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Review Article

# TLR Signaling Pathway Regulation in Association with Autophagy in the Diseased Inner Ear

Jing Zou[1,2,*], Hao Feng[2], Ilmari Pyykko[2]

[1]*Department of Otolaryngology-Head and Neck Surgery, Center for Otolaryngology-Head & Neck Surgery of Chinese PLA, Changhai Hospital, Second Military Medical University, Shanghai, China.*

[2]*Hearing and Balance Research Unit, Field of Oto-laryngology, School of Medicine, University of Tampere, Tampere, Finland*

**Corresponding author: Prof. Jing Zou, Department of Otolaryngology-Head and Neck Surgery, Changhai Hospital, Second Military Medical University, Changhai Road #168, Shanghai 200433, China,Email: zoujinghb@hotmail.com , Jing.Zou@uta.fi*

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## Abstract

Inner ear diseases, such as Meniere's disease and sensorineural hearing loss, significantly impact the work capacity and life quality of affected patients because of the lack of efficient treatments. The causes and mechanisms underlying Meniere's disease and idiopathic sensorineural hearing loss remain unknown. Inflammation secondary to infection or insufficiency of blood supply are accepted as critical steps toward inner ear impairment. Advancements in the understanding of inflammatory signaling pathways have provided an avenue to discover the molecular mechanisms underlying inner ear disease. The present review summarizes advances in knowledge of pattern recognition receptor (PRR) pathway activation, especially with respect to Toll-like receptor (TLR), which plays an important role in the pathological process of inner ear disease. Hyaluronic acid (HYA) is an important molecule in the inner ear and accumulates in the pathological inner ear. HYA receptors are up regulated in the inner ear under pathological conditions. As the most deleterious regulatory mechanism of the TLR signaling pathway, autophagy is beneficial for the cochlea by suppressing potential excess inflammatory reactions via the degradation of key proteins such as A20 and nuclear dot protein-52.

**Keywords**: Hearing Loss; Vertigo; Receptor ; Inflammation;Immune Reaction; Autophagy

## Introduction

Inner ear diseases, such as Meniere's disease and sensorineural hearing loss, significantly impact the work capacity and life quality of affected patients because of the lack of efficient treatments. In China, the overall prevalence of hearing disability is 2.11% [1]. It has been estimated that hearing loss in the USA causes annually costs of 144 billion$ [2]. Balance problems, dizziness and vertigo form another symptom entity related to falls that interferes with quality of life and especially in the elderly drives the individual participants to enter controlled care units in residential homes. In 2011 the total extrapolated costs caused by vertigo and dizziness at emergency department in the USA has been estimated to be $3.9 billion and from that the otologic/vestibular causes were 25.7% ($768; $757 million) [3]. The etiology of Meniere's disease and idiopathic sensorineural hearing loss is unknown. Viral infection, metabolic disorder, immune reaction, inflammation, impaired quality of sleep, and stress are reportedly involved in the occurrence of Meniere's disease and idiopathic sensorineural hearing loss [4-10]. However, the mechanisms underlying inner ear diseases have not yet

been elucidated. Meniere's disease and idiopathic monosymptomatic auditory diseases, such as sudden deafness and other sensorineural hearing loss may have similar mechanism [5]. The delayed endolymphatic hydrops is a good example of linking the pathogenesis of Meniere's disease and other sensorineural hearing loss [11]. A study of mining immune epitopes in the inner ear identified 3036 and 106 unique epitope matches, respectively, the majority of which were infectious epitopes [12]. This suggests that infection-associated reaction may play an important role in the inner ear diseases.

Our previous work of gadolinium-enhanced MRI enabled the visualization of endolymphatic hydrops in an animal model, providing a critical tool for clinical investigation of Meniere's disease [13, 14]. Clinical studies based on gadolinium-enhanced inner ear MRI have demonstrated that endolymphatic hydrops exists in most Meniere's disease patients, but with frequent exceptions [15, 16]. Protein accumulation in the inner ear was detected using MRI in Meniere's disease patients, which possibly leaked through the impaired blood-inner ear barrier [17]. However, endolymphatic hydrops was also detected in idiopathic monosymptomatic diseases including sudden deafness, fluctuant hearing loss, tinnitus, and vertigo that were not diagnosed as Meniere's disease [15].

