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Early control of cochlear viral load limits cochlear inflammation and prevents virus-induced sensorineural hearing loss

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Abstract

Human cytomegalovirus (HCMV) is the most common viral infection acquired *in utero* and a leading cause of neurodevelopmental abnormalities, including sensorineural hearing loss (SNHL). In previous studies using a murine model of HCMV induced SNHL, hearing loss was correlated with virus-induced cochlear inflammation but not cochlear viral load. However, these previous findings were determined at the time of auditory testing, a time poiont well past critical periods of auditory development. In the current study, cochlear virus load early in auditory development could be correlated with the magnitude of virus-induced cochlear inflammation, cochlear histopathology and the development of hearing loss. Transcriptional profiling at early times after infection revealed dysregulation of multiple well described deafness-related genes (DRG). Treatment with antiviral antibodies early after infection decreased cochlear virus load, cochlear inflammation, cochlear histopathology, and normalized DRG expression arguing that virus-induced cochlear inflammation can result in pleiotropic effects on the developing auditory system. Finally, this model also demonstrated that sterilizing immunity was unnecessary for prevention of SNHL, thus providing a rationale for inteventions that could limit, but not completely prevent HCMV infection of the developing auditory system.

Keywords Hearing loss, Congenital cytomegalovirus infection, Inner ear inflammation, Viral load

Introduction

Infections with human cytomegalovirus (HCMV) occur in over 50% of populations throughout the world [1]. In immune competent hosts, control of HCMV replication and spread by innate and adaptive immune responses results in minimal clinical evidence of infection whereas immune compromised individuals such as transplant recipients are at risk for significant end-organ disease from HCMV infection [2]. In contrast to most viruses, including other human herpesviruses, HCMV can cross the placenta and infect the developing fetus. Worldwide, HCMV is the most frequently transmitted virus to the developing fetus and results in a congenital HCMV (cCMV) infection in an estimated 0.2–0.5% of all live-born infants [3]. Only about 10% of cCMV infected

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infants present with clinical manifestations, with the most significant of these symptomatic infections involving the central nervous system (CNS) [3, 4]. CNS infection can lead to neurologic damage, developmental delays and other neurologic sequelae [3, 4]. Sensorineural hearing loss (SNHL) is the most common long term sequelae following cCMV infection affecting up to 15% of infected infants regardless of their clinical presentation and has been reported to be the leading cause of non-familial hearing loss in infants and children [3–8]. Though SNHL is a major long term sequelae of cCMV infection, definitive evidence of an effective treatment is lacking and to date, a prophylactic vaccine for cCMV has not been developed. A continued lack of understanding of the mechanism(s) that lead to SNHL after intrauterine HCMV infection constitutes a major hurdle for the development of effective therapies, including antiviral drugs and immunoprophylaxis for prevention and/or treatment of this relatively frequent sequelae of cCMV infections.

Several small animal models have been developed to study fetal infection of HCMV and the associated adverse neurological and audiological outcomes [9–18]. We have developed model that utilizes infection of newborn mice with murine CMV (MCMV), a virus that is genetically closely related to HCMV and that also shares a similar replication program with HCMV, both in vitro and in vivo. In this model, newborn mice inoculated intraperitoneally (ip) with a non-lethal dose of MCMV develop a disseminated MCMV infection in which virus spreads hematogenously to visceral organs such as the liver and importantly, to the CNS [17]. Because newborn mice are similar neurodevelopmentally to a human fetus in the mid-late second trimester of gestation, this small animal model provides an informative experimental system for studies of the impact of viral infections during neurodevelopment, including development of the auditory system [11, 19, 20]. Using this model, we have demonstrated that MCMV spreads to the inner ear shortly after infection and scattered foci of infected cells can be demonstrated in different regions of the cochlea including the stria vascularis, spiral ligament, temporal bone, spiral ganglia, and modiolus; however, viral proteins and/or viral DNA cannot be detected in the sensory epithelium, specifically in the hair cells or surrounding supporting cells in the Organ of Corti [14]. The absence of infection and direct viral cytopathology in the neurosensory epithelium in this model is consistent with findings from histopathological studies of temporal bones from HCMV infected fetuses and newborn infants [21–24]. Lastly, even though MCMV infection in the cochlea is focal following virus spread to the inner ear, cochlear damage is not restricted to foci of infection as altered auditory function induced by MCMV infection is generalized as evidenced by abnormal auditory function across all regions of the cochlea [14, 16].

In our previous studies utilizing this model, we demonstrated a correlation between the quantity of virus in the inoculum and cochlear viral load [16]. Similarly, we noted a dose response between the amount of the inoculum and the overall incidence of SNHL in groups of infected mice; however, it should be noted that hearing loss developed in some animals given low viral inoculums indicating there was not an absolute level of virus replication required to induce SNHL in this model [16]. From these findings, it appeared that increasing the size of viral inoculum resulted in less efficient control of virus replication, accelerated kinetics of virus dissemination, higher viral loads in multiple organs, including the inner ear, increased expression of cytokines and proinflammatory molecules in the cochlea, and an increased incidence and severity of SNHL in infected mice [14, 16]. Yet as noted above, a direct correlation between cochlear viral load and SNHL could not be demonstrated when cochlear viral loads were quantified in individual ears with and without hearing loss 4–6 weeks after inoculation, a time interval in which auditory function could reliably be tested [14, 16]. In contrast to the lack of a correlation between cochlear viral load and hearing loss in these previous studies, there was a significant positive correlation between expression of pro-inflammatory mediators such as IFIT1 and TNF in the cochlea of ears with hearing loss compared to ears with normal hearing thresholds suggesting that immunopathological responses contributed to hearing loss in this model [14, 16]. Consistent with these findings, treatment of infected mice with anti-inflammatory agents modulated cochlear inflammation and prevented SNHL without impacting the level of virus replication in the cochlea [14]. Of direct relevance to results in the current study, the efficacy of treatment with anti-inflammatory agents in limiting cochlear histopathology and ultimately, hearing loss, was dependent on very early treatment as delaying treatment until late in the 1st week of life had little impact on hearing loss in infected animals. Thus, data from available studies strongly argued that virus-induced inflammation early in auditory development and not direct viral damage to structures or cells in the inner ear is a major mechanism of SNHL in this model of human cCMV infection. Even though findings from these previous studies are compelling, our studies have not directly addressed the possibilty that eliminating or potentially decreasing cochlear viral load very early in auditory development could limit SNHL by limiting viral cytopathology or, indirectly by decreasing virus-induced cochlear inflammation. This question is of considerable importance in the deveelopment of therapeutic approaches to limit hearing loss following cCMV infection, and perhaps more importantly

could provide new insight into mechanisms of SNHL that follow viral infections early in auditory development.

In previous studies, we have detailed the role of murine anti-MCMV monoclonal antibodies (mAbs) in the control of MCMV replication in immunocompromised mice and CNS virus replication in this model [25, 26]. Several anti-MCMV mAbs directed at glycoprotein B (gB), an essential envelope component of MCMV, have been shown to have potent in-vitro virus neutralizing activity, inhibit cell-to-cell spread of virus in vitro, and limit virus spread and replication in vivo [25, 26]. Because of the demonstrated activity of these anti-gB mAbs in vivo, we selected two mAbs for use in a treatment protocol of newborn mice infected with MCMV to determine; (i) the impact of anti-viral antibodies on cochlear viral loads, (ii) if early control of cochlear viral load could modify virus-induced inflammation in the developing auditory system, and (iii) if reduction of cochlear viral load early in auditory development could limit the development of SNHL in this model of cCMV infection. A major goal of these experiments was to determine if sterilizing or near sterilizing immunity in the cochlea during auditory development in infected mice was required to limit cochlear inflammation and the development of SNHL, a question of considerable relevance to the design and evaluation of prophylactic vaccines to prevent or modify congenital HCMV infections. Our results demonstrate that early control of cochlear viral load and associated virus-induced cochler inflammation could prevent SNHL in this model of cCMV infection and unexpectedly, demonstrated that virus-induced inflammation altered the expression of multiple deafness-related genes (DRG) early in auditory development in infected mice. This latter observation has suggested several potential mechanisms of hearing loss following MCMV infection early in auditory development in this model of cCMV infection.

Results

Treatment of MCMV infected mice with anti-MCMV mAbs decreases blood and cochlear viral loads

As noted above, passively transferred murine anti-MCMV mAbs can limit MCMV dissemination in immune-deficient mice and similarly, prevent CNS damage in newborn mice infected with MCMV [25, 26]. We have extended these earlier studies to determine the impact of passively transferred virus-specific antibodies on cochlear viral load, cochlear inflammation, and hearing loss in MCMV infected newborn mice. A mixture of two MCMV gB specific mAbs, each of which has been shown to have virus neutralizing activity and limit cell-to-cell spread of MCMV in in vitro assays were selected for these studies [25]. Two days (PNd2) following MCMV infection of newborn mice, the mixture of the two mAbs or a similar quantity of an isotype control mAb was injected ip followed by a second injection of the mAb mixture or isotype control mAb on PNd5. Infected but untreated mice and mock infected, untreated mice served as controls. Mice were harvested on days PNd4, 8, 14, and 32 and processed to determine; (i) the impact of mAb treatment on viral loads in blood and cochlea and virus-induced cochlear inflammation and, (ii) if early antiviral antibody could limit hearing loss in infected mice as determined by auditory brainstem responses (ABR) prior to harvest on PNd34.

