Published in final edited form as:

Otol Neurotol. 2011 April ; 32(3): 508–515. doi:10.1097/MAO.0b013e31820e6de4.

Murine middle ear inflammation and ion homeostasis gene expression

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Abstract

Hypothesis—Ion homeostasis genes are responsible for movement of ions and water in the epithelium of the middle ear.

Background—It is not well known to what extent disruption of ion homeostasis is a factor in the accumulation of middle ear fluid during otitis media.

Methods—Balb/c mice were transtympanically injected with heat-killed *Hemophilus influenza* bacteria. Untreated and saline injected mice were used as controls. Mice were euthanized at 6, 24, 72 hours and one week after injection, the bullae harvested, and total RNA isolated from the middle ear tissues. Ion homeostasis genes were analyzed with real-time qRT-PCR from the following gene families: Na⁺,K⁺-ATPase, claudins, K⁺ transport channels, epithelial Na⁺ channels, gap junctions, and aquaporins. Inflammatory genes were also analyzed to document inflammation.

Results—All inflammatory genes analyzed were significantly upregulated, more at 6 hours than at 24 hours, with the exception of VEGF and Mapk8. Most middle ear ion homeostasis genes experienced downregulation due to inflammation. This was most prominent in the aquaporin and Na⁺, K⁺-ATPase genes. Significant upregulation was seen in several genes in response to inflammation and saline independently.

Conclusion—The innate immune response to bacteria in the middle ear induces expression of several inflammatory genes. Coinciding with this inflammation is the downregulation of numerous ion homeostasis genes that are involved in ion and water transport and maintenance of tight junctions. This may explain the fluid accumulation within the middle ear seen with both acute and chronic otitis media.

Introduction

Fluid accumulation in the middle ear due to an infectious or inflammatory process is a common scenario faced in clinical otolaryngology practice. Otitis media with effusion is a

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Presented at Association for Research in Otolaryngology, Anaheim, CA, February 6–10, 2010

Conflict of interest: None

sequelae of acute otitis media and is the primary reason for tympanostomy tube insertion in children. This inflammatory process is characterized by bacterial proliferation in the nasopharynx and entry into the middle ear via the Eustachian tube. Inflammation in the middle ear is mediated by toll-like receptor (TLR)-dependent and TLR-independent cellular activation of transcription factors that cause production of inflammatory cytokines and secretion of serous or mucin-rich effusions 1–2. Although numerous inflammatory cytokines are activated 3–5, those of greatest interest are interleukins and tumor necrosis factor- α . Otitis media with effusion causes several problems for the patient: conductive hearing loss (usually temporary), discomfort, possible speech and language delay and potential permanent conductive or sensorineural hearing loss. Thus, controlling this harmful effusion is critical for middle ear integrity and patient health. Fluid in the middle ear is usually ascribed to increased transudation from the vasculature and secretion by the edematous mucosa, further complicated by Eustachian tube dysfunction. However, the mechanisms by which the middle ear resorbs fluid after an infection have not been well-studied. Dysfunction of fluid transport mechanisms may be another underlying factor in the persistent fluid.

Ion and water transport in the inner ear spaces are essential 6. Several ion homeostasis genes are active in the inner ear to maintain potassium, sodium, chloride and calcium concentrations in the endolymph for optimal functioning of hair cells 7. Defects in many of these genes are also associated with deafness, such as those controlling potassium channels (Kcne1, Kcnj1, Kcnj10), Na⁺, K⁺-ATPase (Atp1a1, Atp1a2), chloride channel (Clcnka), gap junctions (Gjb2, Gjb6), tight junction claudins, etc. However, a comprehensive evaluation of these ion homeostasis genes in the middle ear response to acute inflammation has not been done. Sodium (Na⁺) ion and water transport occurs in middle ear epithelial cells 8–10 suggesting an active role for the epithelial sodium channel gene (ENaC) 11. Furthermore, aquaporins are present in the middle ear epithelium and impacted by inflammation 12–13. Thus, disruption of these middle ear active transport processes may underlie the accumulation of fluid during acute and chronic inflammation. Therefore, to better understand the relationship of ion and water transport genes in acute middle ear inflammation, a quantitative RT-PCR study was conducted of a panel of ion homeostasis genes in the mouse model of acute otitis media.

