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Inflammasome activation in mouse inner ear in response to MCMV induced hearing loss

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Abstract

Objective: To identify presence of inflammasome activated in mouse cochlea with sensorineural hearing loss (SNHL) caused by cytomegalovirus (CMV) infection.

Method: MCMV was injected into the right cerebral hemisphere in neonatal BALB/c mice at 2000 pfu virus titers. Auditory brainstem responses (ABRs) were tested to evaluate hearing at 21 days. Histopathological studies were conducted to confirm localizations of MCMV infected cells in the inner ear. Expression of inflammasome related factors was assessed by immunofluorescence, Quantitative real-time PCR and Western blotting.

Results: In the mouse model of CMV induced SNHL, inflammasome related kinase Caspase-1 and downstream inflammatory factor IL-1β and IL-18 were found increased and activated after CMV infection in the cochlea. These factors could further up-regulate expression of IL-6 and TNF-α. These inflammatory factors are neurotoxicity and may contribute to hearing impairment. Furthermore, we also detected significantly increased AIM2 protein that accumulated in the SGN of cochleae with CMV infection.

Significance: We have shown that inflammasome as a novel inherent immunity mechanism may contribute to hearing impairment.

Conclusion: Our data indicate that imflammasome assemble in mouse inner ear in response to CMV infection. We have revealed a novel pathology event in CMV induced SNHL involving activation of inflammasome in mouse cochlea. Additionally, we have shown that inflammasome may be a novel target for prevention and treatment of CMV related SNHL.

Keywords: MCMV; Inflammasome; Caspase-1; SNHL

1. Introduction

Congenital cytomegalovirus (CMV) infection is the leading cause of non-hereditary congenital sensorineural hearing loss (SNHL) (Wang et al., 2013; Ikuta et al., 2015). Previous studies have showed that most congenital CMV infections are asymptomatic, and only 30% of infections are symptomatic at birth (Koyano et al., 2011). SNHL occurs both in symptomatic and asymptomatic CMV infections (Dahle et al., 2000, 1974). It is difficult to prevent congenital CMV induced SNHL through newborn hearing screening (Korver et al., 2009), and treatment

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with ganciclovir, an anti-virus drug, for CMV related SNHL has also failed (Dahle et al., 2000; Fowler, 2013).

Recent studies show that inflammation induced by CMV infection impairs the sensorineural hearing system, which gives a light to understanding the pathology of CMV related SNHL (Schachtele et al., 2011). However, mechanisms and key events regarding how CMV initiates/ignites the inflammation process in the inner ear remains unknown.

The inflammasome is a multiprotein oligomer consisting of caspase 1, PYCARD and the pattern recognition receptors (PRRs) expressed in myeloid cells and is a component of the innate immune system (Martinon et al., 2002). The PPRs initiates inflammasome assembly and determines the exact composition of an inflammasome (Takeuchi and Akira, 2010). A number of inflammasome-forming PRRs have been identified, including the NLR family, pyrin domain containing 1 (NLRP1), NLRP3, NLRP6, NLRP7, NLRP12, CARD domain containing 4 (NLRC4), AIM 2 (absent in melanoma 2), IFI16 and RIG-I (Latz et al., 2013). AIM2 plays an important role in the defense against viral infections (Schroder et al., 2009), particularly in CMV. AIM2 can bind to transfected or pathogen-derived cytoplasmic dsDNA and subsequently recruits Asc by PYD–PYD interaction, which, in turn, binds caspase-1 (Sollberger et al., 2014). The caspase1 is activated in assembling inflammasome and further promotes the maturation the inflammatory cytokines Interleukin 1β (IL-1β) and Interleukin 18 (IL-18), which are some of the earliest and most important alarms to infection (LaRock et al., 2015).

In this study, we reveal a new pathology event in CMV induced SNHL through the activation of inflammasome in mouse cochlea. We further confirm that the DNA sensor AIM2 protein is responsible for CMV activated inflammasome assembling and subsequent ignition of inflammation in the whole inner ear. Our study gives a reasonable interpretation on how CMV initiates inflammation in the inner ear and ultimately induces hearing loss.