Treatment with anti-MCMV mAbs resulted in a significant reduction of viral DNA in the blood of infected mice on PNd4, 8 and 14 compared to untreated and isotype mAb treated, infected animals (Fig. 1A). At PNd34 there was also a decrease in the viral load in blood in the MCMV mAb treated mice as compared to untreated and isotype mAb treated infected animals (Supplemental Fig. 1A). Treatment with MCMV mAbs also reduced the quantity of viral DNA in the cochlea of infected mice on PNd4, 8, 14, and 32 when compared to untreated, infected and/or isotype treated infected control mice (Fig. 1B; Supplemental Fig. 1B). Although there was considerable reduction in cochlear viral loads in anti-MCMV mAb treated mice as compared to untreated and/or isotype treated infected mice, significant amounts of virus could be detected in cochlea from treated animals on PNd8 and 14 as compared to treated animals on PNd4 indicating that sterilizing immunity was not achieved with this treatment protocol and by inference, that MCMV replicated in the cochlea of treated, infected mice (Fig. 1B). Together, these data further demonstrated the activity of this mixture of anti-gB mAbs in vivo, including a reduction in the amount of virus in the blood and perhaps more relevant to this study, a decrease in the quantity of virus present in the target organ of disease in this model, the cochlea. It is also important to note that the even though sterilizing immunity was not achieved with mAb treatment, the decrease in cochlear virus on PNd4, 8, and 14 in mice treated with mAbs took place during a critical developmental period of the auditory system in mice, including onset of hearing [11, 19, 20]. Moreover, the decrease in cochlear virus on PNd4 and 8 in MCMV mAb treated mice paralleled previous findings in which lower viral inoculums were associated with lower cochlear viral loads and importantly, a lower overall incidence of hearing loss in this population of mice infected in the newborn period [16]. These observations raised the possibility that antiviral antibodies could modify the impact of cochlear infection, including hearing loss associated with MCMV infection in this model of cCMV infection.

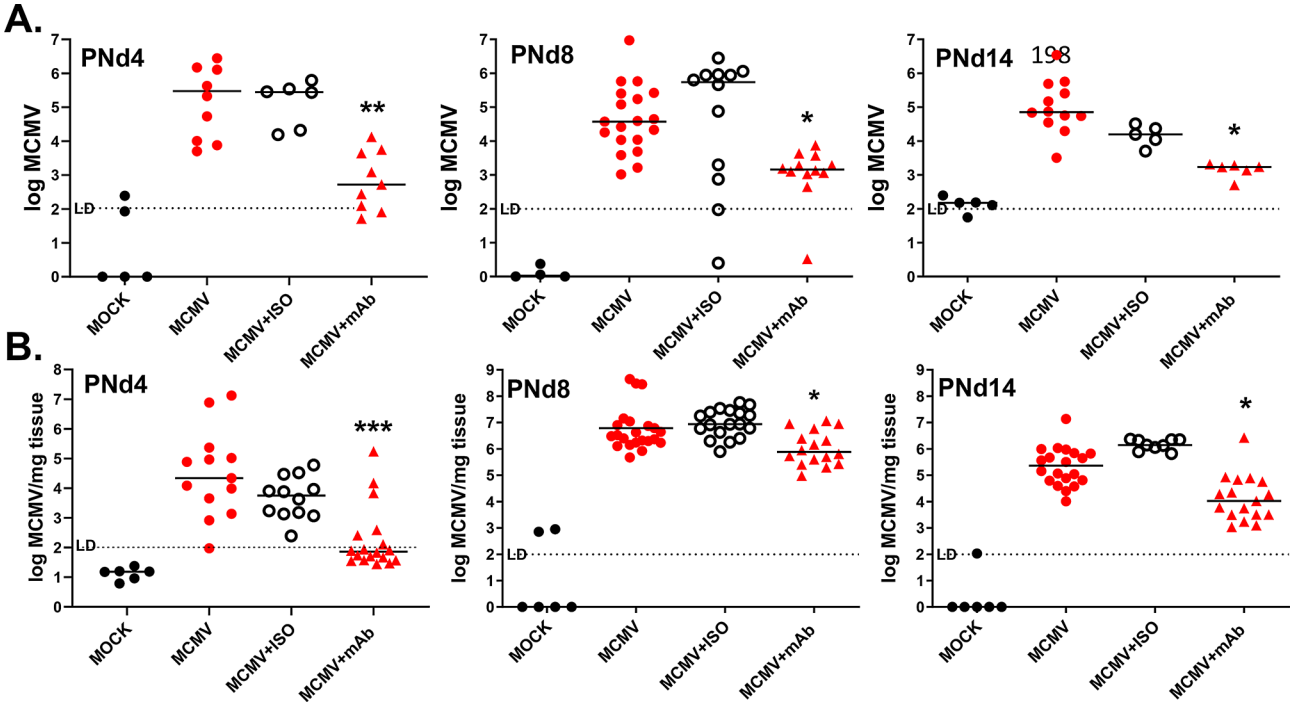

Fig. 1 Treatment with anti-MCMV mAbs decreases viral load in blood and cochleae of mice infected in newborn period. Newborn mice (PNd0) were inoculated ip with 200PFU of MCMV. On PNd2 and PNd5, infected mice were given 100ug of isotype control antibody (MCMV + ISO), 100ug of a mixture murine mAbs 97.3 + M11 (MCMV + mAb) by ip injection, or left untreated (MCMV). A group of uninfected and untreated age matched animals served a mock controls (MOCK). **(A)** Blood viral load from on PNd4, 8, and 14 MCMV infected mice that were treated with isotype control antibodies (MCMV + ISO), mAb treated (MCMV + mAb), untreated (MCMV) and uninfected control mice (MOCK) assayed for MCMV genome copy as described in methods. Each data point represents one mouse. Median of group is shown as horizontal bar with limit of detection (LD) of assay shown as broken line. **(B)** Cochlear viral load on PNd4, 8, and 14 from MCMV infected mice treated with isotype antibody treated (MCMV + ISO), mAb treated (MCMV + mAb), infected and untreated (MCMV), or uninfected (MOCK). Cochleae were processed and MCMV genome copy number determined as described in methods. Each data point represents one cochlea and median indicated by horizontal bar. Experimental groups were compared to MCMV-infected, untreated group to determine treatment effect. Statistical significance was determined by Kruskal-Wallis tests with a Dunn's multiple comparisons post-test ($^{*}P < 0.05$ and $^{**}P < 0.01$). For each experimental group at each timepoint, $n = 3$–11 mice (6–22 cochleae)

Treatment of MCMV infected mice with anti-MCMV mAbs decreases cochlear inflammation

Our previous studies have demonstrated that MCMV infection of the cochlea leads to the increased expression of a large number of pro-inflammatory molecules, including products of interferon stimulated genes (ISG), cytokines, and chemokines [14, 16]. Moreover, virus-induced inflammatory responses appear responsible for MCMV-induced histopathology in the inner ear and hearing loss in this model [14, 16]. To determine the impact of anti-MCMV mAb control of cochlear viral load on virus-induced cochlear inflammation, we quantified expression of an interferon stimulated gene (ISG), IFIT1, and several cytokines previously shown to be upregulated during MCMV infection and identified as surrogates for MCMV induced hearing loss and CNS maldevelopment in this model [14, 27, 28]. The level of transcription of IFIT1 was significantly increased in the cochlea at PNd4, 8 and 14 of MCMV infected but untreated mice (Fig. 2).

Similarly, the expression of TNFα and IFNγ were increased on PNd4, 8, and 14 in the cochleae of MCMV infected mice (Fig. 3). Increased expression of IL-1β was detected at PNd8 and 14 but not on PNd4(supplemental Fig. 2A). Treatment of infected mice with the mixture of anti-MCMV mAbs resulted in a decrease in the expression of TNFα and IFNγ in the cochleae on PNd4, 8, and 14 to levels that did not differ from those in uninfected, control mice (Fig. 3). In addition, treatment of infected mice with anti-MCMV mAbs also decreased the expression of RANTES (CCL5) and ICAM1 in the cochlea at PNd8 and 14 (Supplemental Fig. 2B). These findings indicated that the reduction in cochlear viral load following treatment with anti-MCMV mAbs resulted in a decrease in virus-induced cochlear inflammation.