Materials and Methods

The acute middle ear disease mouse model employed has been described previously 14. Balb/c mice, eight for each end point time, were screened for the absence of middle ear fluid. Acute otitis media (OM) was created by bilateral transtympanic inoculation with heat-killed *Hemophilus influenza* and tissues harvested at 6, 24, 72 hours and one week. With microscope assistance, middle ear tissue was dissected from the inner ear. Middle ear tissues harvested for analysis included middle ear mucosa, bulla walls, ossicles, middle ear muscles, middle ear fluids, but no cochlear tissues. The inner ear was dissected away so that only the cochlear wall common with the middle ear was left, thus minimizing inner ear components but leaving the middle ear wall of the inner ear intact so all middle ear contents were preserved. Left and right middle ear tissues were combined, homogenized, and mRNA extracted for quantitative RT-PCR of key ion homeostasis genes and inflammatory cytokine genes. Eight control mice who had received no bacterial inoculation and who had no evidence of middle ear fluid were collected at each time point. Gene expression in the inflamed middle ears was compared to untreated control mice. To further delineate the difference in response between the fluid vehicle alone versus the inflammatory response to the heat-killed bacteria in the vehicle, another group of 12 animals was trans-tympanically inoculated with phosphate-buffered saline (PBS) and 4 mice collected at three time points (6, 24, 72 hours).

RNA Isolation

Middle ears were isolated and stored in RNA*later* (Ambion, Inc., Austin, TX) at -20°C until RNA was extracted. Tissue RNA was extracted with the Qiagen (Valencia, CA) RNeasy Mini Kit. Tissue was transferred to tubes with 600 μl of extraction buffer and homogenized with a PowerGen 125. RNA was quantified using a NanoDrop and all samples were made up to a concentration of at least 25 ng/ μl .

Quantitative RT-PCR Analyses

Real-time RT-PCR studies used an ABI Step One Plus system (Carlsbad, CA). Total RNA (200 ng) was reverse-transcribed using RT² First Strand Kit (SABiosciences Corp, Frederick, MD) using the manufacturer's instructions. Then samples were prepared for Real-time PCR using the RT² Real-time SYBR Green/Rox PCR master mix. Thermal cycle condition was set as: 95°C 10 min, then 40 cycles: 95°C 15sec, 60°C 1 min followed by a melt curve. Data analysis follows the suggestion of the manufacturer (SABiosciences PCR Array Data Analysis Web Portal). The parameter CT (threshold cycle) is defined as the fractional cycle number at which the reporter fluorescence generated by cleavage of the probe passes a fixed threshold above baseline. The statistical significance and fold change are calculated using the $\Delta\Delta\text{C}_t$ method with the aid of SABiosciences PCR Array Data Analysis Web Portal. Because fold change is a ratio of the two groups' means, it is a single number (ratio); hence no standard deviation can be calculated on the fold change number. The housekeeping gene used for this method was glyceraldehyde-3-phosphate dehydrogenase.

The method utilized custom PCR Arrays (SABiosciences Corp, Frederick, MD) already optimized for reaction conditions, primers, and probe. Recent product developments by SABiosciences have combined the two principles of gene arrays and RT-PCR so quantitative RT-PCR can be done simultaneously on primer arrays of multiple cytokine genes (RT² Profiler PCR Array System) for actual quantification of gene expression. This new technique has been adapted in our laboratory and a standard cytokine profile is assessed in all inflammatory studies. Our custom PCR Array plates were made by SABiosciences Corp (Frederick, MD) to measure expression of ten key inflammation related cytokines: IL-1 α , IL-1 β , IL-6, IL-10, MIP-1 α , Mapk8, MIP-2, KC, TNF α , and VEGF (Table 1). Additional custom PCR array plates were made to analyze 26 ion homeostasis genes (Table 1). These include isoforms of Na⁺,K⁺-ATPase, channels that transport K⁺, Na⁺, Cl⁻, gap junction connexins, water transporting aquaporins, and tight junction claudins. These ion homeostasis genes were selected for their known importance in the function of the inner ear and potential role in control of middle ear fluids during inflammation.