The blood-inner ear barrier is composed of a blood-perilymph barrier and a blood-endolymph barrier, and impairment of the blood-endolymph barrier, especially of the stria vascularis, has been indicated as a critical pathology of inner ear disease [18-22]. The blood-endolymph barrier is mainly comprised of capillary endothelial cells, as well as strial intermediate and marginal cells. The endolymph-perilymph barrier includes the sandwich structure of Reissner's membrane, spiral ligament fibrocytes, and the cells of the stria vascularis. The physiological role of the blood-inner ear barriers is to maintain concentrations of $K^+$, $Ca^{++}$ and $Na^+$ ions within defined ranges. Advancements in the understanding of inflammatory signaling pathways have provided an avenue to uncover the molecular mechanisms underlying inner ear diseases, such as Meniere's disease and idiopathic sensorineural hearing loss. The present review summarizes advances in understanding of the pattern recognition receptor (PRR) pathway, especially with respect to Toll-like receptor (TLR), and of the regulatory mechanisms underlying inner ear diseases. Hyaluronic acid (HYA), a polymer of disaccharides composed of D-glucuronic acid and D-N-acetylglucosamine, is a component of extracellular matrix that induces physiological and pathological signaling pathway through binding with CD44 and TLR4 (23). Our previous study showed that silver nanoparticle exposure induced glycosaminoglycan accumulation and hyaluronic acid up-regulation in the basement membrane [24].

## TLR signaling pathway activation and regulation mechanism

Microorganism invasion, foreign body stimulation, ischemia, hypoxia, and stress all transmit pathological signals to target cells through specific sensors and induce a sequence of correlated changes. PRRs and their associated signaling pathways serve as bridges, linking various pathogenic factors and cellular responses of individuals. As basic sensors of innate immunity, PRRs recognize both specific pathogen-associated molecular patterns (PAMPs) of invading microorganisms and endogenous damage-associated molecular patterns (DAMPs) resulting from sterile tissue impairment, after which they activate signaling pathways and induce a sequence of reactions [25, 26]. PRRs include membrane-associated PRRs (TLR, C-type lectin receptors) and cytoplasmic PRRs (nucleotide oligomerization domain-like receptors (NLRs), retinoic acid inducible gene I-like receptor (RLR)). TLR-1, -2, -4, -5, and -6 detect lipid and protein ligands in the extracellular environment, whereas TLR-3, -7, -8, and -9 recognize endosomal viral DNA and initiate anti-viral reactions. TLR/10 is expressed in lymphoid tissues involved in immune responses such as spleen, lymph node, thymus, and tonsil. TLR-2 and TLR-4 are the major PRRs that recognize extracellular DAMPs. After recognizing PAMPs or DAMPs, TLRs evoke physiological and pathological innate immunity by recruiting a number of cytoplasmic adaptor proteins containing Toll/IL-1R resistance (TIR) domains, including MyD88, TIR-associated protein (TIRAP)/MyD88 adaptor-like (Mal), TIR domain-containing adapter-inducing IFN-β (TRIF), and TLR-associated molecule (TRAM). MyD88 is a common signaling adaptor for all TLRs except TLR-3, and TRIF is a unique signaling adaptor for TLR-3 and TLR-4. MyD88 further promotes downstream signaling pathway activity and activates NF-κB, which translocates into the nucleus and induces the production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6, among others (Figure 1). When interferon regulatory transcription factor-3 (IRF-3) and IRF-7 are activated, target cells will produce anti-inflammatory cytokines, including IFN-α, IFN-β, TGF-β and IL-10. MyD88 and TRIF may produce different responses to various PAMPs/DAMPs, cell populations, and disease stages. In rats with ischemia-reperfusion lesions, MyD88 was found to induce signaling activity corresponding to inflammation and blood-spinal barrier impairment within 12 h, while TRIF induced reactions at 48 h [27].

