

# Influenza Virus Induces Bacterial and Nonbacterial Otitis Media

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Otitis media (OM) is one of the most common childhood diseases. OM can arise when a viral infection enables bacteria to disseminate from the nasopharynx to the middle ear. Here, we provide the first infant murine model for disease. Mice coinfecting with *Streptococcus pneumoniae* and influenza virus had high bacterial load in the middle ear, middle ear inflammation, and hearing loss. In contrast, mice colonized with *S. pneumoniae* alone had significantly less bacteria in the ear, minimal hearing loss, and no inflammation. Of interest, infection with influenza virus alone also caused some middle ear inflammation and hearing loss. Overall, this study provides a clinically relevant and easily accessible animal model to study the pathogenesis and prevention of OM. Moreover, we provide, to our knowledge, the first evidence that influenza virus alone causes middle ear inflammation in infant mice. This inflammation may then play an important role in the development of bacterial OM.

Otitis media (OM) affects >80% of children before the age of 3 years and can cause meningitis, hearing loss, and learning difficulties [1, 2]. OM also represents a significant health care burden, with billions of dollars spent every year in the United States on this disease [3]. Despite the prevalence and importance of OM, many aspects of disease pathogenesis remain incompletely understood.

One of the leading bacterial causes of OM is *Streptococcus pneumoniae* [4]. *S. pneumoniae* is a member of the normal nasopharyngeal flora in up to 80% of children [5, 6]. Although colonization is typically asymptomatic, a viral infection of the upper respiratory

tract can facilitate the dissemination of *S. pneumoniae* to the middle ear. Several different respiratory viruses have been implicated in this process, including respiratory syncytial virus (RSV) [7] and influenza A virus (IAV) [8]. In children with acute OM, *S. pneumoniae* was significantly more likely to be cultured from middle ear fluid samples containing influenza virus, compared with those containing RSV [8]. Moreover, some evidence suggests that vaccination against influenza virus reduces the incidence of OM [9]. The synergistic interaction between *S. pneumoniae* and IAV is also supported by studies in animal models in which infection with IAV results in a dramatic increase in the incidence of pneumococcal OM [10–13].

Many different hypotheses have been proposed to explain how IAV facilitates pneumococcal OM [3]. IAV may damage the Eustachian tube in such a way that it can no longer regulate pressure in the middle ear. This then creates a vacuum that draws in fluid from the surrounding tissue and, thereby, facilitates bacterial growth [14–16]. It is also possible that, by altering the responsiveness of neutrophils [16, 17], IAV facilitates replication of *S. pneumoniae* in the middle ear.

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The majority of studies investigating pneumococcal-influenza OM have been performed in chinchillas [11, 16, 17] and, to a lesser extent, in ferrets [13]. Chinchillas are a useful animal model to study OM because of their enlarged cephalid bulla [18]. This facilitates transbulla inoculation and reduces difficulties associated with obtaining samples of the middle ear fluid [18]. However, the availability and costs associated with the use of such large animals can be prohibitive and knockout strains of these animals are not available, making it difficult to elucidate the specific host factors important in OM pathogenesis [18]. Moreover, the majority of experimental reagents have been developed for use in the mouse.

Studies from our group [10] and those of others [12] show that intranasal infection of mice with IAV facilitates pneumococcal OM. However, this supposition is based solely on the presence of a luminescent signal in the ears of mice infected with IAV and a bioluminescent strain of *S. pneumoniae*, and neither study comprehensively characterized the murine model. The thorough development of this model would represent a relatively inexpensive and easily accessible means for understanding *in vivo* viral-bacterial interactions.