2. Material and method

2.1. Virus and animal model

MCMV (Smith strain) was kindly provided by HongMeng of Shandong University. MCMV was replicated in NIH3T3 cells cultured with Dulbecco's modified Eagle's medium (DMEM, gibbco) containing 10% fetal calf serum, streptomycin (300 g/ml) and penicillin (300 U/ml). The supernatant from the MCMV infected NIH3T3 cells was centrifuged at 1600 × *g* for 10 min, and stored as aliquots at −80 °C until use. MCMV was injected into the right cerebral hemisphere of neonatal (within 24 h old) BALB/c mice using 2000 pfu virus titers, with DMEM as mocking injection. Animal experiments were performed in accordance with a protocol approved by the Ethics Committee of the Experimental Animal Center at Xuzhou Medical University.

2.2. Evaluation of hearing loss

Hearing loss was evaluated at 3 weeks of age by measuring the auditory brainstem responses (ABRs). Anesthesia was induced with pentobarbital sodium. Click stimuli were generated. ABR waveforms were recorded in 10 dB intervals down from 90 dB SPL until no waveforms could be visualized (thresholds).

2.3. Histopathological studies

Mice were exsanguinated by transcardial perfusion with phosphate-buffered saline (PBS) and then fixed with 4% paraformaldehyde in phosphate buffer (4% PFA-PB). The Cochlea was dissected away from the skull and immersed in 4% PFA-PB overnight, and decalcified with 100 mM EDTA in PBS for 2 days at 25 °C. The cochlea was then embedded in paraffin blocks. Cochlear sections were cut and mounted on poly-l-lysin-coated glass slides, and stained with anti-MCMV pp89 encoded by MCMV IE-1 as described previously (Koontz et al., 2008). Decalcified cochleae were embedded in low melting point paraplast and sliced into 6-μ m sections, followed by overnight incubation at 4 °C with one of the following primary antibodies: Caspase-1 (ab1872, Abcam) or AIM2 (ab76423, Abcam). Negative controls were prepared in parallel under identical conditions but incubated without primary antibodies. Sections were washed thrice in PBS and incubated with a corresponding secondary antibody (Alexa Fluor 488 or 594, IgG, Thermofisher) diluted in PBS for 1 h. Nuclei were counterstained with DAPI (Invitrogen). Images of immunolabeled specimens were obtained by confocal fluorescence microscopy.

2.4. Quantitative real-time PCR

The total RNA of mouse cochlea was extracted using Trizol reagent according to the manufacturer's protocol (TIANGEN). Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using the ImProm-II TM Reverse Transcription System (Promega, USA). To assess possible gene amplification, quantitative RT-PCR was done using SYBR green chemistry. β-actin as internal standard was arbitrarily assigned a value of 1.0. The sequence-specific primers in quantitative RT-PCR were indicated as follows: IL-6: F: CTGCAAGAGACTTCCATCCAG, R: AGTGGTATAGACAGGTCTGTTGG; TNF-α: F: CTTCTCATTCCTGCTTGTGG, R: CACTTGGTGGTTTGCTACG; IL1β: F: GAAATGCCACCTTTTGACAGTG, R: TGGATGCTCTCATCAGGACAG; AIM2: F: GGTAAAGGAAGGCAGTGAG, R: GACATTGAGGGTGAAGTAGG; Actin: F: CTGAGAGGGAAATCGTGCGT, R: AACCGCTCGTTGCCAATAGT.

2.5. Western blotting

Tissue from mouse cochlea was lysed by RIPA. Western blotting was performed as described previously (Wang et al.,

2008). Briefly, proteins in the SDS-PAGE gel were transferred onto an PVDF membrane (Millipore, Bedford, MA). Blots were then immunoreacted with anti-Caspase1 (ab1872, Abcam), IL18 (ab71495, Abcam), and AIM2 (ab76423, Abcam), diluted in 5% skim milk, and protein bands were visualized using a chemiluminescence detection system ECL plus (GE Healthcare). Signals in the immunoblots were analyzed through exposure on picture.

3. Results

3.1. CMV infection in cochlea and SNHL

To establish the model of CMV induced SNHL, neonatal Balb/c mice were injected in the right cerebral lobe with either medium or the RM461 strain of MCMV. At 21 d post infection, the age when mouse auditory thresholds are considered mature (Ikuta et al., 2015), both control and MCMV-infected mice were tested for the presence of auditory brainstem responses (ABRs) (Table S1 for details). Fig. 1A shows a significantly increased ABR threshold in a CMV infected mouse compared with a control. Immunohistochemistry using IE-1 as a MCMV marker showed that SGN and SV were sites of viral infection (Fig. 1B).