There are multiple cellular regulatory mechanisms to prevent overactivation of TLR signaling pathways [28] (Figure 2). Autophagy is the most deleterious regulatory mechanism of the TLR signaling pathway, as it supresses potential excess inflammatory reactions via the degradation of key proteins. The principal players in autophagy are A20, also named tumor necrosis factor alpha-induced protein-3 (TNFAIP-3), and nuclear dot protein-52 (NDP-52). Other enzymes, including lysosome-associated small Rab GTPase, Rab7b, and heme oxygenase-1 (HO-1), are also involved in autophagy regulation [29, 30]. A20, a ubiquitin-editing enzyme, exerts negative control by cleaving

Lys63-linked ubiquitin chains and inhibiting REAF6-mediated NF-κB activation and TRAF3-mediated interferon regulatory factor 3 (IRF3) activation. A20 further promotes Lys48-linked ubiquitination of target proteins, including MyD88 and TRIF, resulting in proteasomal degradation. NDP52 negatively regulates TLR signaling under the control of A20. In the case of A20 insufficiency, NDP52 activation is solely controlled by TRAF6 and mediates aggregation of MyD88-TRAF6 and TRIF-TRAF6 complexes. A20 down-regulates NF-κB signaling in conjugation with RING finger protein 11 (RNF11). A20 and NDP52 insufficiencies are involved in the occurence of autoimmune diseases. Systemic lupus erythematosus and coronary artery disease are associated with A20 gene mutation [31], whereas Crohn's disease is associated with NDP52 gene mutation [32].

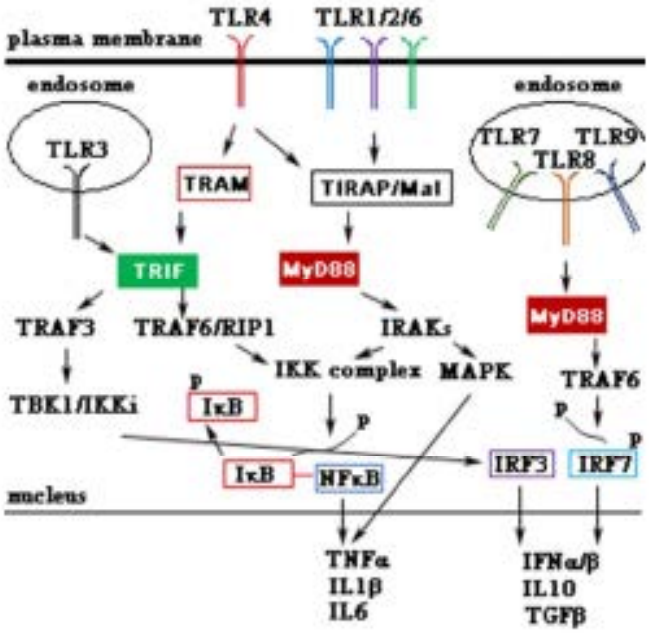


**Figure 1.** TLR signaling pathway. IFNα/β: interferon α/β, IKK: Iκb kinase, IRF: IFN-regulatory factor, MAL: MyD88-adapter-like, MAPK: mitogen-activated protein kinase, RIP1: receptor-interacting protein 1, TBK1: TANK-binding kinase 1, TGF-β: transforming growth factor-β; TIRAP, TIR-associated protein; TRAF, TNF receptor-associated factor, TRAM: TRIF-related adaptor molecule.