The aim of this study was therefore to examine the effect of IAV infection on *S. pneumoniae* in infant mice. Infant mice were selected to reflect the underdeveloped immune system of children, who have the greatest burden of OM [2]. Similar to human infants, murine infants show an inability to respond to T-independent antigens (such as the pneumococcal polysaccharide) and display a variety of different defects in components of the innate immune response [19–23]. Such features may leave children vulnerable to OM by, for example, preventing the development of antibodies necessary for the opsonophagocytosis of the bacteria or reducing the functionality of cell types (such as neutrophils) that may be necessary for the clearance of infection. Thus, using infant mice as a clinically relevant model, we show that coinfection with *S. pneumoniae* and IAV results in a significant increase in middle ear bacterial titers, middle ear inflammation, and temporary hearing loss. We also provide evidence that intranasal infection of infant mice with IAV alone can result in inflammation in the middle ear cavity. This may suggest that virus-induced damage in the middle ear cavity plays a previously unappreciated role in the pathogenesis of pneumococcal OM.

## METHODS

### Viral and Bacterial Strains

The bioluminescent *S. pneumoniae* strain EF3030<sup>lux</sup> (type 19F) [10] or the *S. pneumoniae* strain ML-2302 (type 23F; kindly donated by Prof. Susan Hollingshead, University of Alabama at Birmingham) were used in all experiments. For infection experiments, pneumococci were cultured and stored as described elsewhere [10]. Influenza virus strain A/Udorn/307/72

(H3N2) was used to model infection with IAV. Virus stocks were prepared in embryonated eggs, and titers of infectious virus were determined using 3 independent plaque assays on Madin-Darby Canine Kidney cells [10].

### Infection of Mice

C57BL/6 mice were bred and housed under specific pathogen-free (SPF) conditions at the animal facility of the Department of Microbiology and Immunology at The University of Melbourne, Australia. Germ-free mice were bred and housed in germ-free isolator units at the Bioservices Department of The Walter and Eliza Hall Institute for Medical Research (Kew, Victoria, Australia). Five-day-old C57BL/6 mice were colonized intranasally with  $2 \times 10^3$  colony-forming units of *S. pneumoniae* in 3  $\mu$ L. Alternatively, mice were mock infected with an equivalent volume of phosphate-buffered saline (PBS). At 14 days of age, infant mice were infected intranasally with  $10^{2.5}$  plaque-forming units of egg-grown IAV in 3  $\mu$ Ls or were mock treated with a similar volume and dilution of noninfected allantoic fluid. Six days after IAV or mock infection, mice were killed and the entire bulla from each ear was dissected. All animal experiments were approved by the Animal Ethics Committee of the University of Melbourne and were conducted in accordance with the Prevention of Cruelty to Animals Act (1986) and the Australian National Health and Medical Research Council Code of Practice for the Care and Use of Animals for Scientific Purposes (1997).

### Enumeration of Bacterial Load

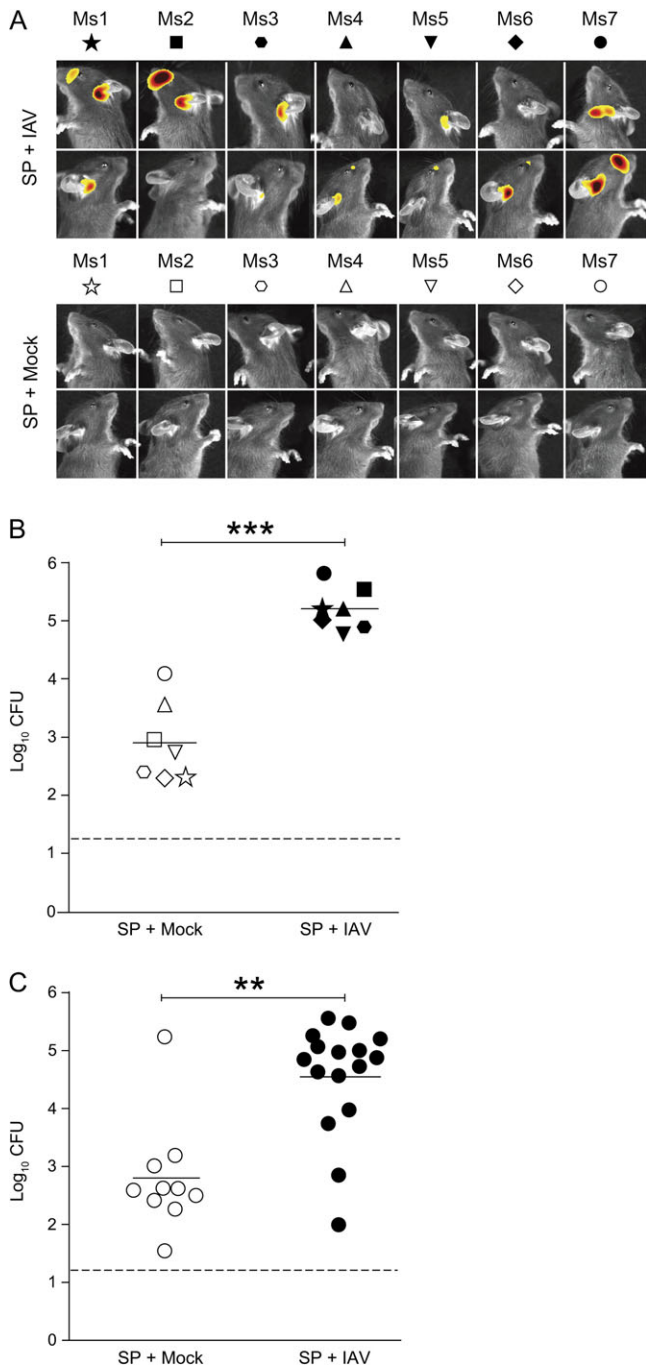
Tissues used to quantify bacterial load were homogenized (Polytron PT2100; Kinematica), and serial dilutions of tissue homogenates were prepared in PBS and cultured on horse blood agar containing 5  $\mu$ g/mL of gentamicin (Pfizer).