3.2. Cochlear inflammation with CMV infection

Inflammation plays a pivotal role in CMV-induced SNHL that has been described in many previous studies (Schachtele et al., 2011; Wong and Ryan, 2015). Changes of inflammatory factors were detected in this study via Quantitative real-time PCR. Fig. 2 shows significant up-regulation of IL-6, TNF-α and especially IL-1β along with CMV infection in mouse inner ear.

3.3. Assembling inflammasome in cochlea in response to CMV infection

Following detection of increasing mRNA levels of IL-1β by Q-PCR, we next intended to determine whether Caspase-1, an upstream kinase for the activation of IL-1β, was responsive to CMV infection in mouse cochlea. Data showed that Caspase-1 was notably up-regulated at both mRNA (Fig. 3A) and proteins level (Fig. 3B) during CMV-induced SNHL. Increased Caspase-1 mainly localized in SGN cells by immunofluorescence data (Fig. 3C). Moreover, cleaved Caspase-1 and IL-18, another substrate of Caspase-1, was also detected in CMV infected cochleae (Fig. 3B), indicating that inflammasome was activated and played an important role in triggering the inflammation during MCMV-induced SNHL.

3.4. AIM2 as a key regulator for assembling inflammasome during CMV-induced SNHL

Increased AIM2 regulating caspase-1-dependent maturation of IL-1β and IL-18 in response to cytosolic double-stranded DNA from CMV infection (Guerville et al., 2015) was detected by Q-PCR and west blot (Figs. 4A and B),

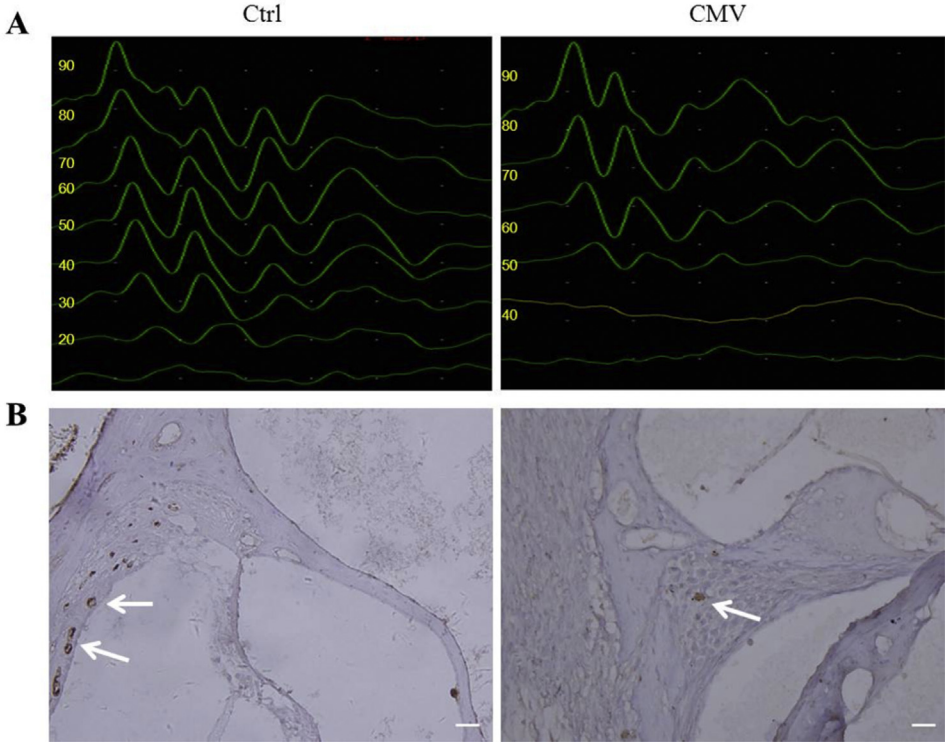

Fig. 1. Inner ear images and SNHL in a CMV infected mouse. A, CMV induced hearing loss detected by ABR thresholds. B, Immunohistochemisty of IE-1 showing the localization of CMV in SV (left) and SGN (right) in the cochlea. Scale bar: 50 μm.

