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Vikrant Borse  
*Washington University School of Medicine in St. Louis*

Tejbeer Kaur  
*Creighton University*

Ashley Hinton  
*Washington University School of Medicine in St. Louis*

Kevin Ohlemiller  
*Washington University School of Medicine in St. Louis*

Mark E Warchol  
*Washington University School of Medicine in St. Louis*

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Programmed Cell Death Recruits Macrophages Into the Developing Mouse Cochlea

Vikrant Borse¹, Tejbeer Kaur², Ashley Hinton³, Kevin Ohlemiller⁴ and Mark E. Warchol¹*

¹Department of Otolaryngology, Washington University School of Medicine, St. Louis, MO, United States, ²Department of Biomedical Sciences, School of Medicine, Creighton University, Omaha, NE, United States

Programmed cell death (PCD) plays a critical role in the development and maturation of the cochlea. Significant remodeling occurs among cells of the greater epithelial ridge (GER) of Kölliker’s organ, leading to tissue regression and formation of the inner sulcus. In mice, this event normally occurs between postnatal days 5–15 (P5-15) and is regulated by thyroid hormone (T3). During this developmental time period, the cochlea also contains a large population of macrophages. Macrophages are frequently involved in the phagocytic clearance of dead cells, both during development and after injury, but the role of macrophages in the developing cochlea is unknown. This study examined the link between developmental cell death in the GER and the recruitment of macrophages into this region. Cell death in the basal GER begins at P5 and enhanced numbers of macrophages were observed at P7. This pattern of macrophage recruitment was unchanged in mice that were genetically deficient for CX3CR1, the receptor for fractalkine (a known macrophage chemoattractant). We found that injection of T3 at P0 and P1 caused GER cell death to begin at P3, and this premature PCD was accompanied by earlier recruitment of macrophages. We further found that depletion of macrophages from the developing cochlea (using CX3CR1DTR/+ mice and treatment with the CSF1R antagonist BLZ945) had no effect on the pattern of GER regression. Together, these findings suggest that macrophages are recruited into the GER region after initiation of developmental PCD, but that they are not essential for GER regression during cochlear remodeling.

Keywords: cochlea, development, GER, cell death, macrophage, thyroid hormone

INTRODUCTION

The cochlea of the inner ear detects sound vibrations and transmits information to the auditory brainstem. Cochlear development is a complex process, and any defects in developmental patterning can lead to congenital hearing loss (Anniko 1983; Rueda et al., 1987; Kamilya et al., 2001; Korver et al., 2017). The developing cochlea also undergoes considerable cellular remodeling, in which certain cell populations die and are removed from surrounding tissues. In mice, such remodeling occurs during the first two postnatal weeks (Ruben 1967; Anniko 1983; Rueda et al., 1987; Sohmer and Freeman 1995; Kamilya et al., 2001; Dayaratne et al., 2014). Among the most critical remodeling events is the regression of a columnar epithelial structure known as the greater epithelial ridge (GER, also known as Kölliker’s organ). This event occurs via programmed cell death (PCD) (Ruben 1967; Anniko 1983; Rueda et al., 1987; Kamilya et al., 2001; Dayaratne et al., 2014), and creates a large...
cavity known as the inner spiral sulcus. This process is vital for the normal onset of hearing (Ruben 1967; Sohmer and Freeman 1995; Lukashkin et al., 2010; Dayaratne et al., 2014), and genetic deficits that prevent PCD result in profound hearing loss (Kuida et al., 1996; Morishita et al., 2001; Takahashi et al., 2001; Makishima et al., 2011). However, the signals that initiate PCD in the developing cochlea have not been completely identified.

