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The role of monocytes and macrophages in the dynamic permeability of the blood-perilymph barrier

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Abstract

The blood-perilymph barrier serves a critical role by separating the components of blood from inner ear fluids, limiting traffic of cells, proteins and other solutes into the labyrinth, and allowing gas (O₂-CO₂) exchange. Inflammation produces changes in the blood-perilymph barrier resulting in increased vascular permeability. It is commonly thought that compromise of the blood-inner ear barrier would lead to hearing impairment through loss of the endocochlear potential (EP). In fact, the effect of increasing cochlear vascular permeability on hearing function and EP is poorly understood. We used a novel method to measure the integrity of the blood-perilymph barrier and demonstrated the effects of barrier compromise on ABR threshold and EP. We also investigated the contribution of CX3CR1 cochlear macrophages and CCR2 inflammatory monocytes to barrier function after systemic exposure to lipopolysaccharide (LPS). We found that systemic LPS induced a profound change in vascular permeability, which correlated with minimal change in ABR threshold and EP. Macrophage depletion using CX3CR1-DTR mice did not alter the baseline permeability of cochlear vessels and resulted in preservation of barrier function in LPS-treated animals. We conclude that cochlear macrophages are not required to maintain the barrier in normal mice and activated macrophages are a critical factor in breakdown of the barrier after LPS. CCR2 null mice demonstrated that LPS induction of barrier leakiness occurs in the absence of CCR2 expression. Thus, enhanced aminoglycoside ototoxicity after LPS can be linked to the expression of CCR2 in inflammatory monocytes, and not to preservation of the blood-perilymph barrier in CCR2 knockout mice.

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Contributions

Keiko Hirose wrote the manuscript, designed the experiments, conducted the perilymph sampling and endocochlear potentials with assistance from the Salt Lab, reviewed the ABR data and viewed the histologic slides, performed the data analysis, consulted with colleagues on interpretation of the studies and statistical analysis, created the figures, and proofread the final version. Song Zhe Li generated the mice, performed the LPS and DT injections, performed the ABRs, produced the histology specimens and proofread the final manuscript. There are no conflicts to disclose.

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Keywords

Blood perilymph barrier; lipopolysaccharide; macrophage; CX3CR1; CCR2; monocyte; endocochlear potential

Introduction.

The blood-perilymph barrier allows the passage of oxygen, carbon dioxide and other small molecules between the blood and the inner ear while maintaining a separation between these two compartments for tight regulation of the ionic environment. The blood perilymph barrier (BPB), like the blood brain barrier (BBB), relies on the integrity of tight junctions between cells, the specialized endothelial cells that line these spaces, and other specialized cells that reinforce the separation between intravascular and interstitial spaces. It is well known that systemic inflammation alters vascular permeability, including those vessels in the inner ear and the brain. The experiments that we present here evaluate how inflammation and cochlear mononuclear phagocytes influence the BPB. The barrier between blood and inner ear has previously been referred to as the *blood-labyrinthine barrier*. In this paper, we choose to use the term: *blood-perilymph barrier* as this terminology is more specific to vessels that are surrounded by perilymph and are the subject of study in these experiments. We have previously shown that lipopolysaccharide (LPS) causes an increase in vascular permeability as measured by the percent of intravenous fluorescein that enters the inner ear fluids over a specified period of time (Hirose et al., 2014a). We have also shown that low dose LPS is permissive for entry of CCR2 inflammatory monocytes and CX3CR1 myeloid cells in the inner ear (Hirose et al., 2014b).

Monocytes and macrophages have been observed in association with the developing ear and as resident cells in the mature inner ears (e.g., Hirose et al., 2017). Resident macrophages have been demonstrated in hair cell epithelia in a variety of species such as zebrafish, chick, mouse, and human (Hirose et al., 2005; O'Malley et al., 2016; Warchol et al., 2012). Migratory monocytes and macrophages enter the labyrinth in experimental conditions where the inner ear has been damaged or subjected to inflammatory stimuli. Among these conditions are noise damage, ototoxic hair cell loss, programmed cell death by genetic manipulation (*pou4f3-DTR* mice) and low-level sepsis (Bohne, 1971; Dong et al., 2018; Hirose et al., 2017, 2014a, 2014a, 2005; Kaur et al., 2015; Ladrech et al., 2007; Liu et al., 2018; Warchol, 1997; Wood and Zuo, 2017).

