OTITIS MEDIA

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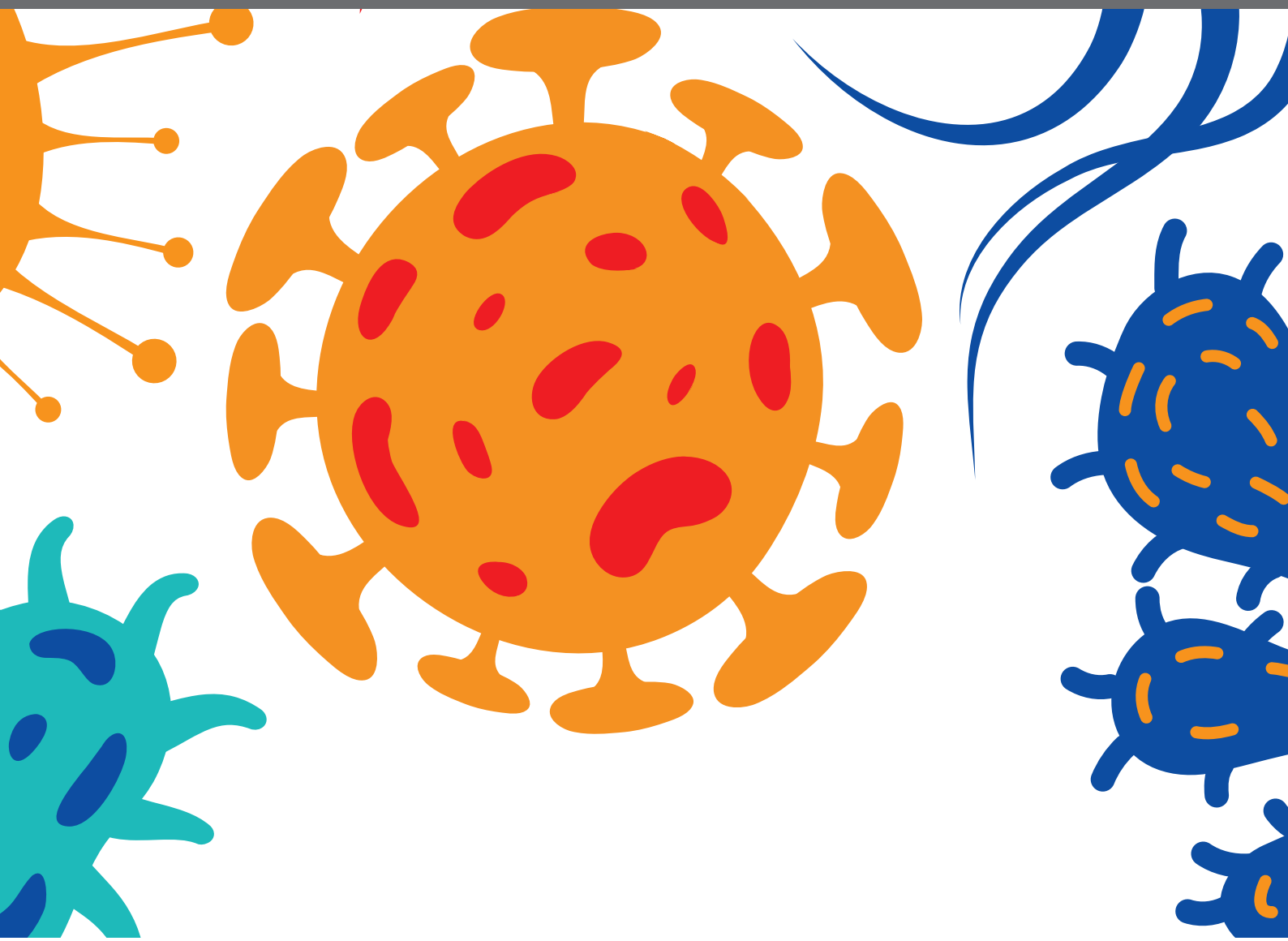

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OTITIS MEDIA

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Editorial: Otitis media

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Editorial on the Research Topic
Otitis media

Middle ear infection remains a significant health concern with 80 percent of young children, primarily between the ages of 6 and 24 months, experiencing at least one otitis media (OM) episode and many suffering multiple recurrences each year (Pichichero, 2013; Mittal et al., 2015; Rosenfeld et al., 2016; Tong et al., 2018). Recurrent acute OM is one of the most common indications for tympanostomy tube placement surgery and antibiotic prescription, with staggering associated treatment costs and impacts on emerging resistance (Pelton et al., 2013). Further burden arises from OM-associated hearing loss that impacts behavior, language development and educational outcomes (Monasta et al., 2012; Schilder et al., 2016; Homoe et al., 2019), with such impacts compounded among global populations with high chronic suppurative OM burden (Leach et al., 2020). Antibiotics remain the preferred treatment to sterilize the bacterial component of disease and abort the inflammatory process in the middle ear; however, infections often persist, resulting in ongoing inflammation and hearing impacts. Despite the vast global disease burden, much remains unknown about OM pathogenesis.

This Research Topic provides insight into the current state of the field, highlighting recent research targeted at critical gaps in understanding of the microbial epidemiology and wider pathobiology of OM. Topics covered extend from ongoing work to define temporal and geographic variability in OM microbiology, through to human and animal model studies exploring host and microbial factors underlying the disease, and implementation of modern computational approaches for improving OM diagnostics.

Temporal and geographic impacts on OM microbial epidemiology

During the COVID-19 pandemic, decreased acute OM presentations were reported for several populations [reviewed in (Marom et al.)]. In a Dutch primary care cohort study (Hullegie et al.), episodes of OM were shown to decrease during the pandemic, despite similar antibiotic prescription rates for acute OM pre- and post-pandemic. A decrease in OM episodes was likely the result of implementation of quarantine measures to control the spread of the COVID-19 pandemic. This observation is a reminder of the potential for public health interventions to disrupt respiratory pathogen transmission and reduce OM disease burden; an observation of particular importance where overcrowded housing is associated with increased OM burden (DeLacy et al., 2020). The direct impact of SARS-CoV2 on middle ear microbiology is not known. As reviewed by Marom et al., a small number of studies have reported SARS-CoV2 detection from middle ear specimens; however, our understanding of this virus in OM remains limited.

Pneumococcal conjugate vaccines impact the population biology of *Streptococcus pneumoniae*; an important otopathogen. Although vaccination has reduced the incidence of disease due to pneumococcal vaccine serotypes, the emergence of replacement serotypes and increased incidence of other otopathogens warrants further investigation. This important point is highlighted in work by Fuji et al. demonstrating the emergence of acute OM-associated *S. pneumoniae* serotype 35B genotypes since 2015. Genomic changes among strains recovered after 2015 - including single nucleotide polymorphisms (SNPs) found in metal binding, ABC transporters and DNA replication genes - may be associated with increased virulence (Fuji et al.).

OM microbiology varies across populations, with population-level effects known to impact key drivers of disease (Sakulchit and Goldman, 2017; DeLacy et al., 2020; Dagan et al., 2021). Geographic variation in OM pathobiology was highlighted by Australian studies examining differences among populations from distinct climatic zones and with variable socio-economic advantage. Clark et al. examined natural IgG antibody titers and avidity to three putative nontypeable *Haemophilus influenzae* (NTHI) vaccine antigens among children living in urban and remote Western Australia. The investigators showed that Australian Aboriginal otitis-prone children had lower serum IgG titers to two of the vaccine candidates compared to that of non-Aboriginal otitis-prone children and non-otitis prone children, potentially indicating a blunting of the immune response among children with early-onset OM (Thornton et al., 2017; Renz et al., 2018; Leach et al., 1994). The results also suggest possible benefits from immunization to boost antibodies against NTHI proteins (Clark et al.). In a second study, Ngo et al. showed NTHI and rhinovirus were the predominant otopathogens within the upper respiratory tract (URT) of children with and without OM from peri-urban and urban South-East Queensland (Ngo et al.). The presence of bacterial otopathogens in the middle ear was more predictive of concurrent URT infection than those associated with virus (Ngo et al.). As with earlier studies (Hall-Stoodley et al., 2006), PCR-based otopathogen detection was more sensitive than culture, potentially related to intracellular and biofilm-associated infections.

Relationships between host genomics and the airway microbiome in OM

Genetic markers of increased OM risk are poorly understood, but candidate genes are emerging. In the study by Elling et al., variants in alpha-1,2-fucosyltransferase (Fut2) were associated with an increased susceptibility to OM, potentially through a shift in nasopharyngeal or middle ear microbiota. The variant was associated with transcriptional changes in processes related to response to infection, increased pathobiont load in the middle ear and decreased nasopharyngeal commensals.

Microbiome shifts were also observed among patients with cholesteatoma. Frank et al. collected middle ear samples from patients undergoing tympanomastoidectomy for chronic OM and showed that factors such as quinolone use and cholesteatoma diagnosis had a substantial effect on middle ear microbiota composition.

Variable gene expression among OM pathogens

The complexity of microbial factors underlying OM are compounded by phenotypic adaption of otopathogens to different airway niches. Janouskova et al. reviewed gene regulation mechanisms that are well-orchestrated among otopathogens during OM in both experimental and in clinical settings. With a focus on phase variable and quorum sensing systems, the authors examined the ability of otopathogens to adapt inside the host to benefit survival and persistence. Whether this translates to increased opportunity for transmission and spread within and between populations remains an open question.

