experimental animals. Postnatal day 2 (sex: unknown) ICR mice (SLC), Ifnar1 null mice (B&K Universal), and Irf5 null mice16 were used in this study. All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. The Animal Research Committee of Nippon Medical School approved all experimental protocols.

Preparation and treatment of cochlear sensory epithelium explant cultures. Cochlear sensory epithelia were resected and cultured as previously described24. All experiments began after an overnight incubation of each cochlea with 300 µl medium to stabilize the explants. Each cochlea was then transferred to 400 µl
Figure 6. Irf5 is involved in differentiation of the SCs into macrophages via Cd200r1 downregulation during virus infection. (a) In Irf5 KO mice, GERCs, but not SCs, migrated during the viral infection. (b) qRT-PCR analysis of F4/80, Mac-1, Iba1, Cd200, and Cd200r1 (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, t-test, Irf5 KO Mock 9 h: n = 4, Irf5 KO TMEV 9 h: n = 5, Irf5 KO Mock 16 h: n = 4, Irf5 KO TMEV 16 h: n = 4). Compared with WT mice during the virus infections, F4/80 transcripts were fewer in the Irf5 KO mice, Cd200r1 transcripts were more in the Irf5 KO mice, whereas Cd200 transcripts in Irf5 KO mice induced by the virus infection were similar to the levels in the WT mice. These findings show that Irf5 activates macrophages by regulating F4/80 expression through Cd200r1 inhibition. Mac-1 expression in Irf5 KO mice did not increase during virus infection compared with WT mice, but Iba1 expression in Irf5 KO mice was upregulated by virus infection to almost the same level as in the WT mice. This indicates that Iba1-positive cells are not regulated by Irf5. (c) F4/80 expression in Irf5 KO mice increased in the GERCs but not in the SCs following virus infection, indicating that Irf5 regulates SC but not GERC differentiation into macrophages. (d) Scheme showing the intra-
and inter-cellular signalling pathways involved in SC differentiation into macrophages. (e) Compared with WT samples where the SC area is mainly infected with TMEV (dsRNA positive), Irf5 KO samples show strong signal intensity of dsRNA over a broad area including the GER during virus infection. (f) There is no statistical difference in numbers of cells between WT (n = 3) and Irf5 KO (n = 3) shown in (e) (P = 0.10412149, t-test).

Cell counts. The numbers of dsRNA and Myo7a double-positive inner and outer HCs in the sensory epithelium were counted in one high-power field (318 × 318 µm) in the basal- to mid-turn of each explant. As described
above, we estimated from the basal- to mid-turn of the cochlea, because Hensen’s cells and Claudius’ cells in the apical turn overlapped with the inner side of the basal-turn.

**Electron microscopy.** Electron microscopy analysis was performed as described previously\(^{50,51}\), with slight modification. Mouse inner ears were observed by transmission and scanning electron microscopy (SEM). The specimens were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 60 min, washed five times in 0.1 M phosphate buffer, and post-fixed with 1% osmium tetroxide for 60 min at 4 °C. For transmission electron microscopy (TEM), the fixed inner ears were dehydrated with a graded ethanol series, and embedded in EponA2 according to the conventional method. Thin sections were cut with a diamond knife, stained with uranyl acetate and lead citrate, and examined with a JEM-1010 transmission electron microscope (JEOL) at an accelerating voltage of 80 kV. For SEM, the fixed inner ears were dehydrated with a graded ethanol series and then freeze-dried (Hitachi ES-2020, Hitachi) in t-butyl alcohol. After coating with osmium tetroxide (approximately 10 nm thick) using an osmium plasma coater (NL-OPC80, Nippon Laser & Electronics Lab.), the specimens were examined with a Hitachi S-4500 field emission SEM at an accelerating voltage of 10 kV.

**RNA extraction, qRT-PCR, and microarray.** For qRT-PCR, the RNeasy Micro Kit (Qiagen) was used to extract total RNA from three cochlear sensory epithelia, which were cultivated under the following conditions for 9 or 16 h: DMEM alone; DMEM with TMEV; DMEM with LPS; and DMEM with poly I:C, respectively. cDNA was synthesized from DNase-treated total RNA using the PrimeScript RT reagent Kit (Takara Bio). Synthesized cDNA was subsequently mixed with TaqMan Universal PCR Master Mix (Applied Biosystems) in the presence of commercial TaqMan primer-probe sets of interest (Applied Biosystems). Real-time PCR quantification was performed using the ABI StepOnePlus Real-Time PCR System (Applied Biosystems). All reactions were performed in triplicate. The relative mRNA amounts were calculated using the \( \Delta \Delta Ct \) method. For the invariant control, we used *actin beta* (*Actb*). For microarray, total RNA was extracted from nine mock explants, nine explants treated with 1000 ng/ml LPS for 16 h, or 14 explants infected with TMEV for 16 h using the RNeasy Micro Kit. TMEV infection for 16 h induced cochlear cell death in HCs and SCs; therefore, a greater number of TMEV-infected cochleae was needed compared with the mock and LPS-treated cochleae to equalize the amount of total RNA. Filgen (a biological technical service company) performed the microarray analysis using the GeneChip Mouse Gene 2.0 ST Array (Affymetrix).

**Live imaging.** TMEV-infected cochleae were cultivated on a collagen-coated 35-mm glass-bottom dish (MatTek) for live imaging. For visualizing activated SCs and GERCs as macrophages, a Leica SP8 confocal laser-scanning microscope equipped with a HC PL APO 63×/1.40 CS2 oil objective was used. Images were captured every 5 min, from 8 to 25 h after TMEV infection, using a 488-nm argon laser with 10% intensity. While capturing images, the explant was maintained in a CO\(_2\) (5%) stage-top incubator (INUBGF-GS12, Tokai Hit) at 37°C. The movies were constructed using Leica LAS AF software (9–14 h and 14–21 h, respectively).

**Statistical analysis.** The data are expressed as the mean ± standard error. Unpaired *t*-tests were used for *Myo7a* and dsRNA double-positive HCs, in which *P*-values < 0.05 were considered statistically significant. Unpaired *t*-tests were used for cell numbers or sum of dsRNA signal intensity in WT and *Irf5* KO cochleae infected with TMEV for 9 h, in which *P*-values < 0.05 were considered statistically significant. In all qRT-PCR analyses, unpaired *t*-tests were used, in which *P*-values < 0.05 were considered statistically significant.

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Author contributions
Y.H. and N.T. designed the experiments. Y.H. performed most experiments. W.N., I.U. and A.T. supported the experiments. H.S. and Y.H. performed electron microscopy. T.K., Y.H. and S-I.K. performed live imaging. N.K., Y.H. and Y.K. performed the GFP-HSV-1-infection assay. T.H., S.K., K.O., H.K., M.Y. and T.F. prepared TMEV. K.O., H.K., M.Y. and T.F. provided valuable advice. Y.H. and N.T. wrote the manuscript.

Competing interests
The authors declare no competing interests.

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