### TLR signaling activation in inner ear disease

It has been reported that TLR-2 and TLR-4 are expressed in a spiral ligament fibrocyte cell line [33], and noise exposure up-regulated the expression of TLR-4 in Corti's organ [34]. A study of comparing genotype and phenotype indicated that allelic variants of TLR10 gene may influence the susceptibility and time-course of hearing loss of Meniere's disease in the European population [35]. Numerous DAMPs in the cochlea are capable of activating TLR-2 and TLR-4, such as heat shock protein and hyaluronic acid. Our previous study demonstrated that silver nanoparticles elevated hyaluronic acid content in rat cochlea and caused hearing loss [24]. Heat shock proteins (Hsps) are mainly expressed in the mitochondria and function to clear other proteins under conditions of stress and degeneration. Hsps redistribute within stressed cells, localizing to the cellular surface to form contacts with TLR-2 and TLR-4 and induce immune reactions and inflammation. Yamanobe and Harris, together with our previous studies, uncovered the existence of an antibody against a labyrinth protein with a molecular weight of 68 kDa [36, 37]. Billings et al. demonstrated that this antibody actually recognizes Hsp70 [38]. Our previous animal model studies of acute middle ear infections caused by Klebsiella pneumoniae showed that Hsp70 expression in normal guinea pigs is extraordinarily low. Klebsiella pneumoniae express Hsp70 in addition to Hsp17 after invading the guinea pig middle ear, which peaks at 3 d after infection [39]. Middle ear mucosa of infected animals was found to express Hsp70, Hsp31 and Hsp17, with Hsp70 expression peaking at 3 d, Hsp31 expression peaking at 5 d, and Hsp17 expression peaking at 7 d after infection [40]. It has been reported that Hsp60 activates macrophages and dendritic cells and induces immune response through TLR-2 and TLR-4, which is one of the mechanisms underlying Behcet's syndrome [41]. It is well known that patients with Behcet's syndrome may have accompanying autoimmune inner ear disease.

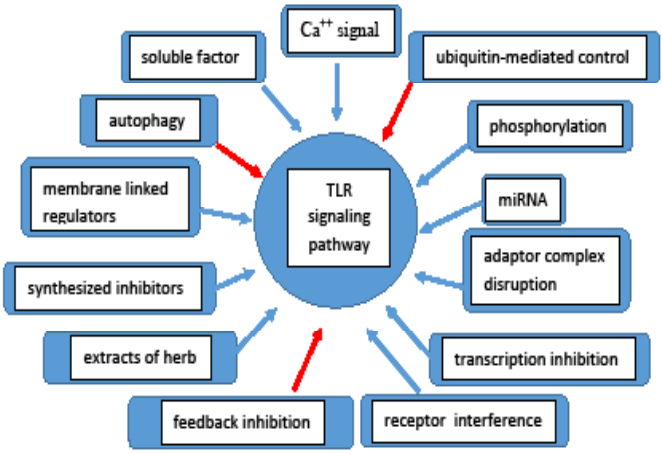


**Figure 2.** TLR signaling pathway checkpoints. Red arrows indicate irreversible regulation points.

As bacteria-released PAMPs, lipopolysaccharides can excessively activate TLR-2 and TLR-4 signaling pathways and may induce inner ear impairment. It has been reported that lipopolysaccharides might be involved in congenital hearing loss [42]. Both lipopolysaccharides and ototoxic drugs are able to up-regulate TLR-4 expression, activate NF-κB, and induce TNF-α, IL-1β, and IL-6 expression in the cochlea. Ototoxic drugs may enhance the interactions between lipopolysaccharides and TLR-4 [43]. Lipopolysaccharides may impair lysosomes in the cochlear stria vascularis in animal models. Lipopolysaccharides have also been shown to induce innate

immune responses in rats and cause hearing loss with elevated TNF-α, IL-1, and IL-6 levels in stria vascularis and spiral ligament cells [44]. In the clinic, serum levels of TNF-α in patients with immune-mediated sensorineural hearing loss are significantly elevated [45]. Genetic studies have shown that individuals carrying the IL1α-889T allele are susceptible to developing Meniere's disease and sudden deafness [46]; the underlying molecular mechanism for this is that the IL-1α gene in IL-1α-889T allele carriers has higher transcriptional activity than in other individuals [47]. Subsequently, an inner ear impairment animal model produced via intratympanic administration of lipopolysaccharide was employed to investigate the efficacy of pharmacological therapy [47].

**Hyaluronic acid (HYA)-induced signaling activity in the inner ear**

Extracellular matrix is a critical substance for cellular communication with the microenvironment. In addition to providing structural support for cells embedded within tissue, the extracellular matrix guides cell division, growth, differentiation, and development through binding to growth factors and hormone. In mammals, there are two main classes of extracellular matrix: fibrous proteins and proteoglycans. Fibrous proteins, including collagen, elastin, fibronectin, and laminin, provide resistance to stretching forces. Proteoglycans, including HYA, are glycosylated proteins, which have covalently attached, highly anionic glycosaminoglycans. The major biological functions of proteoglycans derive from the physicochemical characteristics of their glycosaminoglycan components, which provide hydration and swelling pressure to tissue, protecting it from compressive force.