Immune responses in OM

Advances in understanding middle ear immunology provide opportunities to engineer and test more targeted and effective

OM treatments. A timely review by Massa et al., examines innate immunity in the middle ear in response to bacterial, viral or polymicrobial insult, demonstrating that localized inflammatory responses can persist even after clearance of the bacteria and virus from the middle ear. Otitis-prone children display a strong immune response suggesting that immunomodulatory therapeutics could be of benefit in these patients.

Multiple studies in this collection used *in vitro* models to investigate middle ear responses to infection, with several studies focused on middle ear hyperplasia and leukocyte infiltration – hallmarks of OM pathogenesis. Leichtle et al. showed in patients with chronic disease, persistent mucosal hyperplasia resulted in increased expression of inflammatory and apoptotic genes. Therapeutic approaches to dampen these responses could lessen the consequences of chronic disease. The use of single-cell RNA sequencing identified middle ear cells expressing genes associated with hyperplasia. Sakamoto et al. expanded upon these observations to show that a synthetic analog of viral double-stranded RNA (which simulates a middle ear viral infection) induced heparin-binding epidermal growth factor (HB-EGF) expression in middle ear epithelial, stromal and endothelial cells. Single-cell RNA sequencing also revealed that the eicosanoid leukotriene B4 (LTB4), an arachidonic acid metabolite, mediated leukocyte recruitment during OM (Heo et al.). LTB4 inhibition decreased mucosal hyperplasia and leukocyte infiltration (Heo et al.), suggesting potential therapeutic utility of LTB4 receptor antagonists in OM.

Animal models were also used to investigate how environmental exposures influence host responses. In an LPS-induced model of acute OM, Kim et al. demonstrated that mice treated with LPS and exposed to diesel exhaust particles (DEP) demonstrated mucosal cell hyperplasia and increased expression of TNF-alpha and IL1-beta. Pre-exposure to DEP increased inflammation and lymphangiogenesis.

Emerging OM model systems

Two studies reported the development and use of novel animal models that may allow more detailed mechanistic understanding of middle ear immune functions. Dewan et al. observed that *Bordetella bronchiseptica*, a respiratory commensal and pathogen of mice, ascends the Eustachian tube to colonize the middle ears, providing a model of naturally occurring acute OM. The investigators showed that mice lacking T and B cells failed to clear infection and that adoptive transfer of antibodies cleared lung infection but only partially cleared ear infection.

In a second study, Ma et al. demonstrated that small numbers of *Bordetella pseudohinzii* introduced into the nasopharynx can ascend the Eustachian tube and colonize and persist in the middle ear to model chronic infection. Secretion of a novel pertussis toxin-like factor was associated with prolonged persistence of middle ear pathology leading to hearing loss.

New diagnostic technologies

Data science approaches are increasingly leveraged to extract nuanced understanding of complex biological systems. Large, well-curated datasets are likely to be essential to realizing meaningful outcomes from studies using computational approaches, including artificial intelligence (AI). Current efforts to prepare large scale OM datasets include the Australian BIGDATA study reported by Beissbarth et al. – a compilation of 11 randomized controlled trials, 4 cohort studies, 8 surveys in over 30 remote communities and 5 surveys in urban childcare centers - that is expected to provide powerful insight into OM trends, otopathogen carriage rates and the impact of three sequential pneumococcal conjugate vaccines on pneumococcal serotype replacement among an Australian population at high-risk of OM.

Data science-based approaches are also supporting development of new diagnostic applications. Duan et al. provide an in-depth discussion of this issue and demonstrate a deep learning framework for deriving imaging-based diagnostic results of temporal bone diseases, cholesteatoma, middle ear inflammation and Langerhans cell histiocytosis that outperformed clinical expert-based diagnoses.

There is also vast opportunity for digital technologies to support telemedicine-based OM clinical care, as done during the COVID pandemic (Marom et al.). Though not without challenges, telemedicine consultations are also expected to increase in the coming years, with high potential to enable access to care for populations in geographically remote areas (Kokesh et al., 2011).

Conclusions

Over the past decade we have witnessed the advent of new advanced genomic methodologies, bioinformatics, coordinated multi-systems data analysis, microbiome investigations and the increased use of telemedicine and artificial intelligence platforms for diagnosis of disease. We are now witnessing the impact of these advances in the study of otitis media. The collection of articles in this research topic encompasses the breadth of work being done to advance interventions in public health, clinical diagnosis, and therapeutic development; work that is critically needed to realize our collective goal of reducing, and ultimately eliminating, the global OM disease burden.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
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Transition of Serotype 35B Pneumococci From Commensal to Prevalent Virulent Strain in Children

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In our community-based prospective cohort study in young children, we observed a significant increase in pneumococcal serotype 35B nasopharyngeal (NP) commensal colonization during the 2011–2014 timeframe, but these strains were not associated with disease. Beginning in 2015 and continuing through to the present, the serotype 35B virulence changed, and it became the dominant bacteria isolated and associated with pneumococcal acute otitis-media (AOM) in our cohort. We performed comparative analyses of 250 35B isolates obtained from 140 children collected between 2006 and 2019. Changes in prevalence, clonal-complex composition, and antibiotic resistance were analyzed. Seventy-two (29%) of 35B isolates underwent whole-genome sequencing to investigate genomic changes associated with the shift in virulence that resulted in increased rates of 35B-associated AOM disease. 35B strains that were commensals and AOM disease-causing were mainly associated with sequence type (ST) 558. Antibiotic concentrations of β-lactams and ofloxacin necessary to inhibit growth of 35B strains rose significantly (2006–2019) ($p<0.005$). However, only isolates from the 35B/ST558 showed significant increases in MIC_{50} of penicillin and ofloxacin between the years 2006–2014 and 2015–2019 ($p=0.007$ and $p<0.0001$). One hundred thirty-eight SNPs located in 34 different genes were significantly associated with post-2015 strains. SNPs were found in *nrdG* (metal binding, 10%); *metP* and *metN* (ABC transporter, 9%); *corA* (Mg^{2+} transporter, 6%); *priA* (DNA replication, 5%); and on the enzymic gene *ldcB* (LD-carboxypeptidase, 3%). Pneumococcal serotype 35B strains was a common NP commensal during 2010–2014. In 2015, a shift in increasing number of AOM cases occurred in young children caused by 35B, that was associated with changes in genetic composition and antibiotic susceptibility.

Keywords: *S. pneumoniae*, serotype 35B, antibiotic resistance, β-lactams, fluoroquinolones, whole genome sequencing, comparative genomics, acute otitis media

INTRODUCTION

Streptococcus pneumoniae (*SPN*) is a common bacterial cause of non-bacteremic pneumonia, sinusitis, otitis media, bacteremia, and meningitis (McCoy and Pettigrew, 2003; Bradley et al., 2011; Mufson et al., 2012). Although pneumococcal conjugate vaccines have had a dramatic protective effect against invasive pneumococcal disease (IPD) (Whitney et al., 2006; Pilishvili et al., 2010), non-IPD (Eskola et al., 2001; Black et al., 2002; Esposito and Principi, 2014), and nasopharyngeal (NP) carriage of vaccine serotypes (Kayhty et al., 2006; Whitney et al., 2006; O'Brien. et al., 2007; Pilishvili et al., 2010), strains expressing serotypes not included in the vaccines have emerged as replacements to colonize the NP and cause disease (Cohen et al., 2015; Kaur et al., 2016; Yildirim et al., 2017; Pichichero et al., 2018).

Since 2006, our group has been tracking *SPN* NP carriage and used acute otitis media (AOM) as a sentinel disease in children to monitor prevalence of pneumococcal serotypes (Pichichero and Casey, 2007; Pichichero et al., 2008; Casey et al., 2010; Casey et al., 2013; Kaur et al., 2016; Kaur et al., 2017; Pichichero et al., 2018; Kaur et al., 2020). After implementation of PCV-13 in 2010, we reported that serotype 35B *SPN* became one of the most frequent colonizers in young children (Kaur et al., 2016). However, we rarely detected 35B in middle-ear fluids (MEF) collected by tympanocentesis at the time of AOM prior to 2015. From 2006 through 2016, we found 35B isolates required increased concentrations of multiple β-lactam and fluoroquinolone antibiotics to inhibit growth (Kaur et al., 2020). Our observations in children were not geographically limited. Increased isolation of 35B strains from CDC IPD surveillance was also reported (Chochua et al., 2017; Varghese et al., 2019). During 2015–2019, we observed a striking increase in the detection rates of *SPN* 35B during AOM. Serotype 35B became the most commonly detected serotype among asymptomatically colonized young children and the most common cause of AOM during 2015–2019 (Kaur et al., EJCMID, pending revisions). The shift from NP commensal to AOM pathogen of the serotype 35B led us to perform the comparative analyses described here that included determination of antibiotic susceptibility, molecular characterization, and genetic analysis from whole-genome sequence (WGS) data. The objective was to describe dynamics of circulation of serotype 35B and understand the genetic changes associated with the emergence of a more virulent serotype 35B that was associated with pediatric disease.

MATERIALS AND METHODS

Pediatric Study Population, Sample Collection and Processing, Antibiotic Susceptibility, Multi-Locus Sequence Typing

This study involved subjects who participated in our prospective, longitudinal analysis of NP colonization and pneumococcal infections in young children from June 2006 through June 2019 (Casey et al., 2010; Casey et al., 2013; Kaur et al., 2016; Kaur et al., 2017; Kaur et al., 2020). The focus in this study was pneumococcal serotype 35B for 2006–2019.