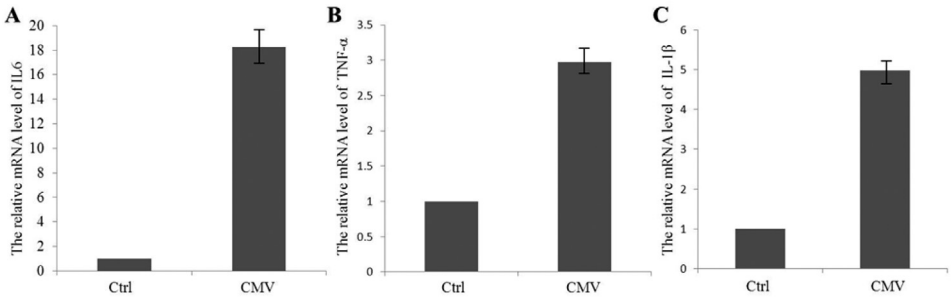

Fig. 2. CMV induced inflammation in mouse inner ear. Increased inflammatory factors including IL-6 (A), TNF-α (B) and IL-1β (C) in mouse cochlea induced by CMV infection.

mostly in SGN cells in CMV infected inner ear (Fig. 4C), similar with a previous study that confirmed Aim2 as an important anti-microbial sensor and a key determinant of protective immunity to viral pathogens (Rathinam et al., 2010).

4. Discussion

In this study, we found CMV positive cells mainly in the SV and SGN cells in CMV infected mouse cochleae (Fig. 1B). This is similar with the colonization of CMV in the human inner ear (Teissier et al., 2011). Moreover, CMV related inflammation also has been detected both in our model (Fig. 2) and in other studies (Harari et al., 2004). Inflammation is a key component of the immune response during infections with different pathogenic microorganisms. Inflammation can be protective by preventing microorganism colonization, replication, invasion, and dissemination. Insufficient inflammation commonly leads to increased susceptibility to infection or prolonged disease. Conversely, excessive inflammation is a driver of several autoimmune diseases and host tissue injury, complicating infectious diseases. Inflammation must therefore