All animal procedures in the study were approved by the OHSU Institutional Animal Care and Use Committee, Protocol # A149, P.I. Dennis R. Trune.

Results

Inflammatory Genes

Significant elevation of nearly all inflammatory cytokine gene levels was seen at one or more time points (Fig 1A–C; Table 2). This confirmed that a strong inflammatory process was caused in the mouse middle ear after the inoculation with heat-killed *Hemophilus influenza* bacteria. Some cytokine expression increased only a few fold, while other cytokines were upregulated several hundred fold (Fig. 1). TNF α and IL-1 α were the least impacted, showing an increase in expression of less than 8 fold at 6, 24, and 72 hours, although both had returned to normal expression at 1 week (Fig. 1A). VegFa and Mapk8

were actually downregulated at the 72 hour endpoint and were the only cytokines to show suppression. These two cytokines were not run at 1 week.

An order of magnitude greater expression was seen in several cytokines, some reaching 50–60 fold change (Fig. 1B). MIP-1 α , IL-1 β , and IL-10 were in this range of activity. MIP-1 α was upregulated 50 fold by 6 hours and over 60 fold at 24 hours. It was still overexpressed by 14 fold at 72 hours, returning to normal by 1 week. On the other hand, IL-1 β was overexpressed at all 4 time points, while IL-10 increased during the first three days and was back to normal by 1 week. This latter cytokine is considered anti-inflammatory, so it serves to downplay the overexpression of the other pro-inflammatory genes.

The greatest upregulation of genes was seen in MIP-2, IL-6 and KC (Fig. 1C, Table 2). MIP-2 was expressed 1083-fold at 6 hours and 400 fold at 24 hours. It was still significantly increased at 1 week, up nearly 3 fold. IL-6 also was most upregulated at 6 hours, showing over 550 fold expression. KC, a proinflammatory chemokine, also was increased over 600 fold at 6 hours. Like many others, it gradually declined over time, but was still up nearing 4 fold at 1 week.

PBS alone caused a much weaker inflammatory response at all time points (6, 24, 72 hours) compared to PBS plus heat-killed (HK) bacteria. PBS cytokine levels compared to the no treatment mice showed no significant elevation at 6 hours in any group, while there were several cytokines that were statistically elevated at 24 hours (MIP-2, IL-6, IL-1 β , MIP-1 α , KC) and less so at 72 hours (IL-10, IL-1 α). Of the cytokines that were statistically elevated at 24 and 72 hours, none were at the levels of the heat-killed bacteria inoculated mice (Table 2).

Ion Homeostasis Genes

The panel of 24 inner ear ion homeostasis genes was applied to the middle ear tissue as a screen to determine which ion regulatory genes are present and potentially affected. It had not previously been determined if any of these genes are actually present in the middle ear tissues. Therefore, we first confirmed their presence in the middle ear by looking at PCR expression level of these genes in the untreated control mice. The level of all genes was above background expression of housekeeping genes according to the manufacturer's cutoff values. Thus, all channels and transporters investigated are presumed present in the middle ear. Several of them were impacted by inflammation, although the impact often was different among members of the same family of genes.

Aquaporin gene family

Aquaporin (aqp) channels move water and several have been described for the middle ear and inner ear. These different channels in the middle ear show varied response to the inflammation (Fig. 2A). Aquaporin 3 was significantly upregulated by inflammation, reaching levels 5–6 times normal by 72 hours and still increased at 1 week. Of interest is the fact that it did not show any significant effects at 6 hours, moderately elevated (3 fold) at 24 hours, and strongest expression at 3 days. This implied it was manifesting a later stage response, possibly due to increased fluid or other fluid transport issues. On the other hand, aqp1 and 5 were significantly downregulated by inflammation, showing reduction by 40–60% in the early stages. Even at 1 week, aqp 1 was still suppressed. Aquaporin 2 showed a trend of downward expression, particularly early in the inflammation, but these differences did not reach statistical significance. Similar patterns were seen when comparing the control to PBS. Aquaporin 1, was suppressed at all time points, however, aquaporin 3 was even more elevated with PBS alone than with HK bacteria inoculation (Table 3).