Under the study protocol, children were enrolled from community-based pediatric practices, mainly from middle-class, suburban communities in the Rochester, New York area (USA) at 6 months of age at seven time points (6, 9, 12, 15, 18, 24, and 30–36 months of age). All enrolled children had received the full primary series of PCV7 or PCV13 before the enrollment. During these well-child check-ups, NP washes (instilling and withdrawing ~2 ml of saline in each nostril with bulb syringe) were prospectively collected and cultured for detection of pneumococci. The details of the study design have been previously described (Casey and Pichichero, 2004; Pichichero and Casey, 2007; Casey et al., 2010; Kaur et al., 2011; Casey et al., 2013; Kaur et al., 2016; Kaur et al., 2017; Pichichero et al., 2018; Kaur et al., 2020). Pneumococcal AOM was used as the sentinel disease to distinguish commensals from virulent strains. For precision, clinical AOM infections were microbiologically confirmed by culture of tympanocentesis fluid. Children were treated with antibiotics, most often with amoxicillin/clavulanate unless the child was allergic to penicillin, in which case cefdinir was typically used.

Written informed consent was obtained from parents prior to enrollment in the study, and the IRB of the Rochester Regional Health System approved the study.

Standard microbiology processing and identification techniques were used for isolation of bacteria including *SPN* from NP and MEF samples. Serotypes *of SPN* were determined using pure cultures of *SPN* by Quellung reaction using Latex pools and serotype-specific pneumococcal antisera (Serum Staten Institute, Denmark). Antibiotic susceptibility testing was conducted for 69% of the (173/250) serotype 35B clinical isolates obtained. When an NP and MEF isolate at onset of AOM were both serotype 35B, only the MEF isolate was included in antibiotic susceptibility analyses as described (Kaur et al., 2020). The susceptibility of *SPN* to antibiotics was determined with VITEK-2 AST-GP68 or GP74 susceptibility cards (bioMérieux) in the clinical laboratories of Rochester General Hospital. Benzylpenicillin, amoxicillin, ceftriaxone, cefotaxime, meropenem, ertapenem, ofloxacin, levofloxacin, moxifloxacin, erythromycin, telithromycin, vancomycin, linezolid, tetracycline, chloramphenicol, and trimethoprim-sulfamethoxazole (TMP-SMX) susceptibility was tested by automated modified broth microdilution. *SPN* strain ATCC49619 was used as a control for each batch of testing. Pneumococci were classified as susceptible, intermediate, or resistant based on 2021 CLSI breakpoints, with oral cutoffs used for penicillin (CLSI, 2021).

Multi-locus sequence typing (MLST) was conducted for 62% (154/250) of the serotype 35B isolates using either standard PCR and Sanger sequencing for seven housekeeping genes (n=82 isolates) or by *in silico* analyses based on their WGS assemblies (n=72 isolates). Sequence types (STs) of *SPN* by PCR method were determined as previously reported (Casey et al., 2013; Kaur et al., 2016; Pichichero et al., 2018; Kaur et al., 2020). For extraction from WGS assemblies, "Sequence query" on

PubMLST was used (Jolley and Maiden, 2010), and STs associated with 35B were extracted using the goeBURST algorithm from PhyloViZ (https://online.phyloviz.net/index) to estimate the clonality of the bacterial population (Feil et al., 2004; Francisco et al., 2012). Strains that shared greater than five out of seven MLST alleles were classified as belonging to a clonal complex (CC) (Tyrrell, 2011). Due to cost constraints and other technical issues (*SPN* could not be recovered from saved culture stocks), antibiotic susceptibility (69%) and MLST typing (62%) were confined to a subset of available *SPN* 35B isolates, selected from our repository to be representative of the time frame and sample types of this project.

WGS and WGS-Based Analysis

WGS was mainly performed by Illumina. Subsequent sequencing was conducted using PacBio (Pacific Biosciences) for some isolates to ensure higher contiguity and out of convenience, due to multiplexing requirements of our sequencers. Barcoded Illumina sequencing libraries (insert sizes ~150–600 bp) were produced using Nextera XT kits according to the manufacturer's protocols, and pooled libraries were sequenced on a NextSeq500 at 2 × 150 nt, targeting 200-fold genomic coverage per library. Barcoded PacBio SMRTbell libraries (insert sizes ~3–20 kb) were produced according to protocol for sequencing on the PacBio Sequel instrument, targeting 100-fold genomic coverage per library. We performed WGS sequencing on a subset of our isolates (**Technical Appendix 1**) to identify potential genetic factors within ST558 associated with the emergence of serotype 35B isolates in pediatric AOM cases and to validate a putative 15A-to-35B capsular serotype switch. This included ST558 isolates from pre-2015 (13 isolates) and post-2015 (36 isolates), ST14687 (one isolate), ST156 (seven isolates), ST10493 (eight isolates), and the novel 35B/ST14683 (one isolate) associated with 15A/ST63 (five isolates) collected for our patient cohort over the same time period to act as a basis for comparison.

Genome Assemblies and Annotation

All sequence data and assemblies have been deposited at NCBI under PRJNA734910. Illumina reads had technical sequences, such as barcodes and adapters, removed by Trimmomatic (v 0.33) (Bolger et al., 2014). Paired ends that overlapped were merged into single reads using COPE (v 1.1.2) (Liu et al., 2012). Merged and unmerged reads had errors corrected using ErrorCorrectReads.pl from allpathslg. Corrected reads were assembled using Spades (v 3.7.0) (Bankevich et al., 2012). Heavily contaminated assemblies were removed by dropping those that were not *SPN* according to Taxator-tk. This was done before removing bad contigs because, in our experience, small amounts of contamination can be removed by contig filtering, but this is more difficult as the level of contamination increases. Poor quality contigs were removed by removing those in which more than 97% of the contig was a single nucleotide, the total length was less than 256 bases, or the coverage per million reads remaining after Trimmomatic was less than 4. Genomes were removed from further analysis if they had more than 130 contigs remaining.

PacBio reads were assembled using Falcon (smrtlink v 7.0.1) (Chin et al., 2016). Contigs were circularized using Circlator (v 1.5.5) (Hunt et al., 2015), and then the resulting assemblies were polished using Arrow (smrtlink v 7.0.1). Contigs were removed if their normalized coverage was less than 0.06 or their quality score was less than 60. The remaining contigs had errors corrected using the corrected Illumina reads (the reads after running ErrorCorrectReads.pl) and Pilon (v 1.23) (Walker et al., 2014). Assemblies for some strains (PP6, PP9, PP11, PP12, PP13, PP14, PP15, PP16, and S589) were not corrected because they were not sequenced on Illumina. Finally, circular contigs were permuted to start at dnaA and linear contigs (only S602) were split into two pieces such that one piece started with dnaA. For subsequent analyses, PacBio assemblies were used if available; otherwise, Illumina assemblies were used.

Before annotation, the species of each assembly was confirmed using Taxator-tk (v 1.2) (Droge et al., 2015). The genes in each assembly were annotated using Prokka (v 1.13) (Seemann, 2014), and then gene clusters were identified using Roary (downloaded from github on January 22, 2019) (Page et al., 2015). Gene clusters with at most one copy per genome were aligned using Prank (v 0.100802) (Loytynoja, 2014) and then concatenated, adding gaps for genes absent from any alignments. This alignment was used as the input alignment to make a phylogenetic tree with RaxML (v 8.2.4) (Stamatakis, 2006). The tree was midpoint rooted using ETE (v 3.0) (Huerta-Cepas et al., 2016). To search for gene presence/absence differences associated with the time periods of interest, we gave the roary gene clusters and the phylogeny to Scoary (v 1.6.16). Both the gene clusters and the phylogeny were filtered so the only contained strains were 35B and ST558.

To find SNPs between the 35B, ST558 strains, we called variants using Snippy (v 4.4.5) (Seemann, 2015). Isolate PP6 was used as the reference strain. For each query strain, we used Illumina reads; otherwise, we used the PacBio assembly for that strain (strains without Illumina reads are listed above). The list of variants was filtered for significant hits using the filtered phylogeny and Scoary (v 1.6.16). These top hits were mapped to genes using Prokka's gene annotations and SnpEff (v 4.3) (Cingolani et al., 2012). Finally, the gene clusters from Roary were used to map hits between strains. The genes that were not identified by Prokka were further identified by BLAST search.

Analysis of Capsule Switch From Serotype 15A to 35B

The best candidate for the parent strain was identified by examining the phylogeny and using BLAST (v 2.2.28) with the sequences flanking the capsule. The candidate for the capsule donor was identified by using BLAST with the sequence of the capsule and the surrounding region. Each strain was then aligned to S558 (the strain with the capsule switch) using Mauve (v 2015-02-13) (Darling et al., 2004). In order to improve the quality of the alignments, contigs were first reordered using Mauve's contig mover. SNPs and indels were extracted, and sliding windows of each were computed using a window size of 1,000 bp and an increment of 100 bp. Capsular serotypes identified by serotyping

methods were further confirmed with PneumoCat (v1.2) (Kapatai et al., 2016).

For pilus-1 operon (Regev-Yochay et al., 2010), genes associated with antibiotic resistance [β-lactam: *pbp1a*, *pbp2x*, *pbp2b* (Li et al., 2017), fluoroquinolones: *gyrA*, *parC* (Brueggemann et al., 2002), macrolides: *ermB*, and tetracycline: TETM (Chochua et al., 2017)] were annotated and extracted with PATRIC. (Brettin et al., 2015; Wattam et al., 2018). PBP typing was classified based on the penicillin binding protein gene type list available at (https://www.cdc.gov/streplab/pneumococcus/mic.html?CDC_AA_refVal=https%3A%2F%2Fwww.cdc.gov%2Fstreplab%2Fmic-tables.html) as of February 2021. For fluoroquinolones, where extracted gene sequences have previously reported point mutations on *gyrA* and *parC* were analyzed (Brueggemann et al., 2002). The presence of the *ermB* gene and TETM were checked using PATRIC.