Thyroid hormone signaling is critical for normal cochlear development (Kelley and Forrest 2001; Ng et al., 2013). Hypothyroidism and mutations in the thyroid hormone receptor β gene (THRB) have been linked to hearing loss (Kelley and Forrest 2001; Christ et al., 2004; Johnson et al., 2007; Mustapha et al., 2009; Sundaresan et al., 2016). Notably, thyroid hormone has been shown to initiate regression of the GER during cochlear development (Kelley and Forrest 2001; Peeters et al., 2015). Mutations in THRB lead to delayed GER remodeling (Kelley and Forrest 2001), while ectopic treatment with T3 (3,5-triiodo-L-thyronine) at P0 and P1 induces premature GER remodeling (Peeters et al., 2015).

Programmed cell death also requires the clearance of dying cells. In many developing tissues, the removal of cellular debris is mediated by macrophages, which recognize apoptotic cells via pattern recognition molecules and phagocytic receptors (Henson and Hume 2006; Erwig and Henson, 2008; Davies et al., 2013; Wynn et al., 2013; Varol et al., 2015; Gordon and Plüddemann 2018). Resident macrophages are present in the developing and mature cochlea (Hirose et al., 2005; Brown et al., 2017; Hirose et al., 2017; Warchol 2019), and deletion of macrophages results in hearing loss caused by excessive glial cells, abnormal myelin formation and edema in stria vascularis. Macrophage number transiently increases during cochlear development and declines as the cochlea matures (Brown et al., 2017; Dong et al., 2018), but the role of macrophages in cellular remodeling of the developing cochlea is not known. In this study, we examined the involvement of macrophages in the clearance of dying cells during GER remodeling. We found that macrophages were recruited into the GER after the initiation of cell death, and that those macrophages were engaged in the removal of cellular debris. In addition, we observed that early induction of PCD by ectopic injection of T3 hormone leads to earlier recruitment of macrophages in the GER region. Finally, we found that macrophage depletion did not affect GER regression in the developing mouse cochlea. Together, these findings suggest that developmental cell death leads to macrophage recruitment into the cochlea. Although such macrophages are actively involved in the clearance of cellular debris, they are not essential for GER regression.

MATERIALS AND METHODS

Animals: Studies used CX3CR1-GFP and CX3CR1-DTR mice (Diehl et al., 2013), of both sexes, on a C57BL/6 (B6) background. Samples were obtained at post-natal (P) day 1, P3, P5, P7, P10, P13, P21 and P30, in order to study the role of macrophages in cochlear development and function. Each experimental group consisted of 3-6 animals, taken from multiple litters. CX3CR1-GFP mice express enhanced green fluorescent protein (EGFP) under control of the endogenous CX3CR1 promoter (Jung et al., 2000). We generated CX3CR1<sup>ΔT/R+</sup> mice by breeding CX3CR1-null (CX3CR1<sup>GFP/GFP</sup>) mice with wild type C57BL/6 mice. Similarly, we generated CX3CR1<sup>ΔT/R</sup> mice by breeding CX3CR1-null (CX3CR1<sup>DTR/ΔT/R</sup>) mice with wild type C57BL/6 mice. All mice were housed in the animal facilities at Washington University, School of Medicine, and were maintained on a 12-h/24 h–day-night light cycle with open access to food and water. All experimental protocols involving animals were approved by the Animal Studies Committee of the Washington University School of Medicine, in Saint Louis, MO.

Treatment: At P0 and P1, mouse pups were subcutaneously injected with 1.5 μg of T3 (catalog# T6397, Sigma) (10 μL) or saline (control), in order to induce premature apoptosis and GER remodeling (Peeters et al., 2015). Saline injected animals were used as controls. Temporal bones from these mice were collected between P3 to P30. Also, some mice received subcutaneous injections of Diphtheria toxin (DT, Sigma), or the colony stimulating factor receptor 1 (CSF1R) inhibitor BLZ945 (MW: 398.48, MedChem Express HY-12768/CS-3971), to eliminate macrophages (Diehl et al., 2013; Milinkevičiute et al., 2019). In these studies, a single dose of 5 ng/gm DT was injected at P0, P2, P4, P6, P8, P10 and P12 in CX3CR1-DTR mice. Other mice received a single dose of 200 mg/kg BLZ945 at P2, P4, P6, P8, P10 and P12. Control mice received either saline or DMSO. Mice were euthanized and temporal bones were collected between P3 to P13.