Macrophages are part of a larger family of leukocytes characterized as mononuclear phagocytes. This includes monocytes, microglia, dendritic cells, and tissue macrophages such as those in the inner ear. Tissue macrophages are derived from two distinct lineages. Some originate from early migration of primitive yolk sac macrophages that enter the fetal circulation and populate the peripheral organ early in development (E9.5 in mice). Other tissue macrophages arise from fetal liver or from bone marrow precursors that develop postnatally (Ginhoux et al., 2010; Merad et al., 2002; Schulz et al., 2012; Gomez Perdiguero et al., 2015). Specialized mononuclear phagocytes that are derived from yolk sac precursors include microglia of the central nervous system, Kupffer cells which are macrophages of the

liver, and Langerhans cells of the skin. Other tissues are populated by macrophages that originate in the bone marrow. These macrophages tend to have a shorter half-life and are rapidly replaced, such as intestinal and peritoneal macrophages (Hoeffel et al., 2015; Schulz et al., 2012).

Mononuclear phagocytes can be divided into two distinct populations, based on differential expression of chemokine markers (Geissmann et al., 2003). The majority of CX3CR1 expressing cells are tissue macrophages and CNS microglia. Activated T cells and NK cells are also CX3CR1+ (Jung et al., 2000). The second category of mononuclear phagocytes are the CCR2-expressing monocytes, sometimes referred to as “inflammatory monocytes”. These cells migrate into peripheral tissues from the vasculature and do not typically reside in peripheral organs as resident cells. CCR2 cells can undergo transdifferentiation and serve as precursors to resident macrophages (Bain et al., 2014). CCR2 has been shown to play an important role in the release of monocytes from the bone marrow into circulation (Serbina and Pamer, 2006). Once in circulation, migration of inflammatory monocytes from the blood into peripheral tissues can occur in the absence of CCR2 expression, and we have confirmed this in studies of the inner ear after treatment with systemic LPS (Hirose et al., 2014b). CCR2 monocytes are not observed in the inner ear, even in the presence of damage caused by noise or aminoglycoside ototoxicity. CX3CR1+ cells are numerous while CCR2+ cells are not observed. In other forms of inner ear damage, such as bacterial meningitis, CCR2 inflammatory monocytes enter the inner ear in abundance. Important distinctions have been made between these monocyte/macrophage populations, not only in their surface markers, but also their effector function in immunity. Whether CX3CR1 or CCR2 mononuclear phagocytes are involved in altering vascular barrier properties is unknown. With the advent of a quantitative technique to measure vascular barrier function in the inner ear using perilymph sampling, we sought to evaluate the role of CCR2 and CX3CR1 in transgenic and knockout mice.

Materials and Methods

Animals.

CX3CR1-DTR mice were bred as heterozygotes from founder mice (gift from Dan Littmann, NYU). These mice express diphtheria toxin receptor controlled by the fractalkine receptor (CX3CR1) promoter such that systemic administration of diphtheria toxin induces elimination of cells expressing CX3CR1 (Buch et al., 2005). CCR2^{RFP/RFP} mice were maintained as a colony from founders that were a gift of Israel Charo, UCSF where CC chemokine receptor 2 (CCR2) was deleted and replaced by red fluorescent protein (RFP, Saederup et al., 2010). Heterozygous mice exhibit normal CCR2 function and express RFP in all CCR2 expressing cells. Homozygotes are CCR2 null and express RFP in all cells that would normally express CCR2. In order to create double reporter mice, CCR2^{RFP/RFP} were crossed with CX3CR1^{GFP/GFP} mice (additional gift from Dan Littmann, NYU) such that RFP served as a reporter for CCR2 and GFP a reporter for CX3CR1. Genotyping was performed on all animals using standard PCR with primers that identified the wild type sequence and the knock in sequence as separate bands, thus distinguishing heterozygotes

from homozygotes. All animal experiments were approved by the Institutional Animal Care and Use Committee at Washington University School of Medicine (IACUC).

Targeted elimination of CX3CR1+ cells.

CX3CR1-DTR mice were treated with diphtheria toxin (DT, List Biological Lab Inc., Campbell, CA) three times over seven days using a dose of 200ng/20g mouse. Preliminary work to evaluate the efficacy and timing of macrophage, monocyte, and microglia depletion was completed as follows: a single dose of 200ng/20gm mouse was given IP and bone marrow, spleen and cochlea (lateral wall and the ganglion) and brain were harvested after intracardiac perfusion under anesthesia. CX3CR1 expressing cells were markedly reduced in number and density in the bone marrow and in the spleen one day post injection. In our experiments with LPS and perilymph sampling, CX3CR1-DTR mice were treated for three doses prior to LPS (every other day) and a final injection given during the second day of LPS. Blood labyrinth barrier testing was performed 24 hours after the second dose of LPS and the fourth dose of DT.