### In Vivo Imaging

*In vivo* imaging was performed using an IVIS-Spectrum camera (Caliper Life Sciences) as described elsewhere [10]. Imaging was performed for 7 minutes for each ear, and images were edited using the Living Image software package, version 3.0 (Caliper Life Sciences).

### Auditory Brainstem Response

The hearing status of each mouse was evaluated using a click-evoked auditory brainstem response (ABR) [24]. In brief, each mouse was anesthetized [10] and placed in a sound-attenuated room. A loud speaker was placed 10 cm from the pinna of the test ear, and computer-generated clicks over a range of 0–100 dB peak equivalent sound pressure level were presented randomly. The contralateral ear was plugged with an ear mould compound (Otoform), and percutaneous recording electrodes were positioned at the vertex of the skull (positive) and on the nape of the neck (negative), with a ground on the snout. ABRs were differentially recorded, and the signals were amplified by  $10^5$  and



**Figure 1.** Influenza virus facilitates pneumococcal otitis media (OM). *A*, Representative ( $n \geq 21$  per treatment group) in vivo images of *Streptococcus pneumoniae* (SP) EF3030<sup>Lux</sup> in the middle ears of mice 6 days after intranasal (i.n.) infection with influenza A virus (IAV) or mock. *B*, Titers of *S. pneumoniae* EF3030<sup>Lux</sup> (SP) in the middle ears of mice after i.n. infection with IAV or mock. Each symbol indicates the bacterial titers for a specific mouse shown in *A*. *C*, Titers of *S. pneumoniae* ML-2302 (SP) in the middle ears of mice after i.n. infection with IAV or mock. Data are pooled from at least 2 independent experiments, and bacterial counts are represented as the mean titer derived from the left and right ear of each mouse. Triple asterisks indicate a highly statistically significant difference ( $P < .001$ ) between treatment groups using a 2-tailed Mann–Whitney *U* test. Double asterisks indicate a strongly statistically

bandpass filtered (150 Hz–3k Hz). Stimulus intensity was adjusted in 5-dB steps around threshold levels. The signals were digitized, and the mean value from 2 sets of 100 trials were determined. The 2 mean ABRs were subsequently analyzed to determine threshold with use of Igor Pro6.04 software (WaveMetrics). Threshold was defined as the minimum stimulus intensity that produced a response amplitude of at least 0.2 mV in the 2–3-ms period after the click stimulus [25]. The threshold increase observed as a result of infection was calculated by subtracting the mean threshold of mock-infected, age matched mice ( $n = 20$ ) from each data point. When this resulted in a negative value, the threshold increase was assigned a value of 0.