Genotyping: Genotyping of CX3CR1-GFP mice (GFP heterozygous and GFP homozygous) and CX3CR1-DTR mice (DTR heterozygous) was performed by Transnetyx (Cordova, Memphis, TN), following previously described methods (Jung et al., 2000; Diehl et al., 2013).

Histological Methods

Animals were euthanized either by quick decapitation or deep anesthesia (Fatal Plus, 50 mg/kg). Temporal bones were isolated and fixed overnight with 4% paraformaldehyde (in PBS) at 4°C. After fixation, cochleae were isolated and washed 3x for 5 min each in PBS, pH 7.4 at room temperature. All specimens, except those collected at P5, were then incubated for ~24 h in 10% EDTA in PBS, pH 7.4 at room temperature. Specimens were thoroughly washed 3x for 5 min each in PBS, pH 7.4 at room temperature and were then dissected into whole mount samples. Other samples were prepared as frozen mid-modiolar sections (30 μm thickness). Those cochleae were incubated in 10% EDTA in PBS, pH 7.4 at room temperature for 3–5 days and then washed 3x for 5 min each in PBS, pH 7.4 at room temperature. Cochlear whole mounts and mid-modiolar sections were immunolabeled for GFP-expressing macrophages (rabbit anti-GFP antibody, catalog #A11122, Invitrogen, 1:500 or chicken anti-GFP antibody, catalog #1010, Aves, 1:500), neurons were labeled with mouse monoclonal anti-β-III tubulin (catalog #MMS-435P, Covance, 1:500), combined with mouse anti-Neurofilament (Catalog #2H3, Developmental Studies Hybridoma Bank, 1:100) antibodies, and apoptotic cells were labeled using rabbit anti-cleaved caspase-3 antibody.
Macrophages were identified by strong GFP and CD45 labeling. Pyknotic nuclei were identified by small size and intense DAPI nuclear labeling, and apoptosis was verified by immunolabeling for cleaved caspase-3. All macrophages, pyknotic nuclei and hair cells counts were obtained manually, within the designed area of complete z-stack using Fiji software.

**Statistical analysis:** All data analysis and statistical tests were carried out using GraphPad Prism version 6.0d. Data are presented as mean ± SD. Student’s t-tests or analyses of variance (ANOVA), followed by post hoc tests, were applied as appropriate. Results were considered statistically significant when p < 0.05.