To further establish the relationship between cochlear viral load and cochlear inflammation, the expression of IFIT1, TNFα, and IFNγ were plotted as function of cochlear viral load on PNd4 (Fig. 4). A significant correlation between viral load and the expression of proinflammatory molecules was noted (Fig. 4). Notably, the impact of anti-MCMV mAb treatment on cochlear viral load and the corresponding decrease in virus-induced

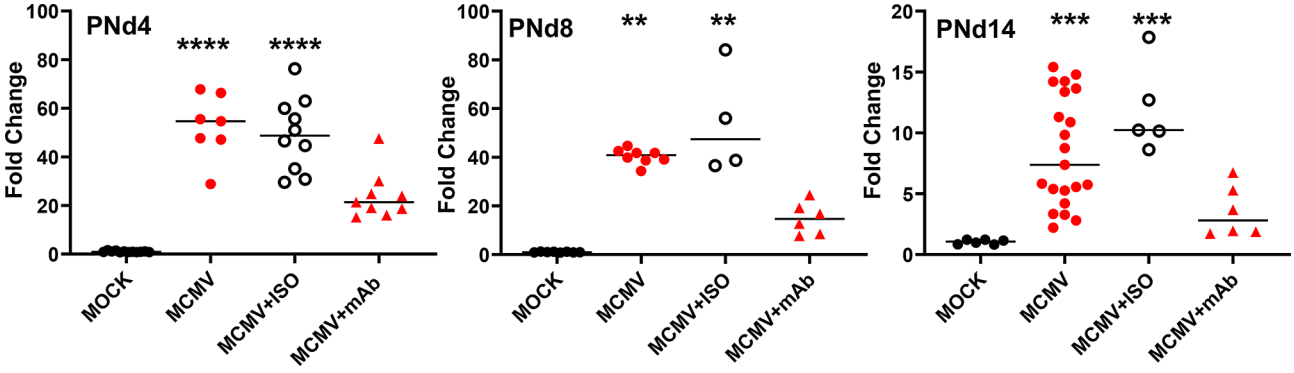

Fig. 2 Treatment with anti-MCMV mAbs decreases expression of IFIT1 in the cochleae of MCMV infected mice. Expression of IFIT1, an interferon-stimulated gene, induced by viral infection at PNd4, 8, and 14 was quantified in individual cochlea as described in methods. Statistical analysis was performed by Kruskal-Wallis tests with a Dunn's multiple comparisons post-test ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$). All experimental groups were compared to mock infected mice (MOCK) with data presented as medians. Median values for MCMV and MCMV + mAb groups were statistically different at all time points ($p<0.05$;Mann-Whitney test). For each experimental group at each time point, $n=2$–12 mice (3–24 cochleae)

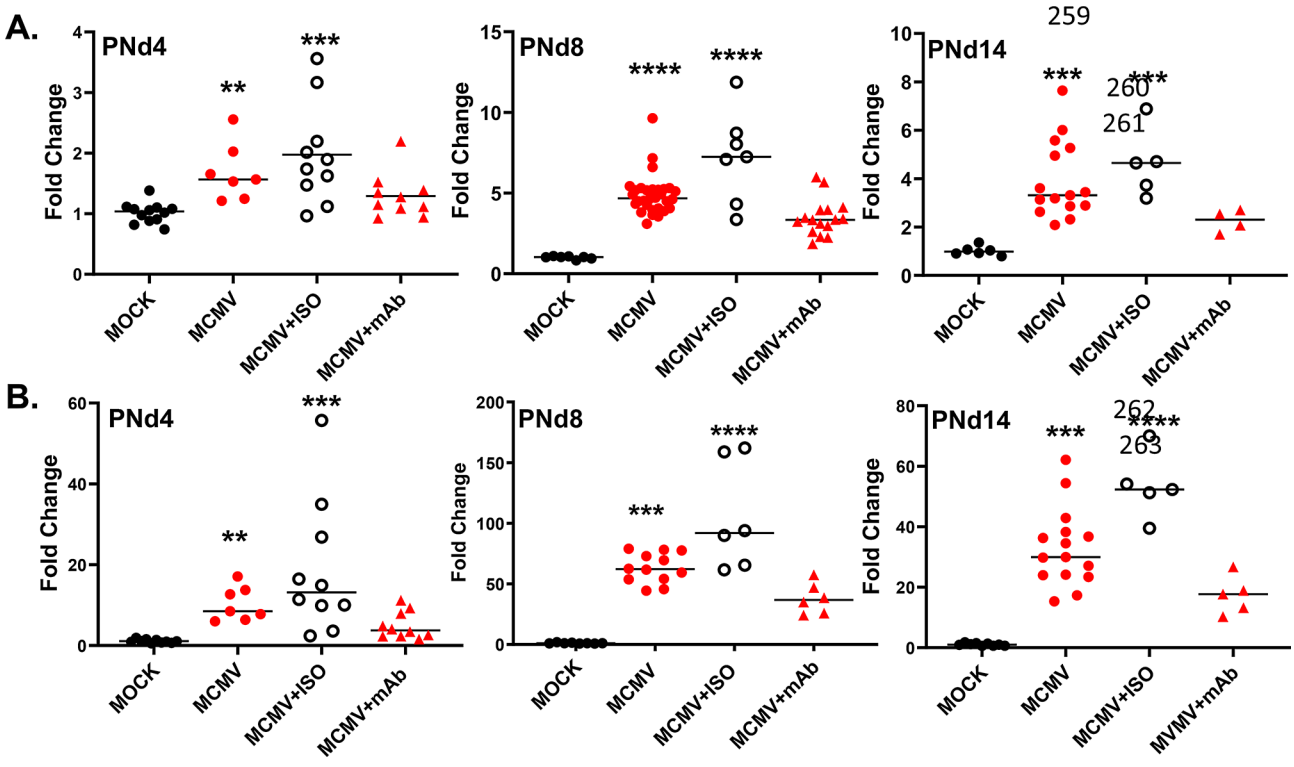

Fig. 3 Decreased viral load in the cochlea is associated with a decrease in the expression of pro-inflammatory molecules in the cochlea. Cochleae from mice at PNd4, 8, and 14 were collected as described in Fig. 2. Expression of inflammatory mediators **(A)** TNFα and **(B)** IFNγ were quantified by qRT-PCR at each time point. Each data point represents an individual cochlea with data being displayed as medians. Comparison between all groups and mock with Kruskal-Wallis with a Dunn's multiple comparisons post-test performed to determine statistical significance ($^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, $^{***}P<0.0001$). Median values for MCMV and MCMV + mAb groups were statistically different at all time points ($p<0.05$;Mann-Whitney test). For each experimental group at each time point, $n=4$–15 mice (8–30 cochleae)

inflammatory responses in the cochlea was further illustrated by these data (Fig. 4). When viewed together with findings presented above, these results indicated that reduction of cochlear viral load by antiviral antibodies could modulate virus-induced inflammation in the inner ear during a critical period of auditory development in this model of cCMV infection. Lastly, the reduction in virus-induced imflammation was not dependent on sterilizing immunity in the cochlea, suggesting that some degree of regulation of cochlear inflammation was operative during this early developmental period.

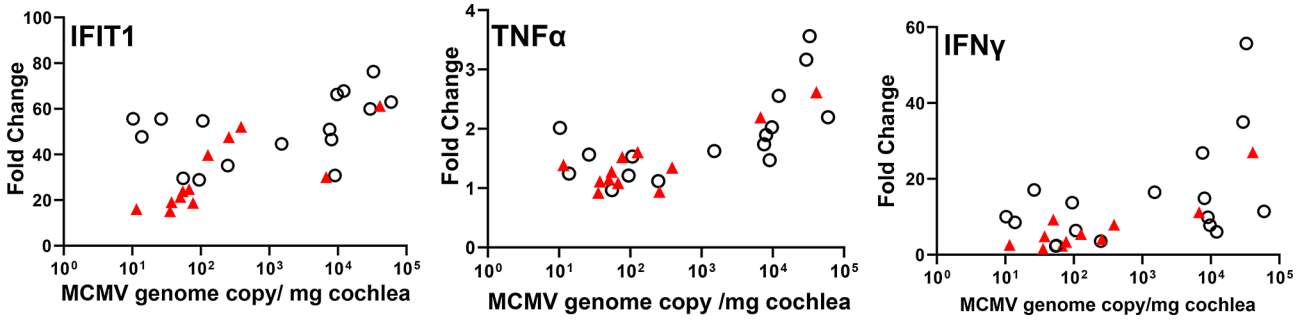

Fig. 4 Positive correlation between cochlear inflammatory response and the viral load. Cochlear expression of IFIT1, TNFα, and IFNγ in PNd4 MCMV infected mice was plotted as a function of cochlear viral load from the same animal to establish correlation between gene expression of inflammatory mediators and respective viral load. Red triangles (△) represent samples from mAb treated mice and open circles (○) represent samples from isotype control antibody treated or untreated but infected mice. Each data point represents an individual cochlea/matching cochlear viral load. Note that all viral load values, even those with values below the LD of the assay, were included in this analysis to limit bias from selection of only specimens with values above the LD of the assay. A Pearson's correlation was performed to determine the r value with a 95% confidence interval for each gene. For correlation with IFIT1: $r = 0.6147$, $P = 0.0005$; TNFα: $r = 0.6579$, $P = 0.0001$; and IFNγ: $r = 0.5249$, $P = 0.0041$. Each correlation, $n = 13$ - 14 mice (26 -- 28 cochleae)