LPS priming.

Mice assigned to the LPS group received 1.0mg/kg of lipopolysaccharide (Sigma-Aldrich, St. Louis, MO) in sterile saline via intraperitoneal injection for two consecutive days.

Perilymph sampling.

Mice were injected with 0.3 ml of sodium fluorescein (10mg/ml, Sigma, St. Louis, MO) intraperitoneally. Mice were anesthetized with ketamine (100 mg/kg) and xylazine IP (20 mg/kg), tracheostomized, and placed in a head holder in the right lateral decubitus position. The posterior semicircular canal was exposed, allowed to air dry, and cyanoacrylate glue was applied, followed by two-part silicone (Kwik-Cast, WPI, Sarasota, FL, see Salt et al. 2006; Salt et al. 2003). The bone overlying the membranous canal at the center of the silicone cup was curetted with a pick. As clear perilymph emerged, it was collected by capillary action into numbered and calibrated glass capillary tubes that were marked at 0.5 μ L. Five samples were collected sequentially, each sample requiring 15–30 seconds to accumulate (an efflux rate of $\sim$ 1–2 μ L/min). The first two samples that emerge from the opening in the posterior canal are representative of fluid from the vestibular system and the cochlea. The next three samples are comprising CSF that has entered the inner ear through the cochlea aqueduct (Hirose et al., 2014a; Mynatt et al., 2006). A separate sample of blood was collected by direct venipuncture. The amount of inner ear fluid or blood in each capillary tube was measured. Inner ear fluid and serum samples were diluted in 100 microliters of saline. The stock fluorescein solution was diluted using 1:10 serial dilutions and these were used as standards for quantitative spectrophotometry. Samples and standards were read on a Spectramax i3 spectrophotometer (Molecular Devices, Sunnyvale, CA). A fitted curve was used to convert fluorescence intensity units to fluorescein concentration for the experimental samples. Fluorescein concentrations of perilymph (from inner ear fluid samples) were calculated as a percentage of fluorescein concentration of the serum (derived from the blood sample).

In order to control for the absorption rate of fluorescein from the peritoneal cavity into circulation, we measured the kinetics of fluorescein uptake into the blood by sampling at 10 minute intervals after an IP injection of 0.3 ml of 10mg/ml fluorescein. We found that fluorescein reached peak serum concentration 30 minutes after injection and that this level was maintained for 3–4 hours.

Endocochlear potential.

The endocochlear potential (EP) was measured in anesthetized and tracheostomized mice. The head was stabilized in a custom head holder. An incision was made in the post-auricular skin, the bulla was opened, and the lateral surface of the cochlea was exposed. A hole was created with a pick in the left otic capsule lateral to the stria vascularis of the basal turn. Glass capillary electrodes (40–80 MΩ) filled with 0.15 M KCl were mounted on a micromanipulator and manually advanced until a stable positive potential was obtained that did not change with increased electrode depth. The signal from the glass pipette electrode was recorded on an intracellular amplifier. Endocochlear potentials were recorded in mice 24 hours and 7 days after the last LPS treatment.

Mouse ABR.

Auditory brainstem responses (ABR) were tested in all of the mice. Mice were anesthetized with intraperitoneal xylazine (20 mg/kg) and ketamine (100 mg/kg). ABRs were evoked through tone pips and recorded via subcutaneous electrodes placed behind the pinna, the vertex of the head and, the ground electrode placed on the back. Stimuli were 5-msec tone pips (0.5-msec rise-fall with a $\cos^2$ onset envelope, delivered at 40/second). The response was amplified (10,000x), filtered (100 Hz to 3 kHz), and averaged using custom computer software (System 3; Tucker-Davis Technologies). The sound level was raised in 5-dB steps from 15-dB SPL to 95-dB SPL. At each level, 1024 responses were averaged, with stimulus polarity alternated. Response waveforms were rejected if the peak-to-peak voltage exceeded 15 μ V. We tested responses at 5.6, 8, 11, 16, 22.63, 32, 45.2, and 64 kHz to obtain thresholds. Threshold was determined by a single observer, who noted the lowest sound level at which a recognizable waveform was present in a series of tracings stacked from lowest to highest sound pressure level. Waveforms were confirmed as auditory evoked responses by their increasing amplitude and decreasing latency with increasing intensity of the stimulus. If hearing threshold was not detected at 95 dB, a threshold value of 100 dB was assigned, which indicated a “no response” ABR in this system. Mice were tested at 1, 14, and 28 days after completion of LPS treatment. Differences in hearing thresholds between mouse groups were compared using non-parametric tests.