**RESULTS**

**Recruitment of Macrophages Into the GER Region of the Developing Mouse Cochlea**

The mouse cochlea undergoes considerable structural changes during the first two postnatal weeks (Ruben 1967; Anniko 1983; Rueda et al., 1987; Kamila et al., 2001; Dayaratne et al., 2014). One critical event is the death of columnar epithelial cells of the GER, leading to the opening of the inner sulcus. This process progresses along the tonotopic gradient of the cochlea, starting at the base (high frequency region) and then moving towards the apex (low frequency region) (Ruben 1967; Anniko 1983; Rueda et al., 1987; Kamila et al., 2001; Dayaratne et al., 2014). Numerous macrophages are also present in the developing cochlea (Dong et al., 2018), but the role of macrophages in cellular remodeling is unknown. To resolve this issue, we first characterized the patterns of programmed cell death and the corresponding recruitment of macrophages in the GER of developing cochlea, using CX3CR1<sup>GFP/+</sup> mice, which express GFP in macrophages, microglia, monocytes and related cells (Diehl et al., 2013; Kaur et al., 2015a). Specimens were fixed at various developmental timepoints and prepared as whole mounts or mid-modiolar frozen sections. Immunolabeling was used to enhance GFP fluorescence in macrophages, and to identify apoptotic cells (cleaved caspase-3), and neurons (TUJ1+NF). DAPI staining facilitated the identification of pyknotic nuclei (a hallmark of apoptosis). Increased numbers of dying cells with pyknotic nuclei and/or immunolabeled for activated caspase-3 were observed in the basal GER region between P5-10, with the number peaking at P7 (Figures 1B–E,G). Over the next 2–3 days, the appearance of pyknotic nuclei progressed from the base toward the apex, and the number of pyknotic nuclei in the apical GER peaked at P10 (Figures 1D,G). Macrophages were also observed in the GER between P7-13. Large numbers of macrophages initially appeared in the basal GER and then spread toward the apex. The numbers of macrophages in the basal and apical GER regions peaked at P10 and P13, respectively (Figures 1B–E,H). The spatial distribution of macrophages followed a similar base-to-apex pattern that was observed for developmental cell death, with a temporal delay of ~3 days (Figure 1B’). By P13, very few apoptotic cells were present within the GER region (Figures 1E,E’), but macrophage numbers in the GER remained elevated after cell death had terminated (Figure 1H). We also observed cleaved-caspase 3-labeled (i.e., apoptotic) cells being engulfed by macrophages.
FIGURE 1 | Progression of apoptosis and macrophage recruitment in the developing cochleas of CX3CR1<sup>GFP+</sup> mice. (A) Schematic representation of the patterns of apoptosis and macrophage recruitment in the GER region. (B–E) Sensory epithelium of the developing cochlea. Apoptotic cells were first observed in the basal region at P5 [white arrow, (B')] and the apex at P7 [white arrow, (C)]. Cell death continued at later time points [white arrows, (C'), (D), (E'), (E'')] and was accompanied by the recruitment of macrophages [magenta arrows]. (F) A representative image of GFP-labeled macrophages engulfing pyknotic nuclei and activated caspase-3-labeled cells in the GER region at P10. (G) Number of pyknotic nuclei (a hallmark of apoptotic cells) in the GER region, as a function of developmental time. Cell death in the basal region peaked at P7, and in the apical region at P10. (H) Number of macrophages in the GER region, as a function of developmental time. The number of macrophages in both the basal and apical regions peaked at ~3 days after the time of maximal cell death. Images in (B–E') were taken using a ×20 objective lens and the image in (F) was taken using a ×63 objective lens. Labels: Red-cleaved caspase 3, Green-GFP + macrophages, Blue-DAPI. White arrows: cleaved caspase-3-labeled (apoptotic) cells, magenta arrows: GFP + macrophages. Abbreviations: OC: organ of Corti, GER: Greater Epithelial Ridge, *p: p < 0.05, relative to P1. (N = 3–6).
(Figure 1D). Similar patterns of cell death and macrophage recruitment were observed in mid-modiolar sections of the developing cochlea (Figures 2A–E). Macrophages in the neonatal cochlea possessed both ameboid and ramified morphologies, while those in the mature cochlea were predominantly ramified (Figures 2F, G). The neonatal cochlea also contained larger numbers of macrophages than were present in mature cochleae. Taken together, these observations indicate
that macrophages are recruited into the cochlea in response to developmental cell death and are actively involved in clearing dying cells from the GER region.

Fractalkine Signaling Is Not Required for Macrophage Recruitment Into the Developing Cochlea