Claudin gene family

Claudins are present in the middle ear and make up the junctional complex sealing mucosal epithelial cells. These claudins appeared to be generally upregulated by inflammation, with the strongest expression seen in claudins 3 and 4 (Fig. 2B). Both were upregulated in the early stages of inflammation, although neither reached statistical significance at 6 hours in spite of a 3–4 fold increased expression. By 1 week these two claudins had returned to normal, even slightly below normal for Claudin 3. Claudin 14 did not appear to be affected in any significant manner. Claudins did show trends for upregulation when exposed to PBS, but none of the values were statistically significant (Table 3).

Chloride Channel

The chloride channel Clcnka that is common in the inner ear is also located in the middle ear (Fig 2B). It did not appear to be significantly affected by inflammation or fluid, although at 1 week it was expressed at 2.6 times normal (Table 3). Clcnka showed a significant elevation at 24 hours to PBS alone (Table 3).

Na⁺,K⁺-ATPase gene family

Several isoforms of this transporter are commonly found in the inner ear, largely responsible for exchanging Na⁺ and K⁺ in the stria vascularis and lateral wall. Several of these isoforms were also found in the middle ear (Fig 2C), nearly all of which were downregulated by inflammation. Some were still depressed at 1 week, suggesting a long term effect of inflammation is to suppress the exchange of these ions in the middle ear space. This may relate to the comparable suppression of most aquaporins above. When analyzing the response to PBS, the ATPase family was also downregulated (Table 3).

Gap junction gene family

Gap junction proteins are involved in the transport of K⁺ in the organ of Corti and lateral wall. Some of them are also present in the middle ear, although they showed variable impact of the inflammation (Fig. 2D). Gja1 (connexin 43) was upregulated slightly, but significantly, at 72 hours, while Gjb2 (connexin 26) was essentially unaffected. Gjb3 (connexin 31) appeared to be downregulated early and overexpressed later, although none of these changes reached statistical significance. Gjb6 (connexin 30) was generally suppressed during early inflammation, suggesting transiently compromised K⁺ transport. Gjb2, Gjb6 and Gja1 were also downregulated in response to PBS alone; however Gjb3 was upregulated at 24 hours to a more significant degree than in response to HK bacteria (Table 3).

K⁺ channel genes

Several K⁺ channel genes are present in the middle ear and in hair cells of the inner ear. In general they were unaffected by inflammation (Fig 2E). Kcnq4 was significantly downregulated at 72 hours, although it returned to normal expression by 1 week. Similar response was seen comparing the response to PBS with little significant change seen at any time point, except for downregulation in Kcnq1 at 72 hours and Kcnj10 at 6 hours (Table 3).

Epithelial Na⁺ gene family

Several epithelial sodium channels are present in the middle ear, as well as one of the enzymes that controls them, transmembrane protease, serine 3 (tmprss3). One of these channel proteins (Scnn1β), as well as tmprss3, were depressed at 24 hours (Fig. 2F). The other Na⁺ channels showed a trend of suppression at 24 hours, although this change was not statistically significant. Similar response was seen comparing response to PBS with little significant change seen at any time point (Table 3).

Discussion

Ion Homeostasis Gene Response to Inflammation

Elevation of cytokines in OM has been studied and early phase cytokines are known to stimulate the innate immune response to the bacterial challenge in the ME. IL-1 and TNF α are two of these cytokines. IL-6 was one of the cytokines most upregulated in our study, reaching 550-fold at the 6-hour time point. IL-6 is thought to be important in developing chronic OM 15. IL-10 is known to be the major anti-inflammatory cytokine, responsible for transition from acute inflammation to clearance of inflammation, possibly via negative feedback on TNF- α . Cytokines studied showed an expected early response to bacterial inoculation with upregulation highest at 6 and 24 hours and returning back towards normal levels at one week.