Statistical Analysis

The difference in the case (AOM) to colonization ratio (the number of isolates from MEF at the time of AOM/the number of isolates from NP at the time of health) was calculated for each respiratory year (June–May). The ratio between pre-2015 (June 2006–May 2015) and post-2015 (June 2015–May 2019) was analyzed by the Mann-Whitney test. The change in ratio among ST558 was analyzed by Fisher's exact test. The trend in antibiotic MICs was computed using a linear regression model. Comparison of MIC50 values was conducted using the Mann-Whitney test with a Bonferroni correction.

RESULTS

Increasing Prevalence in Children of Pneumococcal Serotype 35B Colonization and AOM

We re-examined 250 serotype 35B isolates (consisted of 11.3% of total *SPN* isolates for the study period) that had been collected from 140 children (18.6% of enrolled children) during 2006 to 2019. Of these, 172 isolates (12% of *SPN* isolates) from the NP of healthy subjects, 60 isolates (11.7%) from the NP at the onset of AOM, and 18 isolates (13%) from MEF were collected. Yearly prevalence of 35B isolates as a proportion of all pneumococci is shown in **Figure 1**. Before introduction of PCV13 in 2010, serotype 35B was rarely isolated. After 2011, the relative prevalence of colonizing serotype 35B strains (from healthy NP) significantly increased from 2.7 to 6.1% of all *SPN* (from June 2006 to May 2011) to 12.3–18.8% (from June 2011 to May 2015). Despite colonization prevalence, AOM infections were infrequent. Beginning from June 2015, the prevalence of 35B increased, and it became the most frequently isolated serotype from MEF during AOM bouts. To see the risk of AOM given the frequency of colonization, we calculated the case-to-colonization ratio changes among 35B isolates over time and found AOM cases (MEF isolates during AOM) as a proportion of controls (NP isolates from healthy colonization) significantly increased from 0.08 for the period before June 2015 to 1.68 for the period after May 2015 (p=0.002). Thus, introduction of PCV13 in 2010 was followed by increased prevalence of NP colonization with serotype 35B strains, and an additional shift to higher prevalence and increased AOM disease burden beginning in 2015.

Serotype 35B Strains Predominantly Belong to a Single Clonal Complex but Capsular Switching Also Occurred

Among 163 35B strains tested, 13 distinct sequence types (STs), including two newly identified STs were identified (**Figure 2A**). Most 35B strains belonged to ST558 (89% of pre-2015 and 70% of post-2015 isolates). Although ST558 isolates were detected at comparable levels in healthy children among pre-2015 and post-2015 isolates (6.0 and 5.6%), their isolation among children causing AOM significantly increased post-2015. When the ratio of AOM disease cases to healthy colonization was calculated only among ST558, values for pre-2015 and post-2015 were 0.09 and 2.18, respectively (p=0.0002), underlining the emergent AOM disease burden caused by ST558 *SPN* strains.

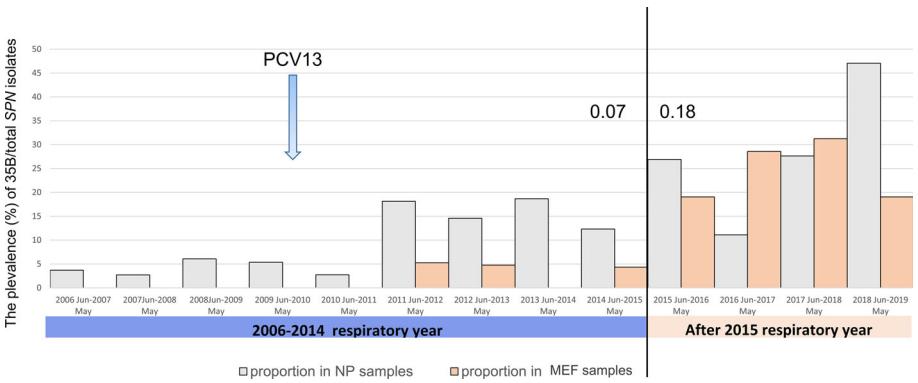

FIGURE 1 | The prevalence of pneumococcal serotype 35B isolation from 2006 to 2019 in the nasopharynx during colonization and AOM and from MEF during AOM disease in 6–36-month-old children. The prevalence (%) of 35B was calculated by dividing the number of 35B isolates with total number of *SPN* isolates. The year is shown by respiratory year (June–May). The case (AOM)-to-colonization ratio (the number of isolates from MEF at the time of AOM/the number of isolates from NP at the time of health) was calculated and is shown for 2014 (0.07) and 2015 (0.18).

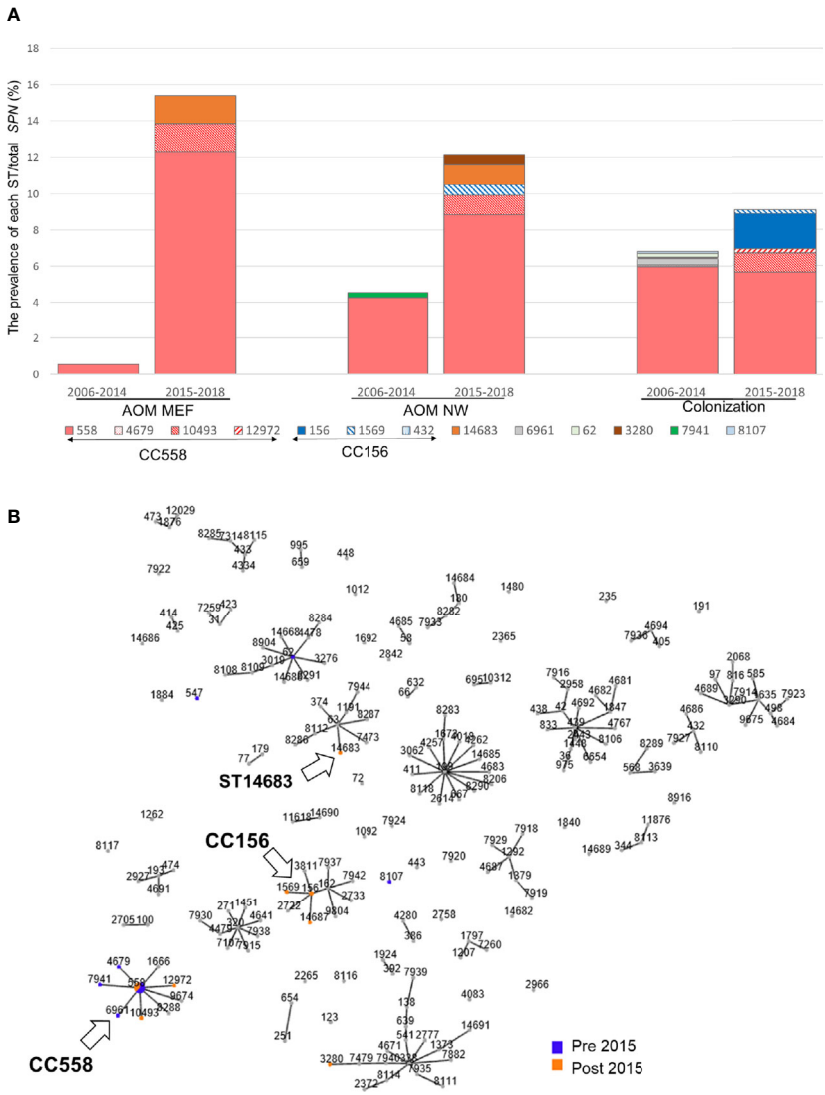

FIGURE 2 | **(A)** Sequence type distribution and prevalence/total *S. pneumoniae* isolates between pre-2015 and post-2015. The prevalence (%) of each ST was calculated by dividing the number isolates with the total number of *SPN* isolates between 2006–2014 and 2015–2018. **(B)** All MLST types and their association with serotype 35B from strains isolated during 2006–2019 in Rochester, NY. Clonal complex that shared greater than five out of seven MLST alleles was linked. Arrow marked 35B/CC558, 35B/CC156, and 35B/ST14683.

To search for evidence of 35B capsular switching, we clustered STs into clonal complexes (CCs) using goeBurst (**Figure 2B**). We found three STs in pre-2015 and two STs observed in post-2015 samples were closely related to ST558 (circled "CC558" consisting of single allele variants from ST558, **Figure 2B**). A more distantly related cluster of three STs separated by a single allele variant including one newly described ST14687 (CC156) was identified; this likely represents a recently described 35B capsule switch between 35B/ST558 and 9V/ST156 serotype strains by Chochua et al. (2017). We also found evidence of a new potential capsular switch. We identified a post-2015 serotype 35B isolate belonging to newly described ST14683, which shares only one allele (ddl) with CC558 and none with those in the "CC156" strains. The ST14683 isolate was detected from two different children. The new ST14683 is a single allelic step from ST63, which was associated with serotype 15A ("CC63") in our cohort.