Apoptotic cells secrete a number of signaling molecules that are chemoattractants for phagocytes (Diehl et al., 2013; Sokolowski et al., 2014; Hirose et al., 2017; Park and Kim, 2017). Examples of such ‘find-me’ signals include lysophosphatidylcholine (LPC), sphingosine-1-phosphate (SIP), nucleotides (ATP and UTP) and the CX₃C motif chemokine ligand 1 (CX3CL1, also called as fractalkine) (Park and Kim, 2017). CX3CL1 is the sole ligand for the CX3CR1 receptor, which is expressed by macrophages, microglia, monocytes and related cells. One function of fractalkine signaling is to mediate chemoattraction between dying cells and macrophages (Sokolowski et al., 2014; Park and Kim, 2017). To investigate the role of fractalkine signaling in macrophage recruitment and GER apoptosis, we compared macrophage numbers and cell death in the developing cochlea of CX3CR1GFP/+ mice (which possess one allele of CX3CR1 and retain sensitivity to fractalkine) and CX3CR1GFP/GFP mice (in which both copies of CX3CR1 had been replaced with the gene for GFP). Cochleae were collected from mice of both genotypes at P5, P7, P10 and P13 and processed as whole mounts. Samples were immunolabeled for GFP (to enhance the fluorescent signal in...
of macrophages. This outcome is consistent with a positive correlation between developmental cell death and macrophage recruitment.

Deletion of Macrophages Does Not Affect GER Remodeling

We investigated whether deletion of macrophages delays or impairs GER remodeling during cochlear development. We treated CX3CR1\textsuperscript{DTR/+} mice and wild type control mice with diphtheria toxin (Diehl et al., 2013) at P0, P2, P4, P6, P8, P10 and P12. Multiple doses of DT were delivered to prevent macrophage repopulation during the experimental period. Cochleae were fixed at P5, P7, P10 and P13 and processed as whole mounts (Figure 6A). Samples were immunolabeled for CD45 (to identify macrophages) and cleaved caspase-3 (to identify apoptotic cells). Cell nuclei were also stained with DAPI. As depicted in Figures 5B–D, DT treatment led to nearly-complete deletion of macrophages from the developing cochlea of CX3CR1\textsuperscript{DTR/+} mice. Macrophage infiltration in the GER region of control animals was normal, but no macrophages were observed in the GER region of CX3CR1\textsuperscript{DTR/+} mice (Figures 5E–L). However, we observed normal patterns of apoptosis and GER regression in the developing cochlea of the CX3CR1\textsuperscript{DTR/+} and control mice (Figures 5E–L). Except in the apical region of P13 cochleae, we observed no significant increase in number of apoptotic cells in CX3CR1\textsuperscript{DTR/+} mice, in comparison with controls. These data suggest that depletion of macrophages may cause a slight delay in the clearance of cellular debris in the apex, but does not otherwise affect GER regression (Figures 5M,N). Interestingly, we also noted that the DT treatment leads to loss of OHCs (Figures 6A,B). We observed missing OHC nuclei and labeling for cleaved caspase-3 in both CX3CR1\textsuperscript{DTR/+} and WT control mice that were treated with DT at P10. No missing nuclei or apoptotic OHCs were observed in mice that did not receive DT.

In addition to the CX3CR1\textsuperscript{DTR/+} mouse model, we also used a pharmacological approach to eliminate macrophages from the developing cochlea. CX3CR1\textsuperscript{GFP/+} mice were given s.c. injections of the CSF1R antagonist BLZ945 (Milinkeviciute et al., 2019) at P2, P4, P6, P8, P10 and P12. (Multiple doses of BLZ945 were delivered, in order to avoid macrophage repopulation.) Cochleae were fixed at P5, P7, P10 and P13, and processed as whole mounts (Figure 7A). Treatment with BLZ945 eliminated nearly all macrophages from the developing cochlea (Figures 7B–D), including the GER region (Figures 7E–L). Notably, we observed normal patterns of apoptosis and GER regression in the developing cochlea of both the BLZ945-treated and control animals (Figure 7M). Macrophage depletion led to no significant difference in the numbers of pyknotic nuclei in the GER, when compared to controls (Figures 7M,N). Collectively, these findings suggest that macrophage depletion has no substantial effect on GER remodeling during development.