Ion homeostasis genes are well studied in the inner ear, but less well so in the middle ear. Location of ion homeostasis genes in the inner ear has been identified by immunohistochemistry, and their function studied in vivo, in vitro, and by knockout mice studies. While less has been published on the function of such genes in the middle ear, it has been proven that the ME epithelium does actively absorb fluid via a Na⁺-dependent process 11. Table 4 reviews potential correlations of known inner ear (IE) and middle ear (ME) functions of these genes.

The present study showed ion homeostasis genes are mostly downregulated in the first 72 hours following bacterial stimulation of the middle ear. Undoubtedly, a finely-tuned balance of different genes and gene products exists to maintain homeostasis of ions and fluids in the middle ear cavity as well as the inner ear. Interestingly, downregulation of ion transport mechanisms in the middle ear would tip the middle ear cavity fluid balance in favor of fluid accumulation. This is certainly seen frequently in the clinical setting of acute otitis media and especially in chronic otitis media with effusion.

The overall time course of the cytokine and ion homeostasis gene changes showed an opposite response to inflammation in the times studied. The cytokine genes demonstrated a strong early response to inflammation, while the ion homeostasis genes showed mainly downregulation at the early time points. However, while the cytokine genes consistently were upregulated early and then returned to more normal levels at one week, the ion homeostasis genes were often still downregulated at one week (Atp α 2, Atp β 2, Cldn 3, Aqp1). Only apq3 was upregulated at one week. Therefore, it would appear that the early response to inflammation in the middle ear stimulates a cytokine production and decreases fluid clearance.

Aquaporins, one of the genes exhibiting significant downregulation, are embedded in the cell wall and regulate water flow. These water channels increase cell membrane permeability to water. If these genes were downregulated during an inflammatory process, perhaps less water is allowed to pass through the epithelial cell and more remains in the extracellular middle ear space.

Na⁺,K⁺ATPases are integral membrane proteins responsible for controlling cell volume and maintaining the electrochemical gradients of Na⁺ and K⁺ ions across the cell membrane. Again, downregulation of these proteins in the acute inflammatory setting may decrease ion movement and increase extracellular water.

Claudins are transmembrane proteins that maintain tight junctions and establish the paracellular barrier controlling flow of molecules in the intercellular space between the

epithelial cells. Thus, alteration of claudins would impact epithelial integrity and decrease their ability to prevent leakage of fluids from an epithelium.

Ion Homeostasis Gene Response to Fluid

The ion homeostasis gene response to a fluid challenge without bacteria (vehicle only) was in large part downregulation, similar to the response to vehicle plus HK bacteria. Aquaporins, Na⁺,K⁺ATPases, gap junction, potassium and sodium channel genes were all downregulated at various time points. The exception to this pattern is aquaporin 3 which is upregulated to both challenges, however, the presence of bacteria seems to delay the response of aquaporin 3 upregulation until 24 hours (Table 3). This would indicate that aqp3 may be downregulated in response to inflammation (early response), but upregulated in response to fluid accumulation (late response). The downregulation seen both with inflammation and with a fluid challenge in the ion homeostasis gene families would indicate that these genes are downregulated in the early response to both fluid and inflammation, compounding the failure to clear fluid from the middle ear space in the setting of middle ear inflammation.

Gene-gene Interaction

Interaction between cytokines and ion homeostasis genes are also known to occur³. When early phase cytokines are upregulated, such as IL-1 and TNF α , there is also an impact on ion flux by virtue of impact of interleukin IL-1 β on ENaC and Na⁺-K⁺-2Cl⁻ cotransporters. Also, there is evidence that IL-1 β (a pro-inflammatory cytokine) inhibits Na⁺,K⁺-ATPase in heart muscle¹⁶, elucidating further this interaction between the cytokines and the ion homeostasis genes. IL-1 β also upregulates the Na⁺-K⁺-2Cl⁻ cotransporter¹⁷ and downregulates ENaC¹⁸ in middle ear epithelial cell culture studies. Undoubtedly, there are many interactions between these two classes of genes involved in the inflammatory response which lead to the clinically observed inflammation and middle ear fluid in otitis media. However, if the control of such effusions could be accomplished quickly with properly targeted therapies, it would eliminate the need for steroids or antibiotics. One example might be the application of mineralocorticoids (oral, transtympanic) for the improved resorption of middle ear fluid¹⁹. Further studies seek to clarify the most relevant ion transport channels in effusion control and develop effective treatments to exploit them.