Genomic Comparisons Confirm 35B Association With ST558 and Two Distinct Capsule Switches Into 9V and 15A Genetic Backgrounds

Genome assemblies were used to produce a phylogenetic tree based on protein-coding genes (**Figure 3**), which found three distinct clades and was consistent with the ST-based results. The largest clade included ST558 and related STs, and strains were isolated from all categories (further discussed below): AOM

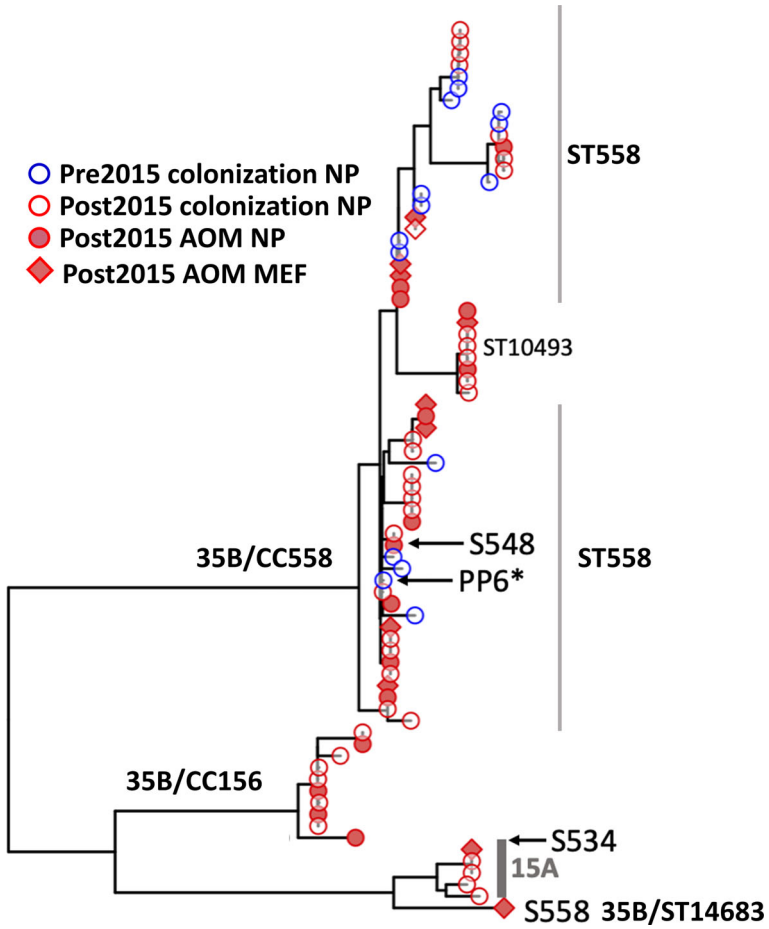

FIGURE 3 | Phylogenetic tree of all 35B isolates based on core protein coding genes from WGS analysis. This phylogeny contains all of the ST558 35B strains sequenced for this study. The strains marked in blue circle were isolated prior to 2015 from colonization and red circle were after 2015 from colonization. Filled red circle represents strains isolated during AOM infections from NP, and filled square represents isolated during AOM from MEF. PP6 used as the reference strain was marked with *. S558 is the 35B/ST14683. The two representative parental strains used for the further analysis to see recombination breakpoints were marked by arrow (S548: 35B/ST558, S534: 15A/ST63).

(MEF and NP) and colonization and pre-2015 and post-2015. The second largest clade contained CC156 isolates that were all collected post-2015, consistent with reports of a recent emergence of this lineage (Chochua et al., 2017). The final branch of serotype 35B consisted of a single ST14683 (S558) strain that was isolated from MEF at an AOM visit that grouped closely with serotype 15A isolates from ST63.

To confirm this novel capsule switch and reconstruct the recombination breakpoints, we produced and aligned nearly finished genomes for the 35B/ST14683 capsule switch strain S558 and two representatives of the parental strain backgrounds (for the donor, 35B/ST558 isolate S548, and for the recipient, 15A/ST63 isolate S534). Comparison of nucleotide divergence across the capsule locus and its flanking sequences shows near-perfect identity between the capsule locus and its immediate flanks with the 35B donor representative, whereas the more distant flanks are nearly identical to the 15A recipient representative (**Figure 4A** and **Supplementary Figure 1**). The precise breakpoints were obscured by high divergence from both parents at intermediate distances from the flanks, including the presence of indels, suggesting the involvement of a third donor or more complex history of divergence within 15A strains. Consistent with past recombination at other loci from 35B/ST558 and other strains into ST14683, most MLST alleles in ST14683 were shared with 15A/ST63 strains, but also included two alleles shared with ST558 and three novel alleles, including a novel potential recombinant allele of *PBP 1a* (**Figure 4B**). This ST isolate was negative for *rrgC* (PI-type 1) that is a common character of 15A and positive for ermB and tetM that were the same profiles as 15A ST63 detected in our cohort.

Associations of Clinical Provenance With Genomic Variation Within ST558

In order to identify potential *SPN* genetic determinants that could be responsible for the increased disease burden associated with the 35B strains post-2015, we focused on genomic variation

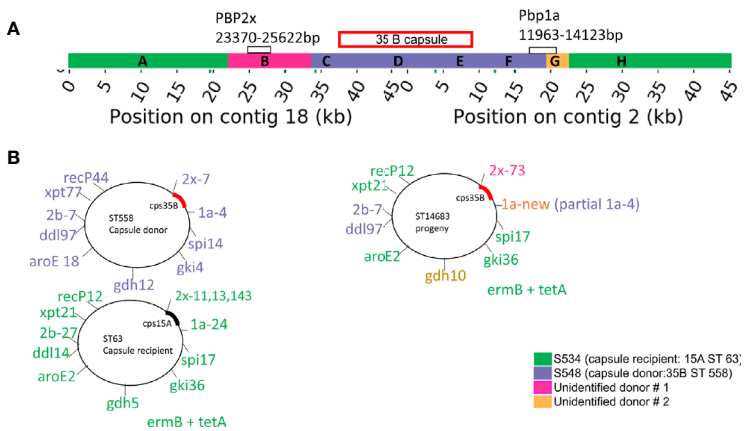

FIGURE 4 | **(A)** Diagrammatic representation of capsule region and adjacent regions from donor, recipient in progeny strain. **(B)** Combination of seven housekeeping genes and PBP genes. The diagram in **(B)** represents our interpretation of the source of each of these regions. The region that is homologous to the capsule donor to the recipient is purple and corresponds to regions C–F in **Supplementary Figure 1**. The regions that are homologous to the recipient strain are orange and correspond with regions A and H in **Supplementary Figure 1**. Regions B (pink) and G (yellow) have limited homology to both the donor and recipient, which probably means that they came from one or more unidentified donors.

within ST558, since most of our sequenced 35B strains belonged to this ST and these had been isolated from across the study period. The ST558 gene phylogeny did not group strains by time period, colonization/AOM status, or NP/MEF isolation during AOM, indicating that emergent 35B disease burden post-2015 is not due to a specific ST558 clone (**Figure 3**). We next sought to identify whether specific genetic variants—either gene presence/absence differences or SNPs—within the ST558 lineage were associated with isolation from disease post-2015. To examine gene presence/absence differences within ST558, 2,072 clusters of orthologous genes were identified, of which 1,804 (87%) were found in >95% of strains. The remaining accessory (or distributed) genes showed no significant associations with time period or health status (**Supplementary Figure 2**). Because the resurgence of *rrgC* (PI-type 1) positive strains were observed among non-vaccine type over time and proposed to be selective virulence advantages (Croney et al., 2013), we specifically checked for *rrgC*. We confirmed that most of ST558 isolates through the entire study period were positive for *rrgC*.

SNP analyses identified 138- post-2015-occurring SNPs that were not detected from strains isolated prior to 2015 that were significantly associated with the post-2015 era (**Table 1**). These SNPs were distributed among 34 different genes. Ten percent of these SNPs were found within the *nrdG* (metal binding) gene; 9% within the *metP* and *metN* genes (members of an ABC transporter); 6% within the *corA* (Mag2+ transporter) gene; 5% within the *priA* gene (DNA replication); and 3% within the enzymatic gene, *ldcB* (LD-carboxypeptidase).

Trends in Antibiotic Susceptibility Among 35B Strains

The MICs for 35B isolates significantly increased over time for 7 of 16 antibiotics tested (**Figure 5**). Six of the seven antibiotics were β-lactams, and one was a fluoroquinolone. For amoxicillin, cefotaxime, and meropenem, linear regression analysis showed that the resistance category changed from sensitive to intermediate over the period from 2006 to 2019. The MIC trend for ertapenem and ofloxacin were close to category change (sensitive to intermediate). To identify STs associated with the increase in antibiotic MICs, MIC50s for each ST were also compared (**Table 2**). In general, STs detected after 2015 showed higher MIC50 in six antibiotics (four different β-lactams, ofloxacin, and TMP/SMX). Ofloxacin and penicillin MIC_{50} among ST558 showed significant increases between the two time periods. Although only two isolates were detected as ST14683, it is noteworthy that this ST showed multidrug resistance against 6 β-lactams, ofloxacin, tetracycline, and TMP/SMX.

β-Lactams and Fluoroquinolone Resistance Determinants

Since we detected increased MICs in β-lactams and ofloxacin among 35B strains over time, we classified β-lactam resistance determinants (PBP types:*PBP1a, 2b* and *2x*) (Li et al., 2017), and fluoroquinolones resistance determinants (*gyrA/parC* genes) (Brueggemann et al., 2002) in our whole genome sequence data. 35B from pre-2015 only associated with PBP type 4:7:7 except two isolates that had one amino acid mutation on *PBP1a* and *PBP2b*. CC558 (ST558, 10493) from post-2015 was also exclusively associated with PBP type 4:7:7. ST156 and ST3280 were associated with PBP type 4-12-7. Our only sequence from ST14683 had a novel *PBP1a* type that was not found in the list of PBP types provided by the US CDC (**Technical Appendix 1**).