DISCUSSION

This study was undertaken to examine the role of macrophages in cochlear development. We found that the spatial and temporal...
patterns of macrophage recruitment into the GER region of the developing cochlea is correlated with the pattern of programmed cell death in that region, i.e. starting from high frequency basal region and then moving towards low frequency apical region. We first observed apoptosis and macrophage recruitment in the GER region at P5 and P7, respectively (Figure 1). In previous studies of cochlear development, apoptosis was first reported to occur at P7 (Kamiya et al., 2001; Peeters et al., 2015). This difference in timing may be due to differences in technique and mouse strain. We also observed macrophages engulfing apoptotic cells and pyknotic nuclei.
nuclei in the GER region and some macrophages that remained in the GER region after the wave of apoptosis had terminated. Our findings demonstrate that macrophages are recruited into the GER region about 2 days after apoptosis has begun. During development, the resident macrophages of the cochlea originate from precursors in the yolk sac and fetal liver, and reach the inner ear via systemic circulation (Kishimoto et al., 2019). The specific source of macrophages recruited into the GER region during cochlear remodeling is not known, but they may originate from circulation, or from other regions of the developing ear and/or surrounding mesenchymal tissue. We also observed two morphologically-distinct populations of macrophages in the developing cochlea (ameboid and ramified), but only ramified macrophages were present in the mature cochlea. The significance of these morphological types is not clear. Microglia (the resident macrophages of CNS) also possess similar morphologies. Microglia with a ramified morphology are thought to be in a ‘resting’ or ‘quiescent’ state, while ameboid microglia are associated with tissue injury (Savage et al., 2019). However, the possible differential roles of

**FIGURE 5** | Depletion of macrophages in the cochlea of neonatal CX3CR-DTR mice. (A) Treatment and sample collection timeline. (B–D′) Cochlear whole mounts showing depletion of macrophages (Green: CD45) following DT treatment. (E–L′) Sensory epithelium of developing cochlea showing occurrence of cell death (red, cleaved caspase-3, white arrows) in normal and DT-treated cochlea. Although DT treatment eliminated macrophages in the cochleae of CX3CR-DTR mice, normal patterns of cell death were observed. (M) Number of pyknotic nuclei (blue: DAPI) in the GER region of developing cochleae. (N) Quantification of macrophages in the GER region of developing cochleae. Treatment with DT led to a large reduction in macrophage numbers. Images in (B–D′) were taken using ×5 objective lens and images in (E–L′) were taken using ×20 objective lens. Abbreviations: OC: Organ of Corti, GER: Greater Epithelial Ridge (*p: p value < 0.05, relative to WT, ns: not significant. (N = 3–6).
ameboid vs. ramified macrophages during cochlear development are not understood.

Apoptotic cells release signaling molecules, known as ‘find-me’ signals, to promote the chemotaxis of macrophages to sites of cell death (Park and Kim 2017). A number of such signaling molecules have been identified. Fractalkine (CX3CL1) is a chemokine that can recruit macrophages to dying cells by activating the CX3CR1 receptor on macrophages (Sokolowski et al., 2014; Park and Kim 2017). Previously, our lab has shown that disruption of fractalkine (CX3CL1/CX3CR1) signaling leads to reduced macrophage recruitment into the sensory region and spiral ganglion of the cochlea after selective hair cell ablation (Kaur et al., 2015a). However, it was not known whether fractalkine also influences macrophage behavior during cochlear development. We investigated the role of fractalkine signaling by quantifying macrophage recruitment into the developing cochleae of CX3CR1-null (CX3CR1GFP/GFP) mice. We observed similar patterns of apoptosis and macrophage recruitment in both CX3CR1GFP/GFP and CX3CR1GFP/+ (control) mice (Figure 1 and Figure 3), suggesting that fractalkine signaling is not required for normal macrophage recruitment into the developing cochlea. This finding suggests that other ‘find-me’ signals, such as LPC, S1P and/or nucleotides (ATP and UTP) might recruit macrophages into the neonatal cochlea. It is notable that spontaneous release of ATP occurs during cochlear development (Dayaratne et al., 2014; Wang et al., 2015; Park and Kim 2017), and this may be one signal that recruits macrophages into the GER.