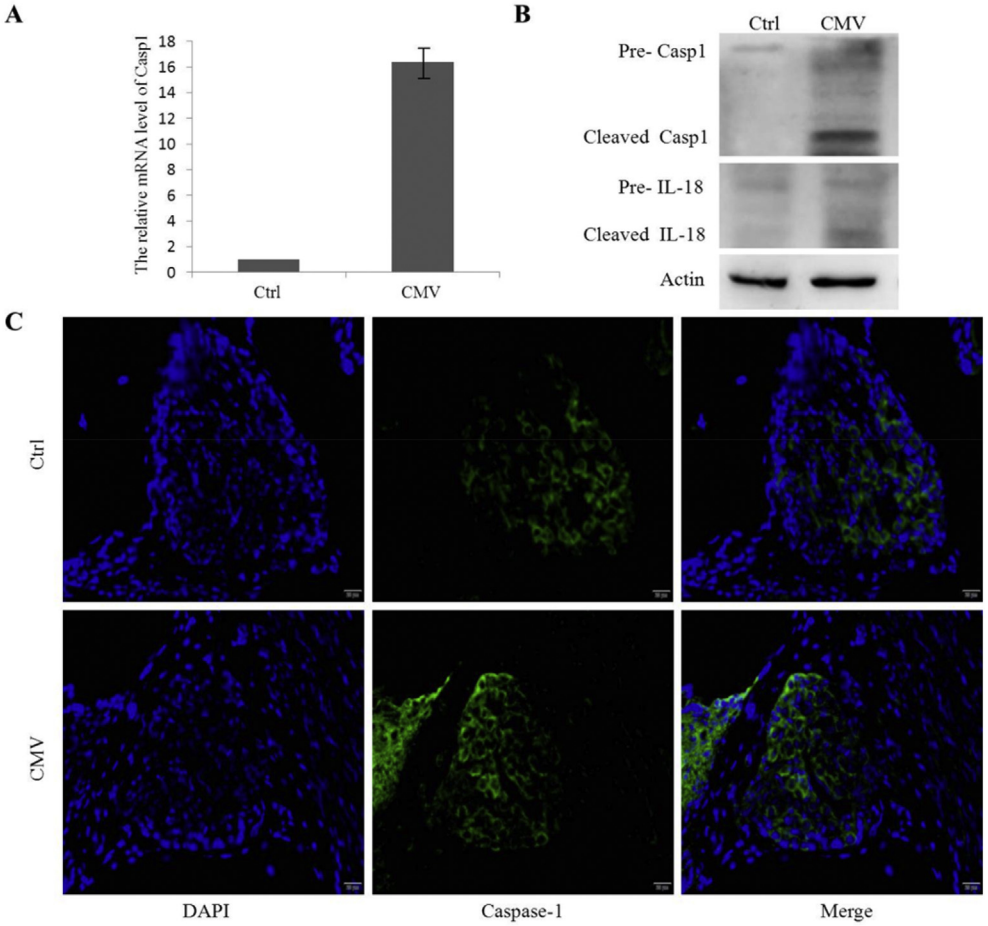

Fig. 3. Inflammasome activation in mouse inner ear after CMV infection. A. Up-regulated mRNA level of Caspase-1 detected by Q-PCR. B. Cleaved Caspase-1 and downstream factor IL-18 detected by WB. C. Immunofluorescence showing increased Caspase-1 in SGN. Scale bar: 20 μm.

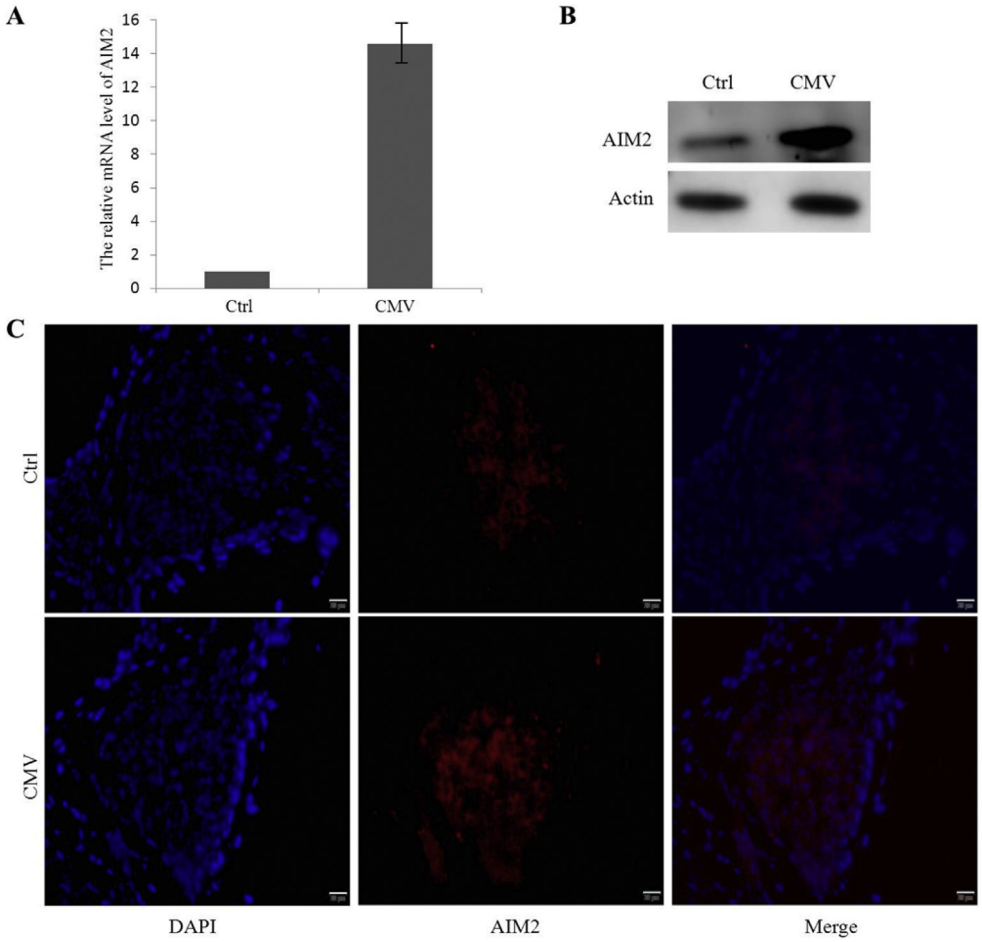

Fig. 4. AIM2 responsible for Inflammasome activation in mouse inner ear after CMV infection. A. Up-regulated mRNA level of AIM2 detected by Q-PCR. B. Up-regulated AIM2 detected by WB. C. Immunofluorescence showing increased AIM2 in SGN. Scale bar: 20 μm.