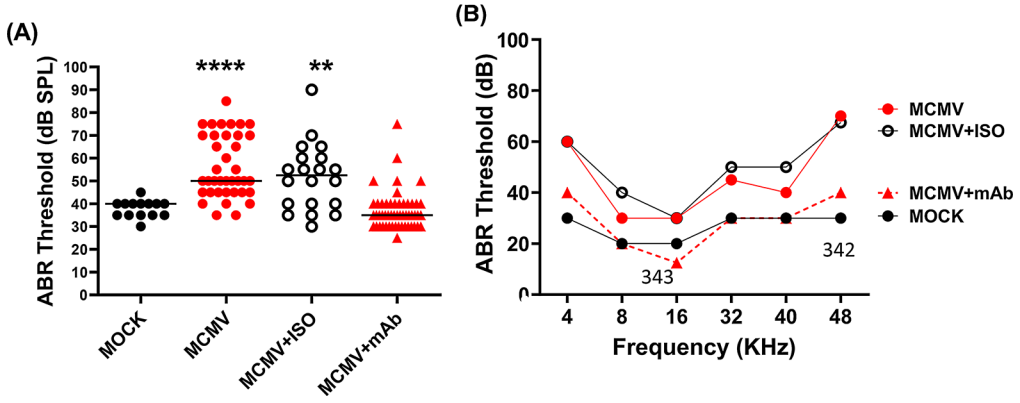

Fig. 5 Treatment with anti-MCMV mAbs limits hearing loss in MCMV infected mice. **(A)** Auditory brainstem responses (ABR) clicks and **(B)** tones were performed on PNd34 in all mice. Significantly more ears in mice infected (MCMV) and isotype control antibody treated (MCMV + ISO) exhibited hearing loss compared to uninfected controls (MOCK) and/or infected mAb treated mice (MCMV + mAb). Noted that hearing loss was across the entire cochlea as indicated by the loss across frequencies from 4–48 kHz. Each datapoint represents an individual ear with median values shown. Statistical significance was determined by a Kruskal-Wallis test followed by a Dunn's multiple comparisons post-test ($^{**}P < 0.01$, $^{****}P < 0.0001$). For each experimental group, $n = 7$–26 mice (14–52 ears)

Decreasing viral load and virus-induced inflammation limits hearing loss and histopathological damage in the cochlea

To examine the impact of early control of cochlear viral load on the development of hearing in infected mice, auditory brainstem response (ABR) testing was performed at PNd34. Two different ABR tests were performed; (i) the ABR click test was used to quantify the hearing threshold and, (ii) the ABR tone test was used to determine hearing thresholds at specific frequencies which are registered at different locations along the cochlea and therefore, can help localize altered auditory function to specific cochlear regions. We found that a reduction of cochlear viral load in anti-MCMV mAb treated mice significantly decreased the incidence of elevated sound pressure level (SPL) thresholds that are indicative of decreased auditory function when compared to thresholds in MCMV infected control mice or infected mice treated with an isotype control mAb (Fig. 5A). The significant elevation of hearing thresholds across all frequencies in untreated and isotype mAb treated infected mice was consistent with previous studies in this model and suggested that the entire cochlea was impacted by infection and that the decrease in damage to auditory pathways following mAb treatment limited was not restricted to a specific area of the cochlea (Fig. 5B) [14]. We have previously shown that MCMV induced hearing loss in this model is associated with decreased output/conductivity from inner hair cells in the cochlea [14]. Wave 1 amplitude in the ABR tracings indicated a decrease in the magnitude of the wave growth in MCMV infected but untreated mice but not in the mAb treated infected mice as compared to controls (Suppl Fig. 3). This finding of delayed growth inwave I amplitude was further illustrated in similar graphs in which MCMV infected but untreated mice with and without decreased

SPL thresholds were plotted separately (Suppl Fig. 3). Together, these data strongly argued that decreasing cochlear viral load during this early period of auditory development could limit virus-induced cochlear inflammation and subsequent hearing loss in MCMV infected mice.

Spiral ganglion neurons (SGN) play a critical role in hearing function by relaying electrical signals produced in the sensory epithelium of the cochlea to auditory pathways leading to the cochlear nuclei in the brainstem [29]. Loss of SGN is a well described histopathological finding associated with several different etiologies of SNHL, including MCMV infection in this model [14]. Furthermore, damage to the sensory epithelium very early in auditory development has been shown to lead to the loss of SGN, suggesting that SGN loss could be a marker for early cochlear damage in this model [30–32]. To determine the impact of control of cochlear viral load early in auditory development on spiral ganglion loss, infected mice that were treated with MCMV specific mAbs or left untreated, were harvested at PNd8 and SGN quantified as previously described and illustrated in the supplemental material (Suppl Fig. 4) [14]. We found a significant decrease in the number of SGN in ears from untreated, MCMV infected mice compared to mice treated with the mixture of anti-MCMV mAbs or control, mock infected mice (Fig. 6). Consistent with findings from the ABR testing described above, there were no significant differences in SGN counts in any specific region of the cochlea between uninfected, control mice and infected mice treated with anti-MCMV mAbs (Fig. 6). These data indicated that the early control of the cochlear viral load not only limited development of hearing loss in MCMV-infected mice but also decreased histopathological changes in the inner ear of infected mice associated with MCMV infection [14, 16]. Thus, modulating either the quantity or kinetics of virus dissemination to the cochlea could limit virus-induced cochlear inflammation and the resulting damage to auditory pathways during a critical developmental period.

Virus-induced inflammation during early auditory development is associated with altered expression of deafness related genes

Previously we have shown that early treatment with anti-inflammatory agents limited hearing loss and cochlear damage in infected mice [14]. This observation together with findings in the current study demonstrating that treatment with antiviral antibodies modified virus-induced inflammation in the developing auditory system, suggested that defining the cochlear transcriptome from infected mice early after infection could potentially identify altered expression of host genes required for the development of hearing. Gene set enrichment analysis (GSEA) of results from bulk RNA sequencing data derived from cochleae of control and infected mice on PNd4 indicated increased expression of antiviral genes and decreased expression of host genes related to tissue formation and tissue remodeling in infected mice as compared to mock infected, control mice (Fig. 7A). When displayed as heat maps, a clear distinction can be appreciated between the expression of these host genes in cochleae from uninfected mice as compared to infected mice (Fig. 7B).

Because of the impact of MCMV infection on auditory development in these young mice, we next surveyed the expression of a group of known deafness-related genes (DRG) in cochleae from infected and control, uninfected mice. Again there was a clear difference between the expression of DRGs in cochleae from uninfected and infected animals on PNd4 (Suppl Fig. 5). Of note, multiple well described DRGs were significantly dysregulated

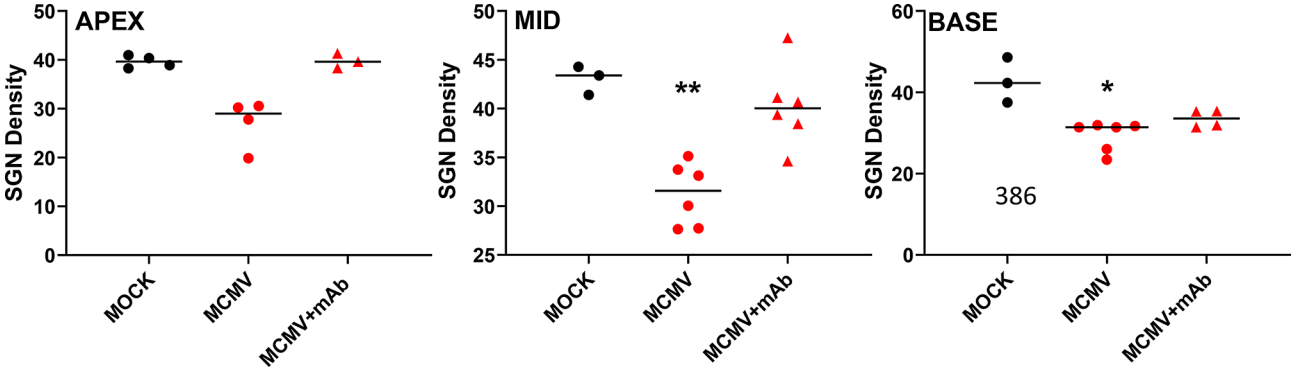

Fig. 6 Treatment with anti-MCMV mAbs limits the loss of spiral ganglion neurons in inner ears of MCMV infected mice. Cochleae were immunostained for tuj-1 (neuronal marker) and imaged by confocal microscopy as described in methods. Spiral ganglion neurons (SGN) were counted in the base, mid, and apex regions of the cochlea. Spiral ganglion from MCMV infected mice contained significantly fewer SGN in the Rosenthal's canal compared to cochlea from uninfected control (MOCK) or MCMV infected mAb treated (MCMV + mAb) mice. Data displayed as medians with each data point representing an individual ear. All experimental groups compared to MOCK using Kruskal-Wallis test with a Dunn's multiple comparisons post-test to determine significance ($^{**}P < 0.01$, $^{*}P < 0.05$). Note that for MCMV group in APEX, p-value was 0.0504 in comparison the other groups. For each group, $n = 2$–3 mice (3–6 cochleae)