Cochlear histology.

Immediately after ABR testing, all animals were injected with a lethal dose of anesthetic agent and a thoracotomy was performed. Mice were perfused with 4.0% paraformaldehyde in 100 mM phosphate buffer through the left ventricle. Both petrous temporal bones were extracted and the round and oval windows opened to allow intralabyrinthine perfusion of fixative. After overnight fixation at 4°C, cochleae were decalcified in a saturated solution of EDTA in phosphate buffer for 5 days at 4 °C. Decalcified cochleae were immersed in cryoprotection solution (20% glycerol in phosphate buffer), frozen in 30% sucrose on dry

ice, and cut into 30 μ m serial sections from round window to oval window on a horizontal sliding microtome. Sections were washed in phosphate-buffered saline (PBS; pH 7.4), dried onto slides and cover-slipped.

Confocal imaging.

Images were acquired by confocal microscopy (Zeiss LSM700) using 20 and 40 x objective lenses and collecting images through z stacks of approximately 10 micron depth. Maximum-intensity projections were derived from each image stack.

Results.

LPS treatment changes the permeability of the blood perilymph barrier.

Both LPS-treated and control mice received fluorescein IP, and perilymph was sampled after 30 minutes. In healthy control mice, a trace amount of fluorescein from the vascular compartment entered the perilymph. The concentration of fluorescein in the perilymph was approximately 2% of the concentration in serum during the time period 30–60 minutes after administration of fluorescein (range: 1–8%, see Figure 1). We also observed that the concentration of fluorescein in the CSF was lower than the concentration of perilymph in all control mice and in nearly all experimental conditions. This relative difference in the fluorescein concentration of CSF compared to perilymph could be due to dilution factors such as a larger volume of CSF and a very small volume of perilymph (about 0.9 microliters/mouse inner ear), a lower density of vessels per volume of fluid, or lower permeability of vessels in the CSF leading to greater exclusion of fluorescein from this compartment.

In a separate group of mice, injections of 1 mg/kg LPS were administered at one day and two days prior to fluorescein injection. This treatment caused a significant increase in vessel permeability and transit of fluorescein into the perilymph: the fluorescein concentration in perilymph rose to an average of 12% of that observed in the serum (range: 8–60% of serum concentration). This outcome was anticipated, based on previously published data on the effect of LPS on vascular permeability. In addition, the gradient between fluorescein levels in the serum and fluorescein levels in perilymph remained: fluorescein was consistently higher in the serum and lower in the perilymph and CSF. Elevated concentrations of fluorescein after LPS were also observed in CSF, although the fluorescein content in perilymph remained higher than in CSF (samples 1 and 2 from the inner ear show higher fluorescein concentrations than samples 3, 4, 5 from the CSF).

Effects of macrophage/monocyte depletion: studies with CX3CR1-DTR.

How these macrophages contribute to the blood-perilymph barrier has been a source of controversy. To resolve this issue, we evaluated the effect of monocyte and macrophage depletion on the integrity of the blood-perilymph barrier in control and LPS treated animals. Experiments utilized CX3CR1-DTR mice, which permit the selective ablation of CX3CR1-expressing cells. These mice received diphtheria toxin for one week, and the reduction of CX3CR1 expressing monocytes, macrophages and microglia was confirmed by imaging of the spleen, brain, and inner ear (Fig. 2). The integrity of the blood-perilymph barrier was then assessed by measuring the levels of fluorescein in the perilymph 30 minutes after IP

fluorescein injection. Depletion in the cochlea was achieved by day 3 (D''), and significant reduction of microglia was noted in the brain by day 8 (E'''). Macrophages in the spleen and in the bone marrow returned 7 days after a single injection of diphtheria toxin. Microglia in the brain, which constitutively express CX3CR1, (Figure 2E) did not repopulate after depletion, which is consistent with other studies (Buch et al., 2005). Cochlear CX3CR1 macrophages were depleted more slowly and repopulated more slowly than CX3CR1 myeloid cells in the spleen in CX3CR1-DTR mice.