### Middle Ear Inflammation

To collect the middle ears for a histological examination of inflammation, mice were killed by carbon dioxide asphyxiation and intracardially perfused with 3 mL of 4% paraformaldehyde (PFA). Middle ear tissues were subsequently dissected and fixed for a minimum of 4 hours in 4% PFA. Middle ears were decalcified in Formalin-EDTA [26], embedded in paraffin, sectioned at 6–8  $\mu\text{m}$ , and stained with hematoxylin and eosin. The percentage of the middle ear cavity containing an inflammatory cell infiltrate was scored by a pathologist (JP) who was blind to the study design.

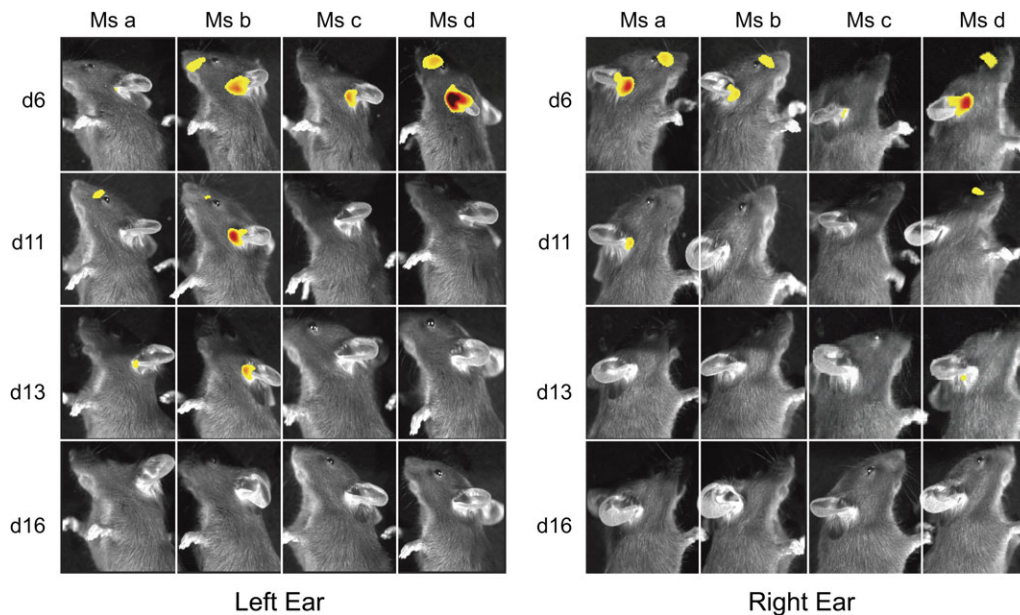
### Immunofluorescence

Heat treatment (10 mmol/L citrate buffer; pH, 6.0) was used for antigen retrieval, and sections were blocked for 10 minutes (Protein block; Dako). Sections were labeled for 1 hour with either *S. pneumoniae* type 19F antiserum samples (Statens Serum Institut) diluted 1:100 in TrueVision reagent (Sapphire Bioscience) or the immunoglobulin fraction from nonimmunized rabbit serum (Rabbit universal negative control; Dako). Sections were subsequently washed with PBS and incubated with sheep anti-rabbit IgG FITC (Silenus Laboratory) diluted 1:300 in PBS and 0.05% bovine serum albumin. Sections were then washed with PBS and incubated in 0.01% Evans blue (Sigma-Aldrich) for 1 minute at room temperature. A final PBS wash was performed, and sections were mounted with 10% (w/v) Mowiol 4–88 and visualized.

### Fluorescent In Situ Hybridization (FISH)

FISH was conducted essentially as described elsewhere [27], with use of 16S rRNA probes labeled with AlexaFluor 546 (Invitrogen). In brief, sections were incubated with 10 mg/mL lysozyme (Sigma-Aldrich) in 0.1M Tris (hydroxymethyl)

significant difference ( $P < .01$ ) between treatment groups using a 2-tailed Mann–Whitney *U* test. The geometric mean is represented by a horizontal line, and dashed lines indicate the detection limit of the assay.