Undoubtedly, middle ear and inner ear ion homeostasis both are tightly regulated by ion channel genes. While we expected upregulation of these genes to be most prominent as a mechanism to fight the inflammation and edema of infection, we actually saw mainly downregulation. Several genes were identified that responded differently to bacterial inflammation and fluid (aqp3, clcnka, gja1,gjb2), elucidating the likely scenario that some genes react to inflammation primarily and some respond primarily to the presence of fluid. We are studying the protein products of these genes from the middle ear in the setting of inflammation to better understand the impact of these changes seen with infection and inflammation. Long-standing middle ear fluid dysregulation, as seen in the hearing impact of middle ear pathology (otitis media with effusion for example), merits further study.

Acknowledgments

Supported by NIH-NIDCD R01 DC009455 and DC005593.

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Fig. 1.

Changes in inflammatory gene expression for various postinoculation times. See Table 2 for fold change values. *, $p < 0.05$.

Figure 1A: Inflammatory genes showing 0–9 fold change after exposure to heat-killed bacteria. TNF α and IL-1 α were significantly upregulated while VegF α and Mapk8 were suppressed and downregulated at 72 hours. VegF α and Mapk8 were not run at 1 week time point.

Figure 1B: Inflammatory genes showing 0–70 fold change. MIP-1 α , IL-1 β , and IL-10 were upregulated after exposure to heat-killed bacteria.

Figure 1C: Cytokines upregulated in the 0 – 1200 fold range. Highest levels of inflammatory gene upregulation were seen in MIP-2, IL-6, and KC. Some were still elevated at 1 week.

Fig. 2. Expression levels of ion homeostasis genes during middle ear inflammation at the various postinoculation times. See Table 3 for fold change values. *, $p < 0.05$.

Figure 2A: Aquaporin genes after exposure to heat-killed bacteria. Aquaporin 3 was significantly upregulated out to 1 week. Aquaporins 1 and 5 were significantly downregulated.

Figure 2B: Claudin genes and the chloride channel gene Clcnka after exposure to heat-killed bacteria. Upregulation seen in Claudin 4 and early in Claudin 3, but downregulation was seen in Claudin 3 at one week.

Figure 2C: Na⁺,K⁺ATPase genes were mostly suppressed after exposure to heat-killed bacteria.

Figure 2D: Gap junction genes after exposure to heat-killed bacteria. Slight overexpression was seen in Gja1. There was a trend of initial suppression of Gjb3, followed by increased expression, although these shifts did not reach statistical significance. The early significant suppression of Gjb6 suggested a transient impact on K⁺ transport.

Figure 2E: K⁺ transport channel genes after exposure to heat-killed bacteria were generally unaffected by inflammation.

Figure 2F: Epithelial Na⁺ channel gene family after exposure to heat-killed bacteria. Scnn1β and tmprss3 were depressed at 24 hours, but appeared to return to normal after that.

Table 1**Key to cytokine and ion homeostasis genes studied:****Ion Homeostasis Genes:****Aqp1, 2, 3, 5** = aquaporins 1, 2, 3, 5**Atp1 β 1, Atp1 β 2, Atp1 β 3** = Na⁺,K⁺-ATPase, Na⁺/K⁺ transporting, beta 1,2,3 polypeptides**Atp1 α 1, Atp1 α 2** = Na⁺,K⁺-ATPase, Na⁺/K⁺ transporting, alpha 1, 2 polypeptides**Clcnka** = chloride channel Ka**Cldn 3, 4, 14** = Claudins 3, 4, 14**Gja1, Gjb2, 3, 6** = gap junction proteins, alpha 1, beta 2,3,6**Kcne1, Kcnq 1, 4** = potassium voltage-gated channel**Kcnj10** = potassium inwardly-rectifying channel**NKCC1** = Na-K-2Cl cotransporter**Scnn1 α , 1 β , 1 γ** = ENaC (epithelial Na⁺ channel), nonvoltage-gated 1 α , 1 β , 1 γ **Tmprss3** = transmembrane protease, serine 3**Inflammatory genes:****IL-1 α , IL-1 β , IL-6, IL-10** = Interleukins (1 α , 1 β , 6, 10)**Mapk8** = mitogen activated protein kinase 8**MIP-2 (Cxc12), MIP-1 α (CC13), KC (Cxc11)** = chemokines [(C-X-C motif) ligand 2, (C-C motif) ligand 3, (C-X-C motif) ligand 1]**TNF α** = tumor necrosis factor α **VegFa** = vascular endothelial growth factor A