Elimination of macrophages in normal (non-LPS treated) mice had no apparent effect on barrier function (Figure 3). The average fluorescein entry into the inner ears of macrophage-depleted mice was 2%, which is comparable to that observed in mice with normal numbers of macrophages. In contrast, depletion of monocytes and macrophages had a marked protective effect on the integrity of the barrier following LPS treatment. Figure 3 demonstrates that LPS had modest effects on barrier function after CX3CR1 macrophages were eliminated. The perilymph of macrophage-depleted mice contained ~5% fluorescein concentration (with respect to serum concentration) versus 12% in LPS injected wild-type mice (compare Figures 1 and 3). The Kruskal-Wallis test was performed to assess differences between the four experimental groups and demonstrated that there was at least one group that was significantly different from the others. The Mann-Whitney U test was applied for post hoc comparison with an alpha level of 0.0083 using Bonferroni's correction. LPS-treated, wild type mice were different from the three other groups (Figure 1). Three other experiments groups (WT control, macrophage-depleted control, macrophage-depleted/LPS treated groups) showed baseline levels of fluorescein extravasation from blood into perilymph. Specifically, after macrophage depletion, LPS induced minimal fluorescein leakiness into the perilymph, and showed no significant difference from the control group (Figure 3). In addition, controls were performed using DTA and LPS in wild type mice, where macrophages were replete. These mice demonstrated increased barrier permeability that was consistent with the LPS effect in the absence of DTA in WT mice.

Effects of deleting the CC-chemokine receptor 2 on BPB integrity.

The CC chemokine receptor 2 (CCR2) is expressed by inflammatory monocytes, which mainly reside in circulation, but make transient, migratory excursions into peripheral tissues. CCR2-expressing monocytes are not observed in the inner ear in control animals, but do appear in the cochlea after systemic LPS exposure (Hirose et al., 2014b). Here, we investigated the effect of CCR2 deletion on the blood-perilymph barrier changes induced by LPS. Figure 4 demonstrates BPB function in CCR2 knockout mice. These mice have normal BPB function when compared to wild type controls (perilymph fluorescein= 1–2% of serum levels). After LPS, a similar breach in the barrier was detected in CCR2 null mice. Genetic deletion of CCR2 did not alter the effect of LPS on the BPB (Figure 4).

Endocochlear potential is minimally affected by loss of barrier function.

Endocochlear potential (EP) has previously been thought to be susceptible to changes in inner ear vessel permeability where increased vessel permeability would result in reduction in EP; however, the empiric evidence for this hypothesis is lacking. EP has not been directly measured in conjunction with a quantitative assessment of inner ear vascular permeability

previously. In these experiments, we measured the EP in a variety of conditions, including those in which vascular permeability is increased and those in which macrophages are activated or depleted. We used the following conditions: control (wild type), macrophage-depleted (CX3CR1-DTR), LPS treated (WT with LPS, where vascular permeability is increased), LPS with macrophage depletion (CX3CR1-DTR with LPS where the increase in vascular permeability is attenuated), CCR2 null (CCR2^{-/-}, where vascular permeability is similar to control, wild-type) and CCR2 null with LPS (CCR2^{-/-} with LPS, elevated permeability). Of these six groups, only one group demonstrated a statistically significant difference in EP. LPS-treated wild type mice demonstrated a 7mV reduction in EP, compared to controls (average EP of 114mV versus 107mV). All other groups demonstrated EPs that were unchanged from wild-type control mice (no statistically significant difference). We observed that in LPS-treated mice, there was a large change in barrier function; however, the EP remained relatively unchanged with an average loss of 7mV (107 vs 114mV) associated with a sixfold increase in barrier permeability. In animals that were treated with diphtheria toxin (DT) to induce macrophage depletion, the EP was unchanged (114, 112, 116 mV in control, macrophage-depleted, and macrophage depleted with LPS).

ABR thresholds are normal in mice with leaky blood-perilymph barriers.

In the present study, we induced leakiness of the blood-perilymph barrier by administering systemic LPS, an intervention that is known to disrupt the blood-brain barrier. We then tested ABR thresholds in mice at the height of barrier leakiness, 24 hours after LPS, and at 7 and 28 days recovery. We found that ABR threshold was unchanged in mice at 24 hours, at the height of barrier leakiness. However, seven days after the cessation of LPS, there was a mild threshold elevation across all frequencies. This threshold elevation resolved by 28 days after LPS (see Figure 6).

Summary:

In these experiments, we tested the effect of depleting CX3CR1 monocytes and macrophages to determine how this intervention would affect the blood perilymph barrier in control mice and how depletion of monocytes/macrophages would affect LPS-induced barrier leakiness. We also tested the effect of eliminating CCR2, the chemokine receptor expressed on inflammatory monocytes, that respond avidly to LPS priming by entering the perilymph spaces. In these experiments, we discovered that CX3CR1 macrophages are not necessary to maintain a normal blood perilymph barrier, and that macrophage-depleted mice demonstrated fluorescein entry into perilymph equivalent to control animals. We also found that depletion of CX3CR1 myeloid cells abrogates LPS-induced leakiness of the blood perilymph barrier and that LPS is less disruptive in the absence of CX3CR1+ mononuclear phagocytes. Deletion of CCR2, on the other hand, did not change barrier properties in either saline or LPS groups when compared to wild type mice. We observed that entry of inflammatory monocytes into the inner ear was not dependent on CCR2 expression, and degradation of the blood perilymph barrier was not an effect mediated by CCR2. We conclude from these observations that when activated by LPS, CX3CR1 expressing cells contribute to breakdown and the opening of the blood perilymph barrier. CCR2 expression is not necessary for the LPS-mediated effects on the blood perilymph barrier.