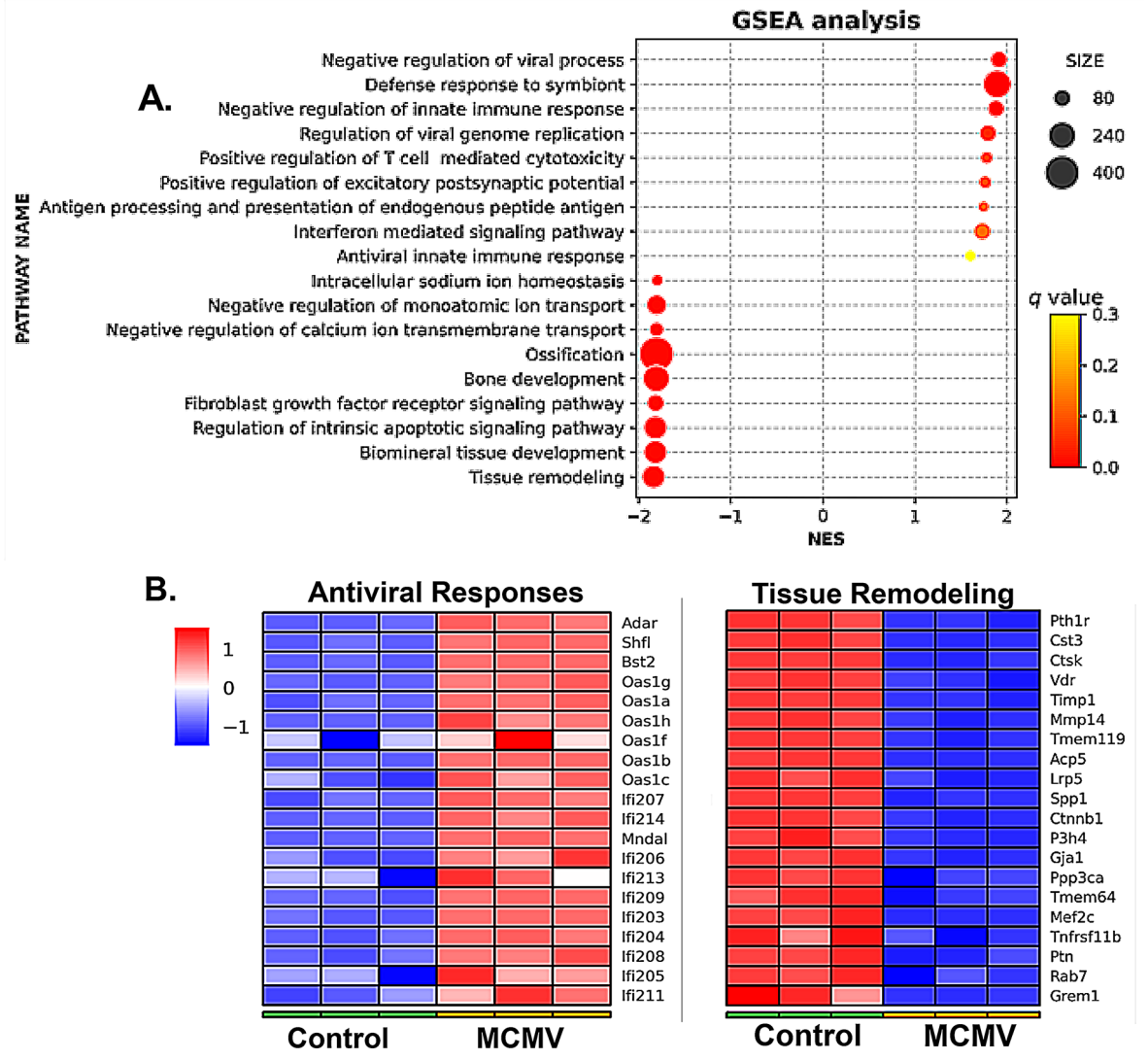

Fig. 7 RNA-seq analysis at PNd4 defines altered cochlear environment in MCMV-infected mice at PNd4. RNA was isolated from cochlea o PNd4 mice and RNA-seq performed as described in the methods. **(A)** GSEA analysis was performed to determine pathways most heavily enriched in MCMV-infected mice. Select pathways with their respective pathway gene set size and q value are shown in this panel. **(B)** Representative heatmaps of pathways related to response to virus and tissue remodeling at PNd4 illustrate the clear difference in gene expression between cochleae from uninfected controls and MCMV-infected mice

in the cochlea of MCMV infected mice, including GJA1, GJB2, TMIE, and KCNE1 (Suppl Fig. 5). These findings suggested that virus-induced cochlear inflammation resulted in dysregulated expression of DRGs that contribute to auditory development, thus providing a potential mechanism(s) for altered hearing in MCMV infected mice.

From the results of transcription profiling at PNd4 in control and MCMV infected mice, we selected candidate DRGs for further analysis by qPCR (Fig. 8). Several of these DRGs have well described phenotypes in genetically engineered mice, including GJB2 (connexin 26), GJA1 (connexin 43), KCNE1, and OTOS (otospiralin) [33–47]. The expression of each of these specific DRGs was reduced in infected mice and infected mice treated with an isotype control antibody whereas treatment of infected mice on PNd2 with anti-MCMV mAbs resulted in near normal levels of expression of these DRGs (Fig. 8). Together with data in previous sections, these findings provided additional evidence that increased cochlear viral load and the corresponding virus-induced cochlear inflammation early in the development of the auditory system altered expression of key DRGs, thus providing a potential mechanism(s) that could contribute to hearing loss and histopathologic findings in this model of SNHL associated with cCMV infection.