In mammals, HYA is synthesized by hyaluronan synthases 1-3 (HAS1–3) and is degraded by hyaluronidase 1-2 (Hyal 1-2) and PH20 [48, 49]. HYA is also an important cochlear molecule and was first reported to exist in the endolymph by Vistrup et al. in 1953 [50]. Afterwards, HYA was demonstrated to be extensively distributed throughout the inner ear and involved in inner ear development and maintenance of inner ear homeostasis [51-53]. Under physiological conditions, HYA exists as high molecular weight (1000-10,000 kDa) polymers. During inflammation, HYA exhibits polymolecularity and exists as low molecular weight fragments. The function of HYA depends on its molecular weight and binding receptor status, the presence of hyaluronidase, and the localization of hyaluronan synthase in the cytoplasm. High molecular weight HYA is involved in maintaining tissue structure and promoting tissue integrity, whereas low molecular weight HYA functions as a DAMP and induces innate immunity and acquired immunity [23, 54]. The pathological low molecular weight HYA fragment (50 kDa) is also capable of binding to CD44 and TLR-2/4 and inducing inflammatory responses or enhancing inflammatory reactions that are pre-evoked by other factors. Low-molecular weight HYA fragments may up-regulate the expression of CD44 and TLR-4 and magnify NFκB activation [23, 55]. Our previous study showed that silver nanoparticle exposure caused accumulation of HYA in rat inner ear [24].

**Autophagy in the cochlea**

Immune responses must be well restrained in a steady state to avoid excessive inflammation. Autophagy is the most deleterious regulatory mechanism of the TLR signaling pathway in a way of suppressing potential excess inflammatory reactions via the degradation of key proteins such as A20 and NDP-52 [29]. Autophagy also plays an important role in an early host anti-fungal response by enhancing NFκB activity through A20 sequestration [56].

In the auditory system, autophagy has been shown to be an active and essential process for the migration of otic neuronal precursors during early inner ear development in chickens by providing the energy required to clear dying neuroepithelial cells [57]. Autophagic stress has been detected in the cochlea of the SAMP8 mouse model, which shows premature age-related hearing loss [58]. The beneficial effect of promoted autophagy in the cochlea can be illustrated by the alleviation of cisplatin-induced ototoxicity following treatment with rapamycin, an autophagy activator, which was shown to up-regulate the expression of both the microtubule-associated protein light chain 3 (LC3) and Beclin-1 [59]. Although still controversial, reactive oxygen species (ROS) have been frequently reported as early inducers of autophagy upon nutrient deprivation [60]. Mitochondria are principal sites of ROS production and are able to induce and regulate autophagy [61]. Approximately 20% of inherited post-lingual hearing loss may be caused by mutations in the mitochondrial genome, although substantial ethnic differences might be present [62]. The predominant pathological mechanism is disruption of redox homeostasis caused by mitochondrial ROS accumulation and cellular injury in the cochlea through apoptosis [63, 64].

**Conclusions and future prospects**

PRRs, especially with respect to TLR signaling pathway activation, play important roles in the pathogenesis of inner ear diseases. HYA is an important molecule in the inner ear that accumulates in the pathological inner ear. HYA receptors are up-regulated in the inner ear under pathological conditions. Autophagy is the most deleterious regulatory mechanism of the TLR signaling pathway and beneficial for the cochlea by suppressing potential excess inflammatory reactions via the degradation of key proteins such as A20 and nuclear dot protein-52. Negative regulation of the TLR signaling pathway via A20 occurs in response to pathogenesis. It is necessary to obtain greater understanding of the detailed molecular mechanisms correlated with PRR signaling pathway activation in the inner ear. It is also important to identify the molecular weight range of HYA in the inner ear that is induced by hazard exposure.

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