Discussion.

The cochlea is a tiny end organ with a constant demand for oxygen, supplied by vessels that allow exchange of gases while providing a tight separation between the blood stream and the inner ear fluids. These vessels carefully regulate the exchange of small molecules, charged particles and cells between the various inner ear compartments. The inner ear has three separate fluid compartments that interface with blood vessels (Figure 7). The perilymph is the fluid that bathes most of the inner ear structures and contacts the majority of the cochlear vessels. The functional separation between the perilymph and blood is the barrier that we have studied in these experiments. The second unique fluid of the inner ear is the endolymph, housed in the scala media with a potassium concentration of 150 mM with a positive potential of +105 to 115 mV in mice. The third fluid compartment is in the intrastrial space that contains another unique fluid that resembles endolymph in charge, but has an ionic composition that resembles perilymph. Neither the endolymph nor the intrastrial fluid is represented by these experiments because we utilize perilymph sampling as a method of evaluating the integrity of the blood-inner ear barrier. Quantifying fluorescein entry into perilymph from the vasculature provides information about communication between these two fluid spaces and does not provide information about blood vessels in the stria vascularis. Nor does it reflect the effectiveness of the barrier separating perilymph from endolymph, or endolymph from serum.

The blood-perilymph barrier, like the blood-brain barrier, serves a critical role in separating the fluid and solutes in the bloodstream from the fluid contents of the inner ear. This barrier is vulnerable and under certain conditions, blood vessels of the inner ear become excessively permeable. It has been postulated that compromise of the blood-perilymph barrier would lead to hearing impairment through loss of the endocochlear potential, although the effect of increasing cochlear vascular permeability on hearing function had not been directly evaluated in a quantitative manner. In conditions where massive breaches in this barrier are created, such as in bacterial meningitis or after creation of a cochleostomy for cochlear implant, maintaining normal function seems highly improbable, when in fact, it is possible. What we demonstrated in these studies is that compromise of the blood-perilymph barrier was not associated with loss of hearing. Furthermore, a significant amount of leakiness was tolerated by the system, with preservation of normal hearing thresholds and near-normal endocochlear potential. With a doubling of % entry of fluorescein from serum into perilymph, only a modest change in EP was noted (7mV drop from 115 to 108 mV), no change in ABR threshold accompanied this change in EP, and the long-term findings of ABR thresholds after a finite period of leakiness demonstrated no evidence of hearing loss in the long term.

Monocytes that express CCR2 are observed in the inner ear with robust inflammatory stimuli such as LPS (Hirose et al, 2014a). In the setting of aminoglycoside ototoxicity, entry of CCR2 monocytes into the cochlea is facilitated by LPS, which is associated with a worse hearing outcome (Hirose et al., 2014b). In these experiments, it was not clear whether the heightened ototoxic injury was caused by increased vessel permeability induced by LPS. The observation of increased fluorescein entry in the inner ear of LPS-treated animals could certainly lead to speculation about interdependencies of the effect of entry of CCR2

monocytes into the inner ear, permissiveness of the leaky barrier, elevated drug concentration in the inner ear fluids, and a poorer hearing outcome. In fact, the increased susceptibility to aminoglycoside ototoxicity is independently influenced by CCR2 expression, as CCR2 null mice do not demonstrate LPS-induced enhanced susceptibility to ototoxic injury (see Hirose et al., 2014b), but they do experience an increase in barrier permeability as shown in Figure 4. We observe that the leakiness of the blood-labyrinth barrier occurs after LPS whether or not CCR2 is present. By extension, we conclude that the attenuation of LPS effects on ototoxicity by deletion of CCR2 occurs even in the presence of a leaky blood perilymph barrier.