Table 2

Fold change of cytokine genes: HK bact vs. controls				Fold change cytokine genes: PBS vs. controls			
Cytokine	6 hours	24 hours	72 hours	1 week	6 hours	24 hours	72 hours
MIP-2	1083.6 *	404.2 *	72.1 *	2.9 *	6.46	24.45 *	9.61
IL-6	557.6 *	30.2 *	9.5 *	1.7	5.49	5.11 *	5.85
IL-1 β	36.1 *	20.3 *	4.5 *	1.6 *	2.19	3.23 *	2.31
IL-10	34.9 *	4.6 *	1.5	1.4	5.42	3.74	9.30 *
TNF	4.5 *	7.8 *	1.7 *	1.0	0.76	0.63	0.92
IL-1 α	5.7 *	5.7 *	3.0 *	0.8	1.52	1.27	2.92 *
MIP-1 α	52.9 *	63.4 *	14.0 *	1.4	1.26	2.19 *	2.2
KC	638.5 *	50.1 *	11.8 *	3.6 *	7.59	4.11 *	4.78
VegFa	0.2	0.7	0.6 *	–	–	–	–
Mapk8	1.0	0.9	0.6 *	–	–	–	–

* p<0.05, HK = heat-killed, Bact = bacteria, PBS = phosphate-buffered saline

Table 3

Fold change of IH genes: HK bact vs. controls				Fold change of IH genes: PBS vs. controls			
	6 hours	24 hours	72 hours	1 week	6 hours	24 hours	72 hours
Aqp1	0.61 *	0.48 *	0.87	0.71 *	0.68 *	0.64 *	0.61 *
Aqp2	0.38	0.41	0.8	0.89	0.99	0.68	1.37
Aqp3	0.94	2.94 *	5.36 *	3.32 *	6.05 *	17.79 *	12.7 *
Aqp5	0.59	0.61 *	0.66 *	0.89	0.69	0.82	0.77
Atp1a1	0.9 *	0.78 *	0.88 *	0.86	0.62 *	0.67 *	0.6 *
Atp1a2	1.23	0.75 *	0.61 *	0	n/a	n/a	n/a
Atp1b1	1.12	0.73 *	0.69 *	0.97	0.76 *	0.72 *	0.6 *
Atp1b2	1.14	0.79	0.75	0.68 *	0.6 *	0.59 *	0.42 *
Atp1b3	1.07	0.93	0.92	0	n/a	n/a	n/a
Clenka	1.46	1.44	1.29	2.59	3.57	3.06 *	4.66
Cldn14	1.23	0.74	1.11	1.17	3.26	2.32	3.67
Cldn3	3.95	2.11	1.4	0.3 *	6.58	3.99	4.67
Cldn4	3.12 *	2.28 *	2.38 *	0.99	1.19	1.4	1.47
Gja1	0.98	1.02	1.59 *	1.3	0.69	0.89	1.03
Gjb2	1	1.04	1.22	0.83	0.45 *	0.78	0.66 *
Gjb3	0.3	1.07	1.13	1.83	1.5	4.1 *	1.23
Gjb6	0.64	0.57 *	0.94	0.94	0.67	0.77	0.53 *
Kcncl	1.16	0.81	0.83	1.26	1.04	1.06	0.63
Kcnj10	1.23	0.84	0.93	0.81	0.66 *	0.7	0.71
Kcnq1	1.02	0.78	0.85	1.28	0.74	0.83	0.59 *
Kcnq4	0.83	0.69	0.58 *	1.08	0.86	0.63	0.69
NKCC1	1.28	1.01	0.9	0.98	0.94	0.8	0.5 *
Scnn1a	1.13	0.79	1	1.26	0.8	0.75	0.63