**Figure 2.** Pneumococcal-influenza otitis media (OM) resolves overtime. Representative in vivo images ( $n \geq 14$  per time point) of mice colonized with *Streptococcus pneumoniae* EF3030<sup>LUX</sup> at various days (d) after IAV infection. The arbitrary letter assigned to each mouse (Ms) is shown.

aminomethane hydrochloride and 0.05 M Na<sub>2</sub> EDTA at 37°C for 2 hours and washed with PBS. FISH was performed on sections in 20% formamide (Amresco) with use of 50 ng/μL of 16S ribosomal probe sequence EUB338 (for the domain bacteria): 5'-GCTGCCTCCCGTAGGAGT-3' [28]. Autofluorescence was quenched using 2.5 mM CuSO<sub>4</sub> in 50 mmol/L CH<sub>3</sub>COONH<sub>4</sub> (Sigma-Aldrich). Nuclei were stained using 1.2 μg/mL Hoechst 33342 (Invitrogen). Sections were mounted using low-fade mounting media and were imaged using a Nikon A1Si confocal laser scanning microscope and high-resolution Plan Apo objectives.

#### Statistical Analysis

Statistical analyses were performed using GraphPad Prism, version 5.00 for Windows (GraphPad Software).

## RESULTS

### IAV Facilitates Acute Pneumococcal OM

To investigate the effect of IAV on the development of pneumococcal OM, infant mice were colonized with a bioluminescent 19F strain of *S. pneumoniae* [10]. At 14 days of age, mice were infected via the intranasal route with IAV or mock. The development of pneumococcal OM was assessed 6 days later by in vivo imaging and enumeration of bacterial load in the middle ear. A strong luminescent signal was observed from the ears of mice coinfecting with *S. pneumoniae* and IAV (Figure 1A). This was not seen in mice infected with *S. pneumoniae* alone (Figure 1A). Enumeration of bacterial titers in the middle ear showed that ~90-fold more bacteria were recovered from the middle ear of

coinfecting mice than from mice infected with *S. pneumoniae* alone ( $P < .001$ , by Mann–Whitney *U* test) (Figure 1B). These findings were not strain dependent, because similar differences in bacterial load were obtained using a nonbioluminescent serotype 23F *S. pneumoniae* strain ( $P = .003$ , by Mann–Whitney *U* test) (Figure 1C). Similarly, these data were not attributable to a systemic infection in coinfecting mice, because no bacteria could be detected in blood samples from coinfecting mice 6 days after IAV infection ( $n = 16$ ; data not shown).

In vivo imaging was subsequently used to track the infection over time in each individual mouse. The luminescent signal from the middle ears of coinfecting mice resolved 11–16 days after IAV infection (Figure 2). Consistent with these data, 16 days after infection with IAV, bacterial titers in the middle ears of coinfecting mice had reduced to  $\sim 10^3$  colony-forming units (data not shown). No mice became moribund during the infection period, indicating the localized nature of the infection.

### Mice Infected With IAV Show Middle Ear Inflammation

To determine whether the increased bacterial load in the middle ears of coinfecting mice had pathological consequences, inflammation in the middle ear was assessed. No obvious inflammation was detected in the middle ear cavity of mock-colonized mice (Figure 3A and 3B) or mice colonized with *S. pneumoniae* alone (Figure 3A and 3B). In contrast, middle ear sections from mice coinfecting with *S. pneumoniae* and IAV showed submucosal edema and a large influx of inflammatory cells (predominately neutrophils) into the lumen of the middle ear cavity (Figure 3A). The inflammation observed in these mice was significantly greater than that seen in mock-infected mice or



mice colonized with *S. pneumoniae* alone ( $P < .001$ , by Kruskal–Wallis test) (Figure 3B). Unexpectedly, mice infected with IAV alone also showed submucosal edema and an influx of predominantly neutrophils into the middle ear cavity (Figure 3A). The mean inflammatory score for these mice was lower than that observed in coinfecting mice, although there was not a statistically significant difference (Figure 3B).