Prior studies have suggested that cochlear macrophages are integrated into the structure of cochlear vessels and are needed to maintain the integrity of the blood labyrinth barrier (Zhang et al., 2012). Our results demonstrate that macrophage elimination via CX3CR1-DTR occurred without compromise of the blood perilymph barrier. Furthermore, elimination of macrophages was accompanied by a normal endocochlear potential and normal ABR thresholds. The depletion of macrophages using CX3CR1-DTR included elimination of the stria macrophages, refuting the idea that macrophages in the stria are necessary to maintain a normal EP. We account for the discrepancy between our findings and the previous experiments by pointing to the method of assessing barrier function. Zhang et al. used cochlear samples that were removed from the animal and then estimated barrier function by assessing dye extravasation in dissected tissues. In our experiments, we sampled inner ear fluids from a live mouse, representative of fluids from the functioning organ. We measured fluorescent intensity of perilymph and blood sampled at the same time from the live animal and calculated the concentrations based on a standard dilution series. Thus, the differential concentration of tracer measured on either side of the barrier represents the functional separation of these two fluid compartments in vivo.

Zhang et al (2012) use the term “perivascular macrophage-like melanocyte” to describe macrophages of the inner ear, specifically to those in the stria vascularis that can contain pigment granules. Macrophages located adjacent to vessels could be called perivascular macrophages, as would be fitting for the macrophages of the stria vascularis. The cytoplasm of stria macrophages may contain pigment granules as a result of phagocytosis of intermediate cells or other cells that contain melanin. We have demonstrated that cochlear macrophages are derived from bone marrow, are sensitive to radiation and are readily replaced by derivatives of transplanted bone marrow precursors (Sato et al., 2008). Cochlear macrophages are myeloid cells that originate from the bone marrow in mature animals. Such resident macrophages are regularly replaced by bone marrow precursors, as has been shown for other tissue macrophages (Bain et al., 2014; Schulz et al., 2012). In contrast, stria melanocytes are derived from the neural crest and establish residence in the inner ear during embryonic development (Steel and Barkway, 1989). Given the differing developmental lineages and phenotypes of macrophages and melanocytes, it is unlikely that a composite of these two cell types is present in the inner ear.

We did observe transient elevation in threshold in mice that received LPS, but not during the time frame when the barrier leakiness was present. Mild threshold elevation was detected at 7 days post LPS and was resolved by 28 days after LPS. Hearing thresholds were unchanged

at the time when barrier leakiness was at its peak (24 hours after LPS treatment). The cause of this transient threshold elevation is unknown. We tested the EP at 24 hours as shown in Figure 5 and we tested again at 7 days after LPS and found that the EP remained stable (data not shown). Thus, threshold shift could not be attributed to dysfunction of potassium recycling or ion channel activity associated with lateral wall inflammation. In summary, LPS-induced cochlear inflammation was associated with short-term, delayed elevation of threshold, detected at 7 days post treatment, not associated with loss of endocochlear potential, and ABR thresholds were restored to baseline at four weeks.

In summary, our data show that elimination of macrophages did not result in changes in blood perilymph barrier function or EP in normal mice. After systemic LPS, breakdown of the blood-perilymph barrier was observed. In animals that were depleted of macrophages, LPS-induced vascular leakiness was reduced, and the BPB was more effectively maintained. Elimination of macrophages resulted in preservation of barrier function, rather than a loss of barrier integrity. It has been postulated that inflammatory cells may contribute to barrier leakiness as assessed by contrast-enhanced magnetic resonance imaging (Floc'h et al., 2014). The findings in these experiments suggest that barrier permeability changes associated with LPS are induced by the activity of cochlear macrophages.

Conclusions.

From these experiments, it is clear that macrophages activated by LPS contribute to compromise of the blood perilymph barrier. Eliminating these macrophages results in preservation of barrier function; thus, macrophages play an active role in opening the vascular-inner ear barrier. In the context of infection and in other non-infectious forms of cochlear inflammation, it is not clear whether maintaining the integrity of the barrier is beneficial to the animal overall. Frequently, in the setting of infection, the effect of increasing barrier permeability and lowering the threshold for cells and molecules to cross the endothelium results in enhanced delivery of important elements of the immune system that contain and eliminate the source of infection. There are instances where strict maintenance of the blood-brain barrier is detrimental to survival because opening the barrier is a critical step in the cascade of events that leads to resolution of infection (Daniels et al., 2017; Durrant et al., 2014). Thus, while we can conclude that CX3CR1 monocytes and macrophages contribute to loss of barrier function, we cannot state that these cells are detrimental to the ear or to hearing in the case of infection or systemic inflammation. Further studies with specific examples of CNS, inner ear, or systemic infection are necessary to determine to what degree these inflammatory cells are beneficial to preserve hearing long term and in what ways they may contribute to loss of function.