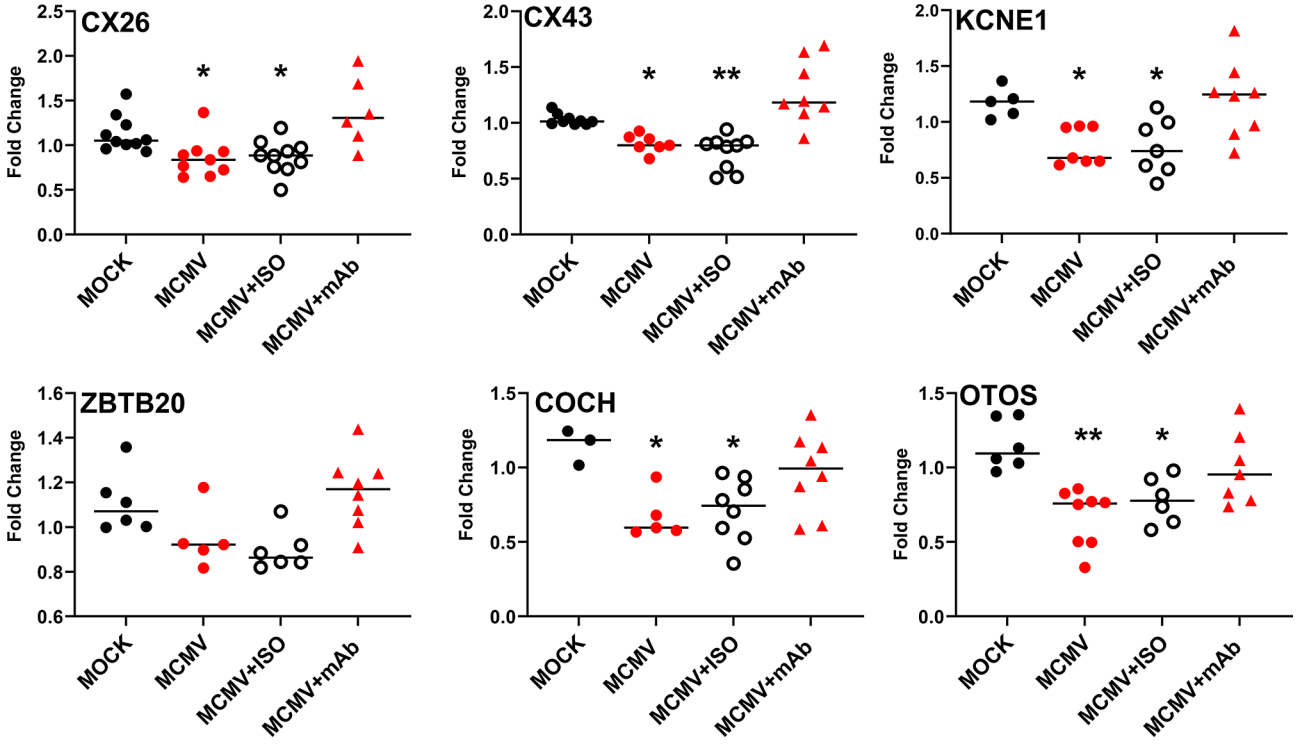

Fig. 8 Treatment with anti-MCMV mAbs prevents decreased expression of deafness-related genes in the cochlea during early auditory development. **(A-F)** Validation of decreased expression of select deafness related genes identified by RNA-seq. Cochleae collected on PNd4 and expression of Cx26, Cx43, KCNE1, ZBTB20, COCH, and OTOS determined by qRT-PCR as described in Fig. 2. Significant decrease of DRG expression in cochleae from MCMV-infected, untreated (MCMV) and isotype control antibody treated cochleae (MCMV + ISO) as compared to cochleae from infected mAb treated (MCMV + mAb) and uninfected control mice (MOCK) for each of the selected genes with exception of ZBTB20. Each data point represents individual cochlea with median of group indicated by horizontal bar. All groups compared to MOCK and statistical significance determined by a Kruskal-Wallis test with Dunn's multiple comparisons post-test ($^{*}P < 0.05$, $^{**}P < 0.01$). For each group, $n = 3$–5 mice (6–10 cochleae)

Discussion

Although extensive epidemiological data has identified several characteristics of maternal HCMV infection during pregnancy that are associated with SNHL in infants infected *in utero* with HCMV, a unifying mechanism that accounts for the development of SNHL and importantly, the variable natural history of SNHL following cCMV infection, remains undefined. The lack of understanding of pathogenesis of this sequelae of cCMV infection has likely contributed to the empiricism that continues to surround the design of treatment trials with antiviral agents and similarly, strategies for vaccine development and deployment to prevent SNHL in infants with cCMV. Results from the current study as well as previous findings in this model system strongly argue that virus-induced cochlear inflammation associated with virus replication during early auditory development has a proximal role in SNHL in infants with cCMV infection [11, 16]. Although earlier studies in this model failed to show a correlation between viral load and virus-induced inflammation in mice with hearing loss, these earlier results were derived from cochlear tissue assayed at the time of initial hearing assessment in infected mice (PNd34) and likely failed to capture contributions of viral load to virus-induced inflammation during early periods in auditory development. Insults to the auditory system early in development lead to several different phenotypes of hearing loss that are not observed when such insults occur later in auditory development as illustrated by different hearing phenotypes observed in engineered murine models with mutations in well described DRGs [33–47]. Consistent with the phenotypic expression of mutations in specific DRGs, severe to profound hearing loss in infants infected *in utero* with HCMV has been more commonly associated with maternal HCMV infections that take place in the 1st and early 2nd trimester of gestation, an interval that could lead to intrauterine transmission and fetal infection during early development of the auditory system in the mid-2nd trimester of gestation [48–50]. In the current study, virus-induced cochlear inflammation was directly correlated with cochlear viral load during early auditory development and therefore, more likely reflected the impact of cochlear inflammation during this early developmental time on the development of hearing. Thus, control of virus replication during early development of the auditory system would seem to be required to limit cochlear damage and prevent hearing

loss in infants infected *in utero*. Vaccine-induced protective immunity in women of childbearing age offers an obvious strategy to limit virus replication in an infected fetus and more recently, data from clinical studies have suggested that treatment of pregnant women undergoing HCMV infection with antiviral drugs represents another strategy to limit damage to developing auditory pathways [51–53]. Alternatively, approaches that specifically target virus-induced cochlear inflammation in infants infected *in utero* with HCMV could potentially provide adjunctive therapy and further decrease the incidence of SNHL in infants with cCMV infection.

In the current study, we selected two mAbs reactive with MCMV gB for treatment of infected newborn mice. These mAbs neutralized cell free virus, efficiently blocked cell-to-cell virus spread, and were of IgG subclasses that could engage activating Fc receptors [25]. Treatment with these antiviral mAbs two days following infection likely limited virus dissemination to the cochlea and/or contributed to the control of MCMV virus replication within the infected cochlea during this early period of auditory development. Although either or both mechanisms could account for lower cochlear viral loads in mAb treated mice, it is likely that the impact of these mAbs on cochlear viral loads observed on PNd4 was secondary to a decrease in the quantity of virus that disseminated to the cochlea. Such a mechanism would be consistent with dependence of cochlear viral loads on the quantity of the viral inoculum that we observed in a previous study [16]. This previous study also demonstrated significant cochlear inflammation as early as PNd4 in infected mice given higher viral inoculums arguing that viral dissemination could result in high cochlear viral loads and virus-induced cochlear inflammation early after infection [16]. Finally, reducing the amount of virus reaching the cochlea could also facilitate early control of virus replication within the cochlea by resident and infiltrating innate effector functions, thus limiting virus-induced inflammation and cochlear damage leading to the development of SNHL.

Cochlear inflammation is frequently associated with hearing loss that follows a number of different insults to the inner ear [32, 54, 55]. Although infectious agents are a well recognized source of cochlear inflammation, cochlear inflammation and hearing loss have also been associated with ototoxic drugs, auto-inflammatory diseases, and physical trauma including noise-induced cochlear damage and mutations in DRGs [32, 35, 54–57]. Even though SNHL is a well known sequelae of congenital, perinatal, and CNS infections that occur in early infancy, mechanisms of SNHL following cochlear inflammation during early auditory development remain less poorly defined. Intrauterine infections with viruses such as Rubella, HCMV, and Zika virus and with the parasite, Toxoplasma gondii, are well described etiologies of SNHL in infants and children [58–61]. Direct pathogen mediated damage to cells of the sensory epithelium has not been consistently demonstrated in studies of temporal bones from cases of congenital and perinatal infections with these agents, suggesting that direct pathogen mediated destruction of sensory epithelia is not a unifying mechanism for hearing loss following infection of the inner ear of a developing fetus or newborn infant [24, 62–64]. Results from several studies of temporal bones from HCMV infected fetuses and infants have demonstrated inflammatory cells infiltrating the cochlea but rarely identified infection and/or viral cytopathic changes in cells of the neurosensory epithelium [21–24]. Furthermore, the severity of CNS damage in autopsy tissue from HCMV infected fetuses has been correlated with the magnitude of the inflammatory cell infiltrate [65]. Thus, findings from these autopsy series are similar to those described in the murine model system used in this study and again argue that indirect effects of viral infection of the inner ear during auditory development such as virus-induced inflammation remain a potential mechanism for SNHL that follows intrauterine HCMV infection.

Previously, we have shown that MCMV infection of the cochlea and virus-induced inflammation in this model system can result in several histopathological findings, including the loss of spiral ganglion neurons as described in this report [14, 16]. In addition, loss of synapses between hair cells and axons of the spiral ganglion neurons in MCMV-infected mice resulting in altered transmission of electrical signals from the sensory epithelium to SGNs have been suggested to be one downstream effect of cochlear inflammation [14]. A similar loss of synapses has been shown in mice following noise-induced hearing loss and interestingly, such findings in non-human primates following exposure to noise have been associated with altered hearing and also proposed as a cause of decreased hearing function in humans [66–69]. Cochlear inflammation has been described as a component of noise induced hearing loss as well as other forms of cochlear damage and can include both the release of proinflammatory cytokines such as TNFα and IL1β and recruitment of $CD45^+$ cells to the cochlea [55, 70–73]. Of note, early treatment of MCMV infected with anti-inflammatory corticosteroids corrected the functional abnormalities in conduction from hair cells to the spiral ganglion cells, suggesting that synapse numbers and function on hair cells in infected mice could be normalized by limiting virus-induced inflammation [14]. Although we have not identified a specific mechanism associated with cochlear inflammation that could account for the histopathological findings in this model, altered conductivity, and SNHL in MCMV infected mice, we would speculate that alterations in connectivity

between the neurosensory epithelium and spiral ganglion neurons at very early time points during auditory development could also reflect altered or delayed development of the neurosensory epithelium. Thus, we would argue that virus-induced inflammation impacts multiple developmental programs in the cochlea that could potentially intersect during early auditory development. This is perhaps best illustrated by the impact of virus-induced inflammation on the expression of multiple DRGs, each of which plays a unique role in hearing development.