DISCUSSION

In this study, we report emergence of serotype 35B as a common NP commensal colonizer during 2010 to 2014 in young children

TABLE 1 | Genes with mutations that are unique to ST558 from post-2015.

Function	Gene name	Moderate	Modifier	The number of SNP
Transporter	corA		8	8
	metP		4	4
	metN	1	7	8
Metabolism	idnO		1	1
	manX_2		1	1
	adk		1	1
Protein biosynthesis	fmt	1	1	2
Transcriptional regulator	YesS*		4	4
Genetic information processing	yqeH	1	1	2
	ftsL		1	1
	priA	3	7	10
	ruvA		1	1
	uvrA		2	2
	rplB		1	1
	rplE	1	1	2
	rplN		1	1
	rplV		1	1
	rsmH_1		1	1
	Putative NrdI-like protein		1	1
Enzyme	nrdG	1	13	14
	nrdD	1	3	4
	ribD		2	2
	fpgS_1		1	1
	kdgA		1	1
	Y1_Tnp*	2	8	10
	ypdF		1	1
	YesM*		1	1
	LdcB*	4		4
	LPXTG-anchored hyaluronate lyase*	1	6	7
Membrane	MptD family putative ECF transporter S*	3	5	8
	DUF1129 domain-containing protein*	5	10	15
Function unknown	YesL*	1	3	4
	ykfA		9	9
	yqeH		1	1
	hypothetical protein		3	3

**The gene name was identified by further BLAST search.*

A total of 49 isolates were analyzed (pre-2015: 13 isolates, post-2015: 36 isolates). PP6 was used as reference. High, moderate, modifier describes the impact of SNP categorized by SnepEff. High: stop_gain, Moderate: missense_variant, Modifier: intragenic and intergenic_variant.

and then a dramatic shift to virulence in 2015, evidenced by a significant increase in serotype 35B *SPN* disease in the form of AOM infections. The basic genetic composition of the 35B strains, reflected by MLST analyses, was and remained predominantly ST558 during the switch of the 35B strains from commensals to otopathogens. WGS analysis identified 138 SNPs within 31 different genes that appeared only following the change in virulence in 2015.

Phylogenetic analysis showed that 35B ST558 from pre-2015 and post-2015 were closely related and strains did not segregate by time period. Our data suggest that most 35B isolates before and after emergence of more virulent strains causing disease in young children were from a single clonal complex containing ST558, although two new clonal complexes, including a new switch into a 15A-like genetic background, also were observed. Although the number of detection was small, this capsular switch event between ST558 or ST156 and 15A/ST63 as progeny of ST14683 is noteworthy. Not only the event occurred between two non-PCV13-covered serotypes, but also ST14683 showed multidrug resistance by acquiring tetracycline resistance and accessory gene (TetM) from 15A in addition to high non-susceptibility against β-lactams and TMP/SMX of 35B.

We have found SNPs in 34 genes, including 13 genes with missense variants, were annotated including Transporter genes, metabolism-related genes, enzymes, e.g., *nrdG*, and protein biosynthesis and processing genes. The *nrdG* gene was shown to be upregulated during anaerobic growth of *SPN* R6 strain (Bortoni et al., 2009), suggesting its importance during infection where the concentration of oxygen is low as occurs in the middle-ear space. The *nrdD/nrdG* genes are known to be essential factors in anaerobic growth and induction of biofilm formation in *Escherichia coli* (Garriga et al., 1996; Cendra Mdel et al., 2012). Bacteria change their metabolism based on their surrounding environment in order to acquire essential nutrients to survive and cause disease (Groisman, 1998; Eisenreich et al., 2010). *metP, metN* genes are involved in a methionine ABC transporter (Merlin et al., 2002), and the *corA* gene is involved in a Mg^{2+} transporter (Kehres et al., 1998); both methionine and Mg^{2+} are important for bacterial growth (Shelver et al., 2003; Fontecave et al., 2004; Groisman et al., 2013), and their availability varies on

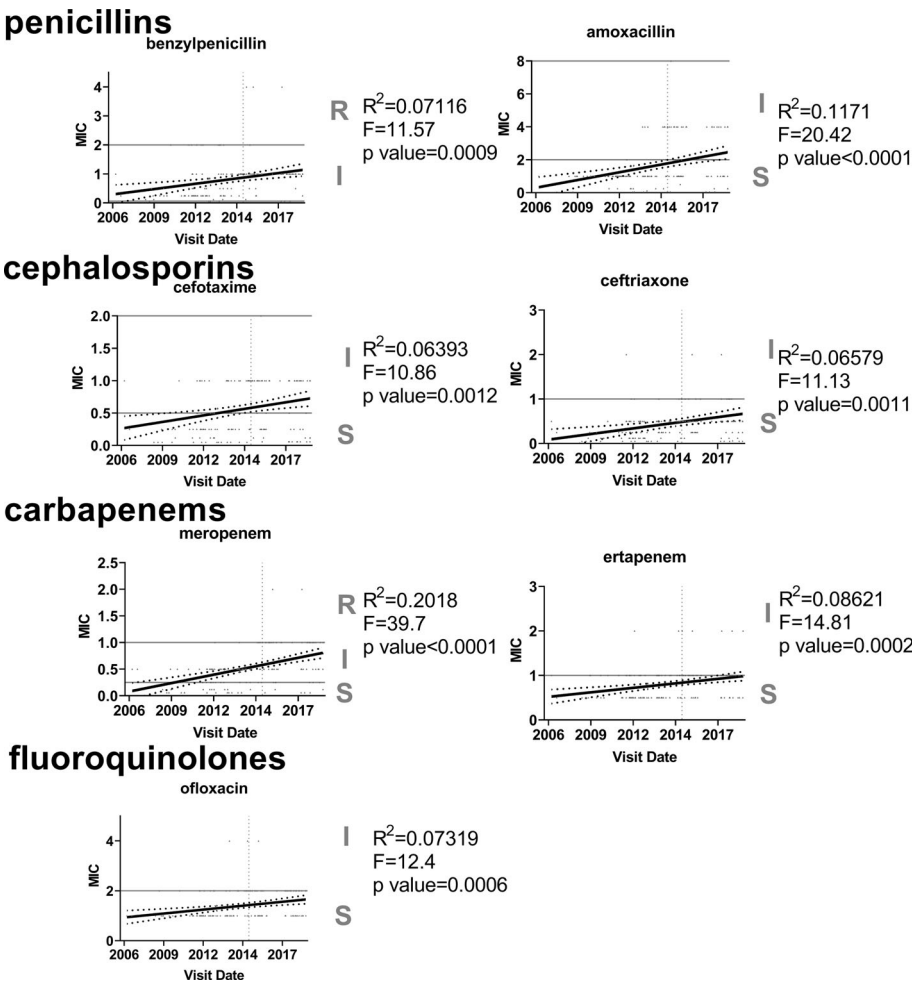

FIGURE 5 | Increasing trend in antibiotic resistance among 35B serotype. Linear regression was used to analyze MIC values (μg/ml) for each antibiotic (n=170). Only seven antibiotics that showed significant increasing trends are shown.

bacteria location (Shelver et al., 2003). Knockout of either of the transporter system showed growth inhibition of *SPN* in various concertation of medium as well as in blood (Neef et al., 2011; Basavanna et al., 2013). ldcB is LD-carboxypeptidases [also known as *dacB* (Cadby and Lovering, 2014)], elucidate the residues essential for peptidoglycan and conformational changes. Deletion of ldcB attenuated D39 *SPN* in murine models of pneumonia and meningitis associated with enhanced uptake by professional phagocytes (Abdullah et al., 2014). Interestingly, 32% of the genes where SNP mutations were found in post-2015 35B strains are known to have functions in genetic information processing such as DNA repair (Ambur et al., 2009). *priA* works in DNA repair by recognizing the DNA replication forks (Nurse et al., 1999; Ambur et al., 2009), and mutated *priA* gene was associated with growth deficiency and more sensitivity to oxidative agents in *Neisseria gonorrhoeae* (Kline and Seifert, 2005). *ruvA* and *uvrA* also function in DNA repair (Iwasaki et al., 1992; Moolenaar et al., 2000). The absence of *ruvA* in *E. coli* leads to a decrease in survival when exposed to fluroquinolone, suggesting the gene functions in phenotypic resistant without becoming genetically resistant (Theodore et al., 2013). The absence of *uvrA* causes hypersensitivity to rifampicin killing (Chao, 1986).

The possible role, association, and importance of the SNPs we identified among recent 35B strains collected from children, and whether they contribute to the virulence of 35B strains, will be the area of future investigation. Sensory-transduction system of *Acinetobacter baumannii* mediates both enhanced virulence by increased protection from serum complement killing and tolerance against antibiotics (Geisinger et al., 2018), consistent with our observation of 35B strains in virulence and antibiotic tolerance.

Among 35B strains, we observed an increase in quantities of six β-lactams and ofloxacin required to inhibit growth. The required MICs for penicillin and ofloxacin significantly increased among 35B strains of ST558 with time, but the

TABLE 2 | MIC50 changes between pre-2015 and post-2015 in each sequence type.