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Highlights:

The blood-perilymph barrier is dynamic, and inflammatory stimuli cause changes in inner ear vessel permeability. We have found that macrophages and monocytes contribute to opening of this barrier and that CX3CR1 expressing cells influence this change while CCR2 expression does not. The endocochlear potential and ABR threshold are effectively maintained in the face of considerable changes in vascular permeability induced by systemic inflammation. We also found that elimination of macrophages, including the striae macrophages, does not result in loss of endocochlear potential or change in ABR threshold.

Figure 1.

Perilymph sampling was used to assess functional integrity of the blood perilymph barrier in wild type mice after LPS treatment for two days. Samples 1 and 2 (shaded in blue) were perilymph samples as determined by volume displacement and samples 3, 4, 5 contained CSF. In control animals, the perilymph concentration of fluorescein was 2% of the concentration in serum (Red: average, grey: individual animals, n=8). After LPS treatment, the percent entry of fluorescein increased. One animal had 56% of the serum concentration of fluorescein in the first perilymph sample. The average % fluorescein in LPS animals was 12.1% and 9.6% re: serum fluorescein in the perilymph samples 1 and 2. We observed a large range in this group (n=20).

Figure 2.

CX3CR1-DTR mice experience loss of macrophages and monocytes after a single dose of 200ng diphtheria toxin (DT). A: Bone marrow of the otic capsule. B: Spleen. C: Organ of Corti and lateral wall. D: Spiral ganglion. E: Cerebral cortex. Time after DT injection: as listed. Depletion of CX3CR1 myeloid cells was slower in the inner ear and slowest in the brain. Insets: DIC image of the organ of Corti and lateral wall (C) and of the spiral ganglion (D).

Figure 3.

Depletion of macrophages and monocytes did not change permeability of the blood perilymph barrier (compare to Figure 1, n=12). Depletion of CX3CR1 macrophages resulted in preservation of the BPB after LPS treatment (n=15, average of 5.8 and 3.8% of serum concentrations in the first two samples compared to 12.1 and 9.6% in the LPS with replete macrophages in wild type mice in Figure 1).

Figure 4.

LPS-induced barrier changes in CCR2 knockout mice. CCR2 knockout mice have barrier properties that mirror wild-type mice both in the control group and in the setting of LPS. CCR2 null mice demonstrate 2–3% perilymph concentration of fluorescein compared with serum, and similar increase in permeability in LPS-treated mice. The barrier permeability changes affected the percent fluorescein in the perilymph (samples 1–2) considerably more than the samples from the CSF (samples 3–5).

Figure 5.

Endocochlear potential (EP) was measured in mice at the time of perilymph sampling, 24 hours after the second dose of LPS. There was a modest reduction in EP after LPS treatment (average EP: **114mV** in control mice, n=13 and **107mV**, n=11 after LPS) at the time when there was substantial barrier compromise. Depletion of macrophages did not result in change of EP (Control: **114mV**, MP depleted **117mV**, n=8). Macrophage depletion prior to treatment with LPS resulted in maintenance of the EP despite LPS treatment (MP-depleted

LPS treated: **115mV**, n=8). EP is minimally affected by substantial changes in vascular permeability. Inflammation may contribute to the minor reduction in EP observed after LPS.

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Figure 6.

ABR thresholds after LPS treatment. On day 1, at the time of perilymph sampling, ABR thresholds were similar between the three groups (control, LPS treated and LPS treated, macrophage depleted groups). There was a slight increase in threshold in LPS-treated animals and in LPS-treated, macrophage-depleted animals, but this difference was statistically insignificant. At seven days after LPS and LPS/diphtheria toxin, larger changes in threshold were noted after LPS and DTA. By 28 days, thresholds returned to levels observed in animals at D1.

Figure 7.

Three separate fluid compartments of the inner ear. The fluid that was sampled in this study is shown in purple (perilymph compartment). The majority of the cochlear vessels are surrounded by perilymph. Measurement of the barrier using perilymph sampling does not evaluate the blood-strial barrier (dark orange), the blood-endolymph or the perilymph-endolymph barrier (light orange).

Table 1.

Mouse treatment protocol. For macrophage depletion, CX3CR1-DTR mice were injected with DTA at days 1, 4, and 7, and mice were then tested at day 8 (arrows above the line). For LPS treatment, mice were injected for two consecutive days and tested one day after the last LPS dose (LPS given at times shown by arrows below the line). For combined macrophage depletion and LPS treatment, mice were treated on days 1, 4, 7 with DTA and days 6 and 7 with LPS and tested on day 8. Testing involved perilymph sampling and cochlear harvest. Additionally, ABRs were tested 24 hours after the last LPS dose (day 8 in the above schematic), 7 days and 28 days after the last LPS dose.