Fold change of IH genes: HK bact vs. controls					Fold change of IH genes: PBS vs. controls				
6 hours		24 hours	72 hours	1 week	6 hours		24 hours	72 hours	
Scnn1β	0.81	0.51 *	0.8	0.91	1.06	0.86	1.24		
Scnn1γ	1.11	0.74	1.27	0.98	1.25	1.26	1.62		
Trprss3	0.95	0.49 *	0.79	1.04	0.63	0.44 *	0.49		

* p<0.05, IH = ion homeostasis, HK = heat-killed, Bact = bacteria, PBS = phosphate-buffered saline

Table 4

Gene Family	Function	Change with Inflammation (up or down-regulated *p<0.05)	IE function	ME function
Aquaporins	<ul style="list-style-type: none"> - water homeostasis - facilitate water transport through cell membranes 	<ul style="list-style-type: none"> - aqp1*, 2, 5* down-regulated - aqp3* up-regulated 	<ul style="list-style-type: none"> - active water transport - osmotic equilibrium in epithelial cells in the Organ of Corti 	<ul style="list-style-type: none"> - ME region-specific functions of the subtypes of aquaporins suggest that they regulate fluid in the ME and Eustachian tube via coordination of the various subtypes
Na ⁺ ,K ⁺ ATPase	<ul style="list-style-type: none"> - Na⁺/K⁺ pump - maintains cell membrane potential - membrane transport of proteins - controls cell volume - signal transduction 	<ul style="list-style-type: none"> - Atp 1α1*, 1α2*, 1β1*, 1β2*, 1β3 down regulated past 6 hours 	<ul style="list-style-type: none"> - circulation Na⁺ to perilymph, K⁺ to endolymph 	<ul style="list-style-type: none"> - Na⁺ absorption and trans-epithelial active transport of fluid
Claudins	<ul style="list-style-type: none"> - ion channels forming tight junctions - regulate barrier to paracellular transport of solutes and ions - major determinant of paracellular permeability 	<ul style="list-style-type: none"> - Cldn3* down-regulated at 1 week - Cldn 4* up-regulated at 6, 24 and 72 hours - Trends for other claudins are also for up-regulation 	<ul style="list-style-type: none"> - maintain tight junctions and physiology of inner ear - prevent intercellular leakage of solutes and ions - barrier function 	<ul style="list-style-type: none"> - maintain tight junctions of mucosal epithelial cells - prevent or control intercellular movement of solutes and ions - barrier function
Gap Junction/Connexins	<ul style="list-style-type: none"> - form links between cytoplasm of 2 cells - means to exchange ions (K⁺, Ca⁺⁺) 	<ul style="list-style-type: none"> - Gjb6* down-regulated at 24 hours - Gja1* up-regulated at 72 hours 	<ul style="list-style-type: none"> - conduits for K⁺ circulation - intercellular signaling 	<ul style="list-style-type: none"> - unknown - cholesteatomas stain positive for Connexins - reported case Cx26 (Gjb2) associated with mixed hearing loss
Epithelial sodium channel (ENaC)	<ul style="list-style-type: none"> - trans-epithelial flux of cations/ 	<ul style="list-style-type: none"> - trends are for down-regulation of most 	<ul style="list-style-type: none"> - movement of Na⁺ out of endolymph 	<ul style="list-style-type: none"> - major ion (Na⁺) transport protein in the middle ear cavity

Gene Family	Function	Change with Inflammation (up or down-regulated) *p<0.05	IE function	ME function
	passive flux of anions which generates an osmotic gradient which generates a water flux	data points (Scnn1b*, Tmprss3 at 24 hours)		- controls thickness of periciliary fluid layer
K ⁺ ion transport channel	<ul style="list-style-type: none"> - K⁺ ion channel - regulates transport of K⁺ in and out of cells 	<ul style="list-style-type: none"> - trends are for down-regulation in all groups - Kcnq4* down-regulated at 72 hours 	- K ⁺ circulation into cells and endolymph	- probably active in ion and fluid transport, but thus far unknown