be carefully regulated for an optimal immune response. However, pathogens usually can exploit the regulatory processes deployed by the host innate immune system to intermediate propagation and invasion (LaRock et al., 2015). For example, inflammation helps break down the blood–brain barrier (BBB) to cause meningitis (Spindler and Hsu, 2012). This phenomenon is consisted with our previous study on LPS helping CMV invade the nervous system (data not shown).

In addition, CMV infected cells discharge/secrete different factors to recruit T-cells and macrophages to eliminate virus infected cells, which can result in neurotoxicity to periphery and central nervous system (Guerville et al., 2015). These observations indicate that inflammation may result in tissue damage and lead to acute complications independent of CMV itself. Thus, even if the pathogen is successfully cleared, the process can be associated with post-infectious sequelae. Therefore, blocking excessive inflammation resulting from CMV infection is important for prevention against CMV induced SNHL.

Our study showed that cleaved Caspase-1 and downstream inflammatory factors including IL-1β and IL-18 were activated in CMV infected cochleae (Fig. 3). These Results indicate that inflammasome assembling plays an important role in CMV induced hearing loss, which is related to initialization of immune responses in the infected cochlea. The IL-18 activation is recently shown to involve AIM2 (Rathinam et al., 2010), which binds its ligand DNA directly and engages ASC to form a caspase-1-activating inflammasome (Fernandes-Alnemri et al., 2009). Herein, we have also characterized Aim2- Caspase-1 pathway as being crucial for innate immunity to CMV in mouse cochlea (Fig. 4).

The possible signaling pathways that are involved in CMV induced inner ear lesions in this mouse model are diagrammatically illustrated in Fig. 5. Briefly, in addition to CMV infection that may directly induce nervous system lesions

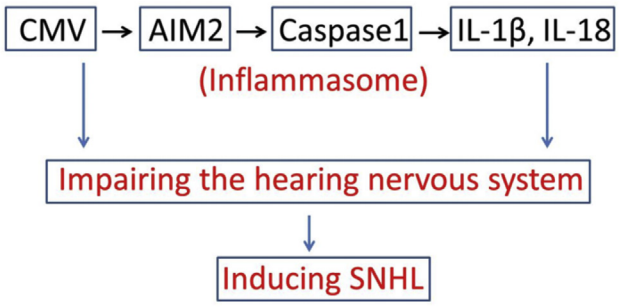

Fig. 5. A diagram of possible pathways about CMV induced SNHL.

affecting hearing, DNA sensor Aim2 engages ASC to form a inflammasome and regulate caspase-1-dependent maturation of IL-1β and IL-18, as well as downstream inflammation in response to CMV infection in mouse cochlea. The perseverative inflammation can potentially result in tissue damage and lead to SNHL.

In addition, the hypothesis on inflammasome playing a key role in hearing lesions resulting from excessive immune reaction is supported by another rare hearing disease – Muckle-well syndrome (MWS), which is caused by a mutation in the NLRP3 gene. The altered gene product cryopyrin leads to activation of inflammasome which in turn is responsible for excessive production of interleukin IL-1β. IL-1β causes the inflammatory manifestations in MWS with progressive sensorineural hearing loss (Kuemmerle-Deschner and Haug, 2013). Therefore, attenuating the activation of inflammasome may be as a new strategy to control hearing impairment induced by excessive immune responses. This strategy illuminates the need in treating CMV induced SNHL to suppress persistent inflammatory state ignited by inflammasome, and to control inflammation in order to avoid organ and other tissue damage.

In summary, this study shows that inflammasome is potentially a novel target for prevention and treatment of CMV related SNHL. Combined with ganciclovir antiviral therapy, other drugs (anakinra, for example) that affect cryopyrin inflammasome and improve sensory deafness in patient with Muckle-Wells syndrome (MWS) (Yamazaki et al., 2008) may be recommended for clinical and laboratory manifestations of CMV induced SNHL.

Conflicts of interest

There are no conflicts of interest in the manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joto.2015.12.001.

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