ST	Antimicrobial resistance phenotype, ug/ml																			
	Number of isolates		Amoxacillin		Cefotaxime		Ceftriaxone		Ertapenem		Meropenem		Ofloxacin		Penicillin		Tetracycline		TMP/SMX	
			Susceptibility range (S; I ; R)																	
	Pre	Post	≤.2; 4; ≥8		≤.5; 1; ≥2		≤1; 2; ≥4		≤1; 2; ≥4		≤.25; .5; ≥1		≤2; 4; ≥8		≤.06; .12-1; ≥2		≤2; 4; ≥8		≤9.5; 19-38; ≥76	
			Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
558	73	51	*1*(58)	*1* (24)	*0.25* (60)	*0.25* (25)	*0.25* (60)	*0.5* (25)	*0.5* (59)	*1** (25)	*0.5* (60)	*0.5* (24)	*1* (60)	*1.5*** (24)	*0.25* (60)	*1***** (20)	*1* (60)	*1* (25)	*10* (60)	*10* (25)
6961	3	ND	*1*(2)	-	*0.38* (2)	-	*0.25* (2)	-	*0.5* (2)	-	*0.5* (2)	-	*1*(2)	-	*0.25* (2)	-	*1* (2)	-	*10*(2)	-
62	2	ND	*1*(1)	-	*0.5* (1)	-	*0.5* (1)	-	*1* (1)	-	*0.5* (1)	-	*1*(1)	-	*1*(1)	-	*1* (1)	-	*10* (1)	-
10493	ND	7	-	*2* (5)	-	*1* (5)	-	*0.5* (5)	-	*1* (5)	-	*1* (5)	-	*2* (5)	-	*1* (5)	-	*1* (5)	-	*10* (5)
156	ND	9	-	*4* (8)	-	*1* (8)	-	*1* (8)	-	*1* (8)	-	*1* (8)	-	*1* (8)	-	*1*(8)	-	*1* (8)	-	*160* (8)
1569	ND	2	-	*4* (2)	-	*1* (2)	-	*1* (2)	-	*1* (2)	-	*1* (2)	-	*1* (2)	-	*2* (2)	-	*1* (2)	-	*120* (2)
14683	ND	2	-	*4* (2)	-	*1.5* (2)	-	*2* (2)	-	*2* (2)	-	*2* (2)	-	*3* (2)	-	*4* (2)	-	*16* (2)	-	*160* (2)
Unknown ST	39	57	*1*(24)	*2* (15)	*0.5* (24)	*1* (15)	*0.38* (24)	*0.5* (15)	*0.5* (24)	*1* (15)	*0.5* (24)	*1* (15)	*1* (24)	*2**(15)	*0.75* (22)	*1* (15)	*1* (24)	*1* (15)	*10*(24)	*10* (15)
All 35B	119	131	*1*	*2*****	*0.25*	*1*****	*0.25*	*0.5****	*0.5*	*1*****	*0.5*	*1*****	*1*	*2*****	*0.25*	*1****	*1*	*1*	*10*	*10*

*Seven antibiotics that showed differences in MIC50 (μg/ml) were analyzed. STs that have more than two isolates are shown. The number of isolates data analyzed for antibiotic susceptibility is shown in brackets. P<0.007 was considered as p<0.05 after the Bonferroni correction. ND, not detected, *<0.05, **<0.007, ***<0.0004, ****=0.0001, *****<0.0001.*

increases were not associated with PBP types and fluroquinolone resistance determinants, respectively. Although the increases in MIC for penicillin and ofloxacin did not change the interpretation category of resistance (penicillin: intermediate, ofloxacin: sensitive), which still support the notion that PBP types and fluoroquinolone genetic determinants are the dominant factors that dictate a strain's resistance to their respective antibiotics (Li et al., 2016). However, our results suggest the existence of additional factors that can modulate antibiotic non-susceptibility at a marginal level. Sequence variations outside the PBPs that influence β-lactam MICs have been reported previously (Sauerbier et al., 2012; Chewapreecha et al., 2014). A recent study has shown that a part of the penicillin-binding protein (*pbp1b*), which does not change resistance to penicillin, but prolongs killing time, increases the risk for pneumococcal meningitis (Li et al., 2019).

The cause of the shift in antibiotic susceptibility to ofloxacin is of interest. Fluoroquinolones are not used in our pediatric population. Together with the unclear reason why we have observed the delayed emergence of 35B among our pediatric population in 2015, we note that in 2014, the CDC recommended use of PCV13 in adults in the USA (Tomczyk et al., 2014), and AOM incidence increase among 35B strains began a year later among our child population, suggesting adult to child transmission of strains. These observations suggest that further study of adult PCV vaccination and adult-to-child transmission of *SPN* may be warranted.

This study had some limitations. Our study was geographically limited. However, 35B emergence has been reported by the CDC as occurring nationwide in the US for both non-invasive and invasive diseases (Chochua et al., 2017). Studies from other groups in France, Israel, and USA have also reported emergence of 35B SPN strains (Cohen et al., 2015; Chochua et al., 2017; Yildirim et al., 2017). We did not sequence all 35B strains, so minority ST populations may have been missed. The causality of genetic differences among 35B/ST558 strains in pathogenesis from pre-2015 and post-2015 was not tested.

In conclusion, serotype 35B strains have become the most frequent pneumococcal disease isolate in young children for the sentinel infection AOM, mainly associated with ST558 since 2015. SNPs in 34 genes were associated only with isolates post-2015. Antibiotic MICs for β-lactams and ofloxacin among 35B strains have increased over time. Emergence and dynamic changes in genetic makeup among SPN strains have occurred since introduction of PCVs, and further changes should be anticipated and studied.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI, PRJNA734910. The SRA accession numbers can be found in **Technical Appendix**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Rochester Regional Health Institutional Review

Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MP and RK contributed to conception and design of the study. NF and RK organized and analyzed the data. NF, RE, JM, and RK contributed to data curation. NF and JM wrote the first draft of the manuscript. RE wrote sections of the manuscript. GE supervised the whole genome sequence data analysis. All authors reviewed contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.744742/full#supplementary-material

Supplementary Figure 1 | The line graphs of SNPs and indels in the capsule donor and recipient strains when aligned with Mauve to S558, the strain with the capsule switch. The x-axis shows the coordinate on contig 18 or the coordinate on the first 35 kb of contig 2. The y-axis shows the number of indels (top graphs) or SNPs (bottom graphs) in each 1 kb sliding window. The orange lines represent the capsule recipient, S534, and the purple lines represent the capsule donor, S548. Different segments of interest are separated by dashed green lines on contig 18 at 19,600, 30,000, and 34,229 bp and on contig 2 at 3,890, 12,200, and 15,000. The segments are labeled **(A–H)** on the top graphs. **(D, E)** are the 35B capsule.

Supplementary Figure 2 | Gene presence/absence difference in ST558 associations with time periods and health status.

Technical Appendix | Clinical information, SRA accession numbers, PBP typing, MLST alleles and Phenotypic antimicrobial susceptibility of each isolate determined with WGS is shown.

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REVIEW
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Innate Immunity in the Middle Ear Mucosa

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Otitis media (OM) encompasses a spectrum of clinical presentations ranging from the readily identifiable Acute OM (AOM), which is characterised by otalgia and fever, to chronic otitis media with effusion (COME) where impaired hearing due to middle ear effusion may be the only clinical symptom. Chronic suppurative OM (CSOM) presents as a more severe form of OM, involving perforation of the tympanic membrane. The pathogenesis of OM in these varied clinical presentations is unclear but activation of the innate inflammatory responses to viral and/or bacterial infection of the upper respiratory tract performs an integral role. This localised inflammatory response can persist even after pathogens are cleared from the middle ear, eustachian tubes and, in the case of respiratory viruses, even the nasal compartment. Children prone to OM may experience an over exuberant inflammatory response that underlies the development of chronic forms of OM and their sequelae, including hearing impairment. Treatments for chronic effusive forms of OM are limited, with current therapeutic guidelines recommending a "watch and wait" strategy rather than active treatment with antibiotics, corticosteroids or other anti-inflammatory drugs. Overall, there is a clear need for more targeted and effective treatments that either prevent or reduce the hyper-inflammatory response associated with chronic forms of OM. Improved treatment options rely upon an in-depth understanding of OM pathogenesis, particularly the role of the host innate immune response during acute OM. In this paper, we review the current literature regarding the innate immune response within the middle ear to bacterial and viral otopathogens alone, and as co-infections. This is an important consideration, as the role of respiratory viruses as primary pathogens in OM is not yet fully understood. Furthermore, increased reporting from PCR-based diagnostics, indicates that viral/bacterial co-infections in the middle ear are more common than bacterial infections alone. Increasingly, the mechanisms by which viral/bacterial co-infections may drive or maintain complex innate immune responses and inflammation during OM as a chronic response require investigation. Improved understanding of the pathogenesis of chronic OM, including host innate immune response within the middle ear is vital for development of improved diagnostic and treatment options for our children.

Keywords: innate immunity, otitis media, non-typeable *Haemophilus influenzae*, influenza A virus, otopathogens, bacteria, viruses, mucosa

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INTRODUCTION

Otitis media (OM) is defined as inflammation of the middle ear and encompasses a range of clinical presentations. Acute OM (AOM) is characterised by otalgia and fever and may occur occasionally, particularly during a child's first 3 years of life. If acute episodes occur a minimum of 3 times in a 6 month period or more than 4 times within a 12 month period the condition is considered to be recurrent (RAOM) (Granath, 2017). Otitis media with effusion (OME) is defined by the presence of middle ear fluid, or effusion, without any symptoms except impaired hearing due to restricted mobility of the tympanic membrane. Chronic suppurative OM (CSOM) involves perforation of the tympanic membrane with persistent fluid discharge for more than 6 weeks (Kong and Coates, 2009; Lieberthal et al., 2013; Massa et al., 2015; Bhutta et al., 2017).