In order to determine the relationship between GER cell death and macrophage recruitment, we studied whether early induction of apoptosis by T3 hormone affected the recruitment of macrophages into the GER. Similar to Peeters et al. (2015), we observed robust early apoptosis in the GER region of T3 treated animals. In addition, we noted that induction of premature apoptosis by T3 injection led to early recruitment of macrophages. Treatment with T3 caused cell death in the GER.
to begin approximately 4–5 days earlier than in normal cochleae and, in accordance with earlier studies, led to profound hearing loss (Peeters et al., 2015). We further found that T3 treatment led to correspondingly earlier macrophage recruitment into the GER, but that the delay between the onset of cell death and the appearance of macrophages was ~2 days in both normal and T3-treated cochleae. These observations suggest a relationship between cell death and macrophage recruitment, whereby dying cells release a chemoattractant that causes macrophages to migrate into the GER within ~2 days. We acknowledge, however, that the present data do not prove that these two events are causally related.

A prior study reported that short-term depletion of macrophages during cochlear development leads to hearing impairment, which was attributed to the presence of excessive glial cells, abnormal myelin formation, and defects in the stria vascularis (Brown et al., 2017). The present study employed two methods for depletion of macrophages: treating CX3CR1<sup>DTR/+</sup> mice with DT (Diehl et al., 2013), and treating CX3CR1<sup>GFP/+</sup> mice with the CSF1R antagonist BLZ945 (Melinkeviute et al., 2019). Similar to earlier studies, we observed that multiple doses of DT and BLZ945 resulted in sustained near-complete depletion of macrophages from the developing cochlea (Diehl et al., 2013; Melinkeviute et al., 2019). We further found that eliminating macrophages did not disrupt the normal pattern of apoptosis in the GER region of the developing cochlea. These results suggest that the cells in the GER region may engage in self-clearance by autophagy and/or by phagocytosis (Monzack

**FIGURE 7** | BLZ945 treatment eliminates macrophages in the developing cochlea. (A) Treatment and sample collection timeline. (B–D) Cochlear whole mounts showing depletion of macrophages following treatment with the CSF1R antagonist BLZ945 (Green-GFP + macrophages). (E–L) Sensory epithelium of the developing cochlea showing apoptotic cells (red-cleaved caspase-3) and macrophages (green-GFP). Normal patterns of dying cells (white arrows) were observed in BLZ945-treated cochleae. (M) Number of macrophages in the GER region of the developing cochlea. Note that BLZ945 sharply reduced the number of macrophages in the GER. (N) Number of pyknotic nuclei in the GER region of the developing cochlea. Images in B–D were taken using a ×5 objective lens and images in E–L were taken using ×20 objective lens. Abbreviations: OC: Organ of Corti, GER: Greater Epithelial Ridge, P: Postnatal Day. (*p: p < 0.05, relative to controls (DMSO), ns: not significant. (N = 3–6).
et al., 2015; Hou et al., 2019). Similarly, in the mature ear, both macrophages and supporting cells have been shown to contribute to the phagocytic clearance of dead hair cells (Kaur et al., 2015b; Monzack et al., 2015). Recruited macrophages may assist GER cells in rapid clearance of dead cells, but they do not appear to be essential for GER regression during normal cochlear development.

In conclusion, the results of this study demonstrate that programmed cell death recruits macrophages in the GER of the developing cochlea and that macrophages engage in the phagocytic clearance of dead cells. However, elimination of macrophages does not affect the patterns of cell death and remodeling in the GER. Thus, although the developing cochlea contains a higher density of macrophages than are present in the mature cochlea, macrophages may not play an essential role in the development of the organ of Corti.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

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ETICS STATEMENT

All experimental protocols involving animals were reviewed and approved by the Animal Studies Committee of the Washington University School of Medicine, in Saint Louis, MO.

AUTHOR CONTRIBUTIONS

VB and MW wrote the main manuscript text, prepared figures, designed and performed experiments, TK, AH, and KO designed and performed experiments.

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