Results from our bulk RNA sequence studies in control and infected animals provided new insight into the impact of virus-induced cochlear inflammation at early times in auditory development. Much of the patterning and specific cell differentiation and localization in the cochlea occurs embryonically in mice and involves the expression of multiple transcription factors at specific timepoints and locations. However, the cochlea, and particularly the neurosensory epithelium and SGNs, are still undergoing remodeling and maturation in mice during the early postnatal period as evidenced by results from single-cell RNA sequencing experiments of the cells of the neurosensory epithelium early in the postnatal period [74]. Bulk RNA sequencing studies of the cochleae of PNd4 MCMV-infected mice revealed dysregulation of many host genes associated with auditory development and importantly, these dysregulated genes have previously been shown to be expressed in different cell types and regions of the developing cochlea. Although the expression of multiple DRGs were decreased during early auditory development, the potential impact of decreased expression of two important gap junction proteins, GJB2 (connexin 26; CX26) and GJA1 (connexin 43; CX43) was of particular interest because mutations in these genes are frequently associated with hearing loss and decreased expression of either of these genes could suggest a mechanism(s) for hearing loss in this model cCMV infection. Both GJB2 and GJA1 have been associated with hearing loss in humans and in engineered rodent models, with mutations in GJB2 (CX26) representing the most commonly identified genetic lesion associated with hearing loss in human populations in all regions of the world [75]. Because deletion of CX26 results in embryonic lethality in mice, murine models have been developed that utilize either conditional deletion of the gene encoding CX26 in the perinatal period or alternatively, targeted deletion of this gene in specific cell lineages in the cochlea [35, 76–78]. Decreased expression of CX26 mRNA before PNd6 to levels between 30 and 50% of wild type and CX26 protein to levels of 12% of wild type resulted in SNHL through a mechanism that is thought to be secondary to damage to multiple areas in the developing cochlea, including decreased viability of the neurosensory epithelium and spiral ganglion neurons, loss of synaptic refinement in outer hair cells, and altered output of outer hair cells leading to loss of signal amplification [35, 39, 76]. In addition, the loss of hair cells was associated with activation of cochlear macrophages and infiltration of inflammatory myeloid cells, raising the possibility that cochlear inflammation could have been additive to the initial damage associated with decreased CX26 expression [31]. The mechanism(s) of damage to auditory pathways at early times in development (< PNd6) in mice with either spatial or conditional deletions of CX26 remains incompletely defined but is thought to include mulitple mechanisms, including a commonly cited deficit in recycling of K^+ in the cochlea that is dependent on the expression of this gap junction protein [35]. In contrast to these findings in studies with decreased CX26 expression during early auditory development, conditional deletion of CX26 after PNd10 resulted in late onset and progressive hearing loss with minimal evidence of loss of cells in the neurosensory epithelium [39, 76]. Together, these phenotypes that follow deletion of CX26 parallel many of the phenotypes observed in MCMV-infected mice and interestingly, in human infants infected *in utero* with HCMV, including both late onset hearing loss and progressive hearing loss [7, 16]. However, it is important to note that in the model of cCMV SNHL described in this report, mice are infected with MCMV in the first 12 h of life and histological evidence of loss of the neurosensory epithelium has not been observed in infected mice, even in mice with profound hearing loss [14]. It should also be noted that phenotypes of MCMV infected mice when compared to engineered mice with CX26 deletions could differ for several reasons including; (i) embryonic loss of CX26 expression following targeted deletions in specific cell types in the cochlea or early conditional deletions of CX26 that often induced CX26 deletions in E19.5 embryos and continued induction through the first 5–6 days of postnatal life are not comparable to MCMV infected mice inoculated on PNd0 raising the possibility that the phenotype of MCMV infected mice could span the phenotypes of early and late postnatal conditional CX26 deletions, (ii) levels of CX26 expression in infected mice likely exceeded those in mice with targeted or conditional deletions of CX26, (iii) variable expression of CX26 in different cell types in the cochlea in infected mice as compared to similar levels of expression in most cell types in mice with conditional deletions of CX26 and/or targeted deletions, and (iv) the duration of decreased CX26 expression in MCMV infected mice is likely limited to control of the virus-indcued inflammation as compared to mice with conditional deletions of CX26. Although any or all of these differences could explain variations in phenotypes between mice infected with MCMV or mice with conditional CX26 deletions, the most likely difference between experimental models

of CX26 conditional deletions and mice infected at birth with MCMV is the timing of decreased expression of CX26 during auditory development. Consistent with this possibility have been the findings of more severe hearing loss with histopathological changes in the neurosensory epithelium in mice with germline deletions of CX26 in specific cell types in the cochlea or conditional deletion mutants that are induced in embryos [39, 76–78]. Of note, the finding that hearing loss and loss of cells of the neurosensory epithelium in mice following conditional deletion of CX26 before PNd5 could be minimized by treatment with steroids was of particular relevance to the current study and was strikingly similar to findings that steroid treatment could prevent hearing loss in MCMV infected mice [14, 79]. Although a mechanism(s) that accounted for the protective activity of steroid treatment in mice with CX26 deletions during early auditory development was not defined in this latter study, the effect of steroid treatment was correlated with a decrease in cochlear inflammation as suggested by the reduction in the number of $CD45^{+}$ cells present in the cochlea after steroid treatment [79]. This rather suprising result raised the possibility that cochlear inflammation directly contributed to hair cell damage and hearing loss following deletion of CX26 in these mice and did not merely reflect innate responses following interactions with damage-associated molecular patterns (DAMPs) produced by damaged cells in the cochlea. Finally, the expression of several other DRGs was also decreased early in auditory development in MCMV infected mice including GJA1 (CX43) mutations. The phenotype of the heterozygous CX43 mutant, $CX43^{G60S/+}$, includes profound hearing loss across all frequencies but importantly, without evidence of loss of cells of the sensory epithelium nor loss of SGN [41]. Of note, this specific CX43 mutation resulted in a significant decrease in CX43 expression to levels of about 20% of WT protein production indicating that complete loss of this protein was not necessary for phenotypic expression of this mutation [41].

Dysregulated expression of multiple DRGs during auditory development in infected mice could have resulted from a global impact of inflammation on cochlear gene expression or alternatively, dysregulated expression of key host genes within pathways critical for auditory development leading to decreased expression of multiple downstream genes that contribute to normal auditory development. Regardless of the mechanism(s) leading to altered DRG expression, a mechanism of SNHL that includes decreased expression of a combination of DRGs would be consistent with the different phenotypes of mice with conditional deletions and/or mutations in DRGs discussed above because of the dependence on the level of expression or perhaps more importantly, the temporal relationship between auditory development and altered expression of specific DRGs. Thus, dysregulation of the expression of several DRGs at specific time periods during early auditory development could also result in variability in the phenotypes of SNHL in MCMV-infected mice and in human infants infected *in utero* with HCMV.

Lastly, an important finding from our studies is the quantitative relationship between cochlear viral load and the resulting virus-induced cochlear inflammation that in turn, leads to SNHL. Although the absolute quantity of cochlear virus required to generate an immunopathological response leading to the development of SNHL has not been established, results from the current study suggest that a threshold of cochlear virus could be required to induce inflammatory responses sufficient to disrupt normal auditory development and hearing loss. This possibility is of considerable significance for the development of prophylactic vaccines and/or passively acquired antiviral antibodies to limit HCMV associated SNHL or alternatively, the optimal time to provide antiviral treatment to limit viral load in pregnant women acutely infected with HCMV. By inference from results presented above, none of these approaches will require sterilizing cochlear immunity but only that antiviral immunity and/or antiviral drugs decrease cochlear viral loads below a threshold necessary to induce an immunopathological response in the cochlea early in auditory development. The addition of anti-inflammatory agents to treatment protocols provided during early auditory development could further improve clinical outcomes, perhaps by reduction of cochlear inflammatory responses during this critical interval in auditory development. Finally, while the variability in the incidence and clinical expression of SNHL in infants with HCMV infections continue to present enormous challenges to the design of clinical trials, the availability of informative small animal models could help further refine the design of interventions to limit this common sequelae of congenital HCMV infections.

Materials and methods

Sex as a biological variable

In this model system of a human infection, sex has not been shown to represent a biological variable. In addition, this model was developed to study human cytomegalovirus infection of the developing human fetus and decades of clinical natural history studies of human congenital CMV infections have not identified sex as biological variable in this perinatal infection.

Animal ethics statement

All animal procedures performed for this study were approved by the Institutional Animal Care and Use Committee (IACUC) (APN: IACUC-09351 and IACUC-20678). All mice were euthanized by asphyxiation with

carbon dioxide prior to experimental procedures. Adult mice were given secondary confirmation of euthanization by cerivial disclocation and young mice by decapitation, in accordance with the UAB Aimal Resource Program (ARP) guidelines. All protocols for tissue collection for these studies were approved by UAB IACUC.

Mice and MCMV infection

Newborn pathogen-free Balb/c mice were produced by breeding of mice purchased from Charles River Labs and used for all experiments. Mice were infected within 12 h of birth by intraperitoneal injection with 200PFU MCMV Smith strain in 30ul phosphate-buffered saline (PBS). Viral stocks were grown in mouse bone marrow stromal cells (M2-10B4; ATCC CRL-1972) and titered by plaque assay. The virus was aliquoted and stored at -80 °C and aliquots thawed individually for immediate use. Blood was collected prior to termination by cardiac puncture, then mice were exhaustively perfused with PBS prior to any further manipulations. Cochleae were collected at PNd4, PNd8, PNd14, and PNd34 and organs were harvested either by flash freezing on dry ice for qPCR or fixed and frozen in OTC for IHC.

Monoclonal antibody treatment

Purified MCMV-specific monoclonal antibodies (mAbs) M11 and 97.3 were obtained from BioXCell (Lebanon, NH) from cloned hybridoma cells producing these murine monoclonal antibodies described by Bootz, et al. [25]. At age PNd2 and PNd5, mice were injected intraperitoneally with either (i) 50ug M11 and 50ug 97.3 in 30ul, (ii) 100ug of IgG2b isotype control monoclonal antibody specific for human CMV gB protein and non-reactive with MCMV, or (iii) no injection. Anti-MCMV mAbs and isotype controls were diluted in PBS. Previous studies have demonstrated that injection with PBS or no injection results in an identical phenotype.

Viral genome copy number quantitation and gene expression

Following removal, cochleae were individually homogenized and nucleic acids (DNA and RNA) were isolated from the tissue by E.Z.N.A Total RNA Kit I (Omega Biotek, Norcross, Ga) with modifications to obtain the DNA for viral genome quantitation as previously described (Sung et al., 2019). Viral genome copy number was determined by qPCR. Experimental samples were compared to a plasmid containing a fragment of MCMV ie1 exon 4 (forward: 5'-GGC TCC ATG ATC CAC CCT GTTA-3' and reverse: 5'-GCC TTC ATC TGC TGC CAT ACT-3') and the probe (5′-AGC CTT TCC TGG ATG CCA GGT CTC A-3′) labeled with reporter dye FAM and quencher dye TAMRA. The plasmid was diluted serially (log10) to generate a standard curve which was used to quantify the experimental sample genome copy numbers in every experiment. Copy numbers are expressed in log10 format and were run in duplicate.

For quantification of RNA transcripts, cDNA was synthesized from total RNA isolated from cochleae using Invitrogen Superscript IV First strand synthesis (ThermoFisher). TaqMan Gene Expression Master Mix (ThermoFisher) was used for qPCR of specific gene targets: HPRT (Mm00446968_m1), IFIT1 (Mm00515153_m1), TNF (Mm00443258_m1), IFNg (Mm01168134_m1), IL1b (Mm00434228_m1), GJB2 (Mm00433643_s1), GJA1 (Mm00439105_m1), KCNE1 (Mm01215533_m1), ZBTB20 (Mm00457764_m1), COCH (Mm00483360_m1), and OTOS (Mm01292235_g1). For each gene, the reporter dye was FAM and the quencher was MGB. HPRT is a housekeeping gene used as an internal control and expression of each gene target was assessed by the $2^{-\Delta\Delta Ct}$ method and as described in previous publications [80]. Values obtained for each experimental group in each target gene were normalized to the control, uninfected group (mock).

ABR hearing tests

Hearing tests were performed as described previously [14, 16]. Mice between PND32 and PND35 were anesthetized by ip injection of a combination of ketamine (50 mg/kg) and medetomidine (0.5 mg/kg), placed in a soundproof chamber and maintained at 34–37°, and needle electrodes were inserted subcutaneously in the hind leg (ground), at the base of the skull, and ipsilateral ear. Ears were tested individually and the ear being tested was moved next to a microphone/speaker. Tucker-Davis Technologies (TDT) software and hardware (BioSig and SigGen) were used to acquire hearing thresholds of uninfected control, MCMV infected, and mAb treated mice. For ABR click tests, broadband click stimuli from 90dB– 20dB descending by 5dB steps were delivered and responses were recorded and measured. Frequency specific (tone) hearing thresholds were determined using tone-pips at 4, 8, 16, 32, 40, and 48 kHz (90-10dB in decreasing 10dB steps). Signals were detected by the needle electrodes, followed by amplification by 10,000x, filtered (300 Hz high-pass, 3 kHz low-pass, 60 Hz notch), and averaged by alternating stimulus polarity. Hearing thresholds were defined as the lowest intensity in which peaks were discernable and expressed as sound pressue level (SPL). Wave I amplitudes were quantified as we have previously described [14].

Immunohistochemistry

After removal, cochleae were submerged in 4% PFA in 1x PBS overnight, decalcified using RDO Rapid Decalcifier (Electron Microscopy Sciences, US), and placed in 15% and 30% sucrose solutions sequentially for 24 h at

each concentration. Cochleae were then flash frozen in OCT and cryosectioned at 10 µm for immunostaining and microscopy. Images were captured as described [14]. Briefly, samples were rehydrated in 1x PBS for 15 min, then blocked in blocking buffer (2% NGS, 0.3% Triton X100, in 1x PBS and 10% normal goat serum) for 3 h at room temperature. Cochleae were immunostained with beta-tubulin (tuj1) (Cell Signaling, Danvers, Mass) overnight in 4 °C. After primary antibody incubation, species and isotype matching FITC-conjugated secondary antibody was applied for 2 h at room temperature followed by nuclear staining with Hoescht dye (Thermo Fisher Scientific) for 15 min.

Confocal imaging

Images were captured on an Olympus FV1000 confocal microscope with a 40x objective (numerical aperture: 0.60; medium: air) at room temperature. FV10-ASW 4.2 software was used for image capture of the immunostained sections. Laser parameters and gain were maintained at the same output for each image to allow comparison of relative expression of selected targets.

Spiral ganglion neuron (SGN) quantification

Confocal images were loaded into ImageJ to quantify tuj1 positive cells in Rosenthal's canal [14, 81]. Density measurements of SGN soma were made by manually counting tuj-1 positive cells in each region of the midmodiolar sections (apex, mid, and base) and dividing that number by the area of Rosenthal's canal for each region with results being expressed as whole number of SGN cells/10000um^2.

Bulk RNA-seq

Cochleae from mock infected and MCMV infected mice aged PNd4 were removed after perfusion of the mouse with PBS, homogenized, and RNA was isolated using the same method as samples collected for qPCR above. Three biological replicates (3 mice, 6 cochlea) were used for each individual mock and MCMV infected experimental group, thus each sample represents total cochlear RNA from 3 mice. RNA sequencing was performed by Hudson Alpha (Huntsville, AL, USA). Quality control was performed using FastQC (v0.12.1). Clean reads were aligned to the mouse reference genome (*GRCm39* primary assembly) with GENCODE version vM32 for genome annotation using STAR aligner with the *--quantMode GeneCounts* option [82, 83]. Differential gene expression analysis was performed using the R DESeq2 package (v1.40.2) [84]. The log2 fold change (LFC) shrinkage was applied using the apeglm method [85]. LFCs were computed for each gene, with positive LFC values indicated upregulation in MCMV infected mice, while negative LFC values indicated downregulation in uninfected controls. The selection of differentially expressed genes for heatmap visualization was based on the 40 genes with the lowest adjusted p-value and Z-score normalization was performed for each gene.To identify the changes at the pathway level, a gene set enrichment analysis based on gene ontology (GO) terms was performed using the GSEA (v.4.1.10) software [86–88]. The analysis specifically targeted the biological process (BP) domain of the GO from MSigDB (v2023.1). Genes were ranked according to the metric *-log10(p-value)* × *SIGN(LFC)* derived from the differential gene expression analysis results [89]. The range of gene set size was between 15 and 500. Multiple testing correction was applied using the Benjamini-Hochberg's method to adjust p-values.

Statistics

All statistical analyses were performed using Prism 6 (GraphPad, San Diego, CA). The Shapiro-Wilks test was used to analyze datasets for normality. Comparisons of multiple groups were subjected to non-parametric testing of significance using Kruskal-Wallis tests with Dunn's comparisons test to determine significance across groups. Data are reported as medians. Values were considered to be statistically significant as indicated: (∗) $P<0.05$, (∗∗) $P<0.01$, (∗∗∗) $P<0.001$, (∗∗∗∗) $P<0.0001$, P-values above 0.05 ($P>0.05$) was considered non-significant. For comparison of median values of MCMV infected untreated mice versus MCMV infected mAb treated mice, Mann-Whitney statistical testing was carried out (GraphPad, San Diego, Ca).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12974-025-03416-4.

Supplementary Material 1

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Author contributions

M. S.: Conceptualization, Data acquisition and curation, Formal Analysis, Investigation, Methodology. M. S.: Data acquisition and curation, formal analysis, methodology. review & editing. T. ML.: Conceptualization, Data acquisition and analysis. JWC.: Data acquisition and analysis. M.M.: Conceptualization, Resources. WJB.: Conceptualization, writing review & editing, project administration, resources, funding acquisition.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare no competing interests.

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