The pathogenesis of OM is largely due to activation of the innate inflammatory responses to viral and/or bacterial infection of the upper respiratory tract (Mandel et al. 2008; Bakaletz, 2010; Mittal et al., 2014a; Parrish et al., 2019). Pathogen-induced inflammation in the nasopharynx (primarily due to viral infections) and eustachian tubes (both viral and bacterial infections) then leads to a range of responses including enhanced mucus secretion (Val et al., 2018), neutrophil extracellular traps (Thornton et al., 2013), damage to the epithelium and enhanced commensal bacterial colonisation (Nokso-Koivisto et al., 2015; Chonmaitree et al., 2016). Nasopharyngeal inflammation also causes a loss of pressure equilibrium with the middle ear (Martin et al., 2017), allowing fluid accumulation and invasion of viruses and commensal bacteria. Unresolved inflammation then leads to the reoccurring or chronic infections and fluid build-up in the middle ear that are the hallmarks of recurrent and chronic forms of OM (Bhutta et al., 2017).

In most children, AOM is resolved by the mucosal immune response of the middle ear and upper respiratory tract, that protects against repeated infections of the middle ear during the early years of development when children are at highest risk for ear disease. Progressively, the mean frequency of AOM episodes experienced by a child falls from 1.97 per year at six months of age to 1.07 per year by 36 months of age (Chonmaitree et al., 2008). Although overall, by three years of age, 60% of children have experienced one or more episodes of OM and 24% have experienced three or more episodes (Kaur et al., 2017). However, the global burden of disease caused by recurrent and chronic forms of OM, that are not as well controlled by the immune system, is considerable, particularly during the first 5 years of life. Globally, CSOM occurs in 4.76% of the population (22% in children under 5 years old) with hearing impairment present in 30 children per 10,000 (Monasta et al., 2012), a prevalence that may rise further in adults (Chung et al., 2016).

Our understanding of the role of the innate immune response in OM is improving as more evidence comes to light that frequent or prophylactic antibiotics are not always effective for chronic or recurrent forms of OM, or even for non-severe AOM, where a "watch and wait" approach to treatment is recommended (Lieberthal et al., 2013). In many clinical practice guidelines, antimicrobial decision-making is based on the clinical severity of AOM (Spoiala et al., 2021), with a previous randomised control trial in high-risk individuals reporting that long-term antibiotics did resolve OME and prevent AOM with perforation (Leach et al., 2008). However, for chronic forms of OM, such as CSOM, the benefits of either systemic or topical antibiotics for rapid resolution are not clear (Chong et al., 2021), and analgesics rather than antibiotics or anti-inflammatories are recommended as the front line treatment for OME (Rosenfeld et al., 2016; Chong et al., 2021).

The innate immune system is a critical first line of defence within the middle ear and is activated in response to pathogen associated molecular patterns (PAMPs) on invading pathogens (Li et al., 2013; Massa et al., 2015). Key characteristics of this defence mechanism include physical epithelial barriers, recognition of non-self-factors such as pathogens, up-regulation of the complement system, initiation of generalised inflammatory responses and activation of specific immune responses to pathogens through upregulation of the adaptive immune system (Massa et al., 2015; Preciado et al., 2017).

BACTERIAL AND VIRAL OTOPATHOGENS

Historically, the middle ear has been considered a sterile environment (Kurono et al., 1996; Lim et al., 2000). However, recent advances in 'OMICS technology have revealed the potential of a middle ear microbiome (Marsh et al., 2020). The mechanisms by which the middle ear microbiome, in concert with that of the nasopharyngeal microbiome, may impact on the development and pathogenesis of OM remain largely unknown. This is a growing area of investigation (Schenck et al., 2016; Enoksson et al., 2020; Marsh et al., 2020), as the presence of a healthy middle ear microbiome is controversial (Johnston et al., 2019) with one key study in adults finding no evidence of bacterial colonisation of the middle ear using microscopy and culture techniques (Jervis-Bardy et al., 2019). The inflammation that defines OM, however, is induced by invading respiratory viral pathogens and dysregulated commensal bacterial populations within the nasopharynx, which then migrate through the upper respiratory tract *via* the eustachian tubes to infect the middle ear. How well these infections are then controlled by host mucosal immune responses, and the nature of each individual's immune response, direct the course of OM disease.

Three bacterial species, *Streptococcus pneumoniae*, non-typeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis* are the dominant bacterial otopathogens globally (Schilder et al., 2016) although individual species and strain dominance may vary according to geographical location (Ngo et al., 2016). These bacteria are commensal in the nasopharynx and typically do not induce localised inflammation or activation of innate immune responses, however their presence within the middle ear stimulates both responses and may result in clinical presentation of AOM.

Functional, effective clearance of bacterial otopathogens from the middle ear, particularly for children experiencing RAOM and

COME, may be disrupted by the presence of bacterial biofilm development on the epithelium of the middle ear (Hall-Stoodley et al., 2006; Thornton et al., 2011). Mucosal biofilms result from host and bacterial interactions, often multiple bacterial species (Thornton et al., 2011), incorporating host and bacterial DNA to develop and stabilise the biofilm. Furthermore, in addition to biofilms, the presence of intracellular bacteria, within the mucosal epithelial cells may also contribute to OM persistence and ineffective clearance of infection through antibiotic use (Bakaletz, 2012; Coates et al., 2008).

Viruses play an important role in the induction of AOM, which often occurs as a complication of upper respiratory infection (URTI) (Pettigrew et al., 2011). Some viruses can cause AOM in isolation, although most cases of AOM are the result of polymicrobial infections (Ruohola et al., 2013; Sawada et al., 2019). All respiratory viruses have been associated with AOM, in that they have been detected in middle ear fluid (MEF) or by nasopharyngeal swab during an episode of AOM, although some are more commonly detected than others (Pettigrew et al., 2011; Sawada et al., 2019). These include rhinovirus (RV), respiratory syncytial virus (RSV), adenovirus (AdV), human metapneumovirus (HMPV), influenza A virus (IAV) and seasonal human coronaviruses (Heikkinen et al., 1999; Chonmaitree and Heikkinen, 2000; Chonmaitree and Henrickson, 2000; Heikkinen and Chonmaitree, 2003; Kalu et al., 2011; Nokso-Koivisto et al., 2015; Chonmaitree et al., 2016; Schilder et al., 2016). In many studies, rhinovirus is the most common virus detected in both MEF and the nasopharynx of children with AOM (Moore et al., 2010; Yatsyshina et al., 2016), while in other studies RSV is most commonly detected (Sawada et al., 2019). Some viruses, however, are considered more "otopathic" than others, in that they are more likely to cause AOM without bacterial co-infection. A study that investigated the seasonality of AOM identified that peak AOM activity was significantly associated with detection of RSV, HMPV and IAV (Stockmann et al., 2013). RSV in particular is known to induce AOM in the absence of bacterial infection of the middle ear (Ruohola et al., 2013; Yatsyshina et al., 2016). There is substantive clinical evidence that IAV and RSV enhance the severity of OM (Heikkinen et al., 1999).

It is becoming increasingly apparent that defects in the host innate immune response of the middle ear drive prolonged inflammation, reduce pathogen clearance, and underlie chronic and recurrent forms of OM. This review explores the innate immune response to both viral and bacterial pathogens in OM, and current knowledge regarding the dysregulation of the host immune response that may underlie the development and recurrence of OM. Animal experimental models, in addition to clinical studies, are discussed, since investigating immune responses to pathogens in isolation in the middle ear is difficult, and mechanistic studies using a range of animal models are essential. However it needs to be noted that murine immune responses may differ to those of humans and so need to be viewed in the context of complementary studies (Bhutta, 2012; Tyrer et al., 2013). A focus of this review is the host innate immune response to NTHi, as the most common otopathogen used to investigate host immune mechanisms using animal models for OM. In contrast, respiratory virus activation of host innate immune responses in OM pathogenesis is less clear, with most fewer animal model studies focussed on the role of IAV and RSV.

PATHOGEN RECOGNITION AND ACTIVATION OF SIGNALLING PATHWAYS WITHIN THE INNATE IMMUNE RESPONSE

Within the middle ear, like other mucosal immune locations, molecular signatures known as pathogen-associated molecular patterns (PAMPs), are produced by both bacterial and viral pathogens and recognised *via* one or multiple microbial pattern recognition receptors (PRRs) (Medzhitov, 2007; Kawai and Akira, 2009; Li and Chang, 2021). These receptors are located throughout the middle ear epithelium, and are expressed on the cell surface, internal cell membranes and within the cytoplasm of structural epithelial and antigen presenting cells within the host innate immune system (Leichtle et al., 2011; Kumar et al., 2013; Kurabi et al., 2016).

Non-self-DNA and RNA sensing within the middle ear mucosa can involve multiple PRRs depending on the invading pathogen and intrinsic host PRR expression. Overall, this activation of multiple PRRs during the initial innate immune response to pathogens provides increased opportunity for multiple synergistic and/or redundant signalling pathways to be activated, often through NF-κB, interferon response factors (IRFs) and AP-1 transcription factor activation (see **Figure 1**). The efficacy of different signalling pathways can influence host susceptibility to disease (Skevaki et al., 2015). Signalling regulation by host and pathogen factors within the middle ear during OM may lead to modification of timing and secretion of various cytokines, chemokines, interferons, and antimicrobial peptides from the mucosal epithelium or cells recruited to the site of activation. Importantly, the concentrations of antimicrobial peptides and proteins, and cytokines within MEF of children experiencing recurrent AOM are known to increase (Seppanen et al., 2019).

Host innate immunity is activated in response to a wide range of bacterial and viral pathogens through transcription factors downstream from the PRRs and can vary to provide stimulus-specific responses (Borghini et al., 2018). This regulation of the innate immune response is essential to optimise pathogen clearance whilst minimising local tissue damage from