### IAV Causes Middle Ear Inflammation in the Absence of Normal Flora

The development of middle ear inflammation in mice infected with IAV alone may indicate that the viral infection induces inflammation in the middle ear. Alternatively, these data may reflect the ability of normal nasopharyngeal flora, in the presence of an IAV infection, to ascend the Eustachian tube and induce middle ear inflammation. To differentiate between these hypotheses, 14-day-old C57BL/6 germ-free mice (which lack commensal flora) were mock or IAV infected. Low-level inflammation was observed in the middle ear cavity of mock-infected, germ-free mice 6 days after mock infection (Figure 4A). Nevertheless, IAV infection of germ-free mice induced a significant increase in middle ear inflammation ( $P = .04$ , by Mann–Whitney  $U$  test) (Figure 4A). These data suggest that IAV-induced middle ear inflammation is not dependent on the presence of normal nasopharyngeal flora and may be caused by the virus. To verify these findings, FISH for 16S ribosomal RNA (rRNA) was performed on middle ear sections taken from IAV-infected SPF-housed C57BL/6 mice. No bacterial rRNA was detected in these sections (Figure 4B). In contrast, 16S rRNA was detected in the middle ear cavity of mice coinfecting with *S. pneumoniae* and IAV (Figure 4B). Bacteria in these sections were restricted to the inflammatory cell infiltrate. To confirm the localization of *S. pneumoniae* in the ears of coinfecting mice, immunofluorescence for pneumococcal antigens was performed. As seen with FISH, bacteria localized to the inflammatory cell infiltrate (Figure 4C). In summary, these data demonstrate that the middle ear inflammation observed in IAV-infected mice is not the result of bacterial antigens in the middle ear. Furthermore, in coinfecting mice, pneumococcal antigens are found in the lumen of the middle ear cavity.

### Hearing Loss in Infected Mice

In humans, the middle ear inflammation associated with OM can lead to hearing loss [2, 29]. Thus, the hearing status of infected mice was measured using a click-evoked ABR [24]. Hearing thresholds of infected mice were compared with thresholds of

mock-infected mice, in which higher hearing threshold differences were indicative of greater hearing loss. Six days after IAV infection, mice infected with *S. pneumoniae* alone showed a mild increase in hearing thresholds, compared with mock-infected mice (Figure 5). Conversely, hearing thresholds for mice coinfecting with *S. pneumoniae* and IAV were  $\sim 20$  dB higher than those in mock-infected mice. This was significantly higher than any hearing loss observed in mice infected with *S. pneumoniae* alone ( $P < .001$ , by Kruskal–Wallis test). Mice infected with IAV alone also displayed hearing loss, consistent with our previous observations that IAV induces middle ear inflammation. However, the threshold increase detected in these mice was significantly lower than that observed in coinfecting mice ( $P < .05$ , by Kruskal–Wallis test). The hearing loss observed in coinfecting and IAV-infected mice returned to similar levels to those in mock-infected mice within 3 weeks (data not shown).

## DISCUSSION

OM is the most frequently diagnosed illness in children [2]. OM can arise when a viral infection enables bacteria from the nasopharynx to replicate in the middle ear. Here we have presented the first comprehensive characterization of an infant murine model for influenza-pneumococcal OM. Similar to children, infant mice have an incompletely developed immune system [19, 20]. Thus, infant mice were used to model the underdeveloped immune system of children, who have the greatest burden of OM [2].

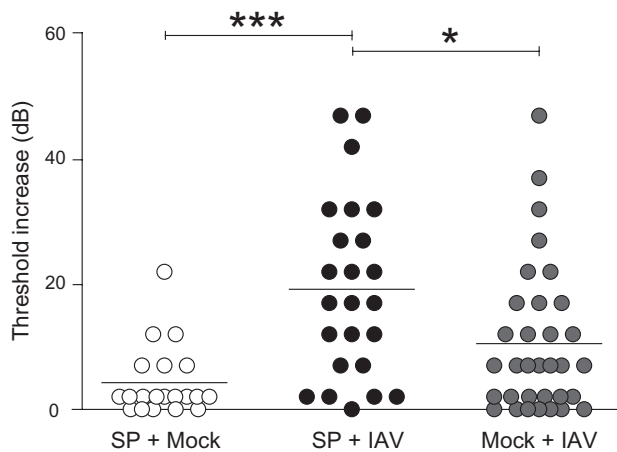
Using this model, we have shown that infection with IAV significantly increased pneumococcal bacterial counts in the middle ear. These data closely mimic the association seen in children between the presence of an IAV infection and the detection of *S. pneumoniae* in the middle ear [8]. The acute and self-limiting nature of this infection also accurately reflects the clinical course of acute OM, which typically resolves in the absence of treatment [30].

In children, the presence of bacteria in the middle ear can lead to inflammation [31]. We reasoned that the increased bacterial load seen in coinfecting mice would similarly lead to increased middle ear inflammation. Indeed, co-infected mice had significantly greater middle ear inflammation, compared with mock-infected mice or mice infected with *S. pneumoniae* alone. There was also a trend toward increased inflammation in co-infected mice, compared with mice infected with IAV alone. Although the

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Figure 3 continued. a single ear from an infected mouse (12 mice treated with *S. pneumoniae* alone, 11 mock-treated mice, 17 mice treated with IAV alone, and 16 mice treated with *S. pneumoniae* and IAV). Triple asterisks indicate a highly statistically significant difference ( $P < .001$ ) between treatment groups using a Kruskal–Wallis test accompanied by Dunn's posttest. The arithmetic mean is represented by a horizontal line. "Mock + Mock" and "SP + Mock" images shown in A were given a score of 0. The "SP + IAV" image in A was given a score of 75, and the "Mock + IAV" image was given a score of 20.





**Figure 5.** Coinfected and IAV-infected mice display hearing loss. Hearing loss in mock- or *Streptococcus pneumoniae* (SP)-colonised mice 6 days after infection with influenza A virus (IAV) or mock. Data are pooled from at least 2 independent experiments, and each data point represents the hearing threshold increase of either the left or right ear of an infected mouse (10 mice infected with *S. pneumoniae* alone, 12 mice infected with *S. pneumoniae* and IAV, and 17 mice infected with IAV alone). The threshold increase was calculated by subtracting the mean hearing loss of mock-infected, age-matched mice from each data point. Triple asterisks indicate a highly statistically significant difference ( $P < .001$ ) between treatment groups using a Kruskal-Wallis test accompanied by Dunn's posttest. Single asterisks indicate a statistically significant difference ( $P < .05$ ) between treatment groups. The arithmetic mean is represented by a horizontal line.

*Moraxella catarrhalis*, and *Haemophilus influenzae* [2]. Thus, if these individuals contract IAV, they are likely to develop viral-bacterial OM. This may account for the lower rate of virus-alone middle ear inflammation observed in the pediatric population.

At present, the mechanisms by which IAV facilitates middle ear replication of *S. pneumoniae* remain unclear. Previous studies have suggested that the ability of IAV to perturb Eustachian tube function may facilitate bacterial replication in the middle ear [41]. In the present study, minimal Eustachian tube damage was observed (data not shown). However, the ability of IAV to induce middle ear inflammation was striking. We therefore postulate that IAV-induced inflammation in the middle ear facilitates bacterial replication. The role of IAV-induced inflammation in pneumococcal pneumonia has been well documented [42, 43]. It has been suggested that the pro-inflammatory response to IAV increases the expression of pneumococcal receptors on epithelial cells and, thus, facilitates bacterial outgrowth [44]. However, in our study, pneumococci were localized to the inflammatory cell infiltrate, rather than the middle ear epithelium. Instead, a possible scenario is that the neutrophil influx into the middle ear cavity after IAV infection results in damage to the cells of the middle ear. This would increase the amount of free nutrients available and help

support increased bacterial growth. Neutrophils may not be able to control this bacterial outgrowth, because IAV is known to suppress neutrophil function [45, 46]. Determining the precise mechanisms underlying disease development in our model remains a key area for further study.

## Notes

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**Potential conflicts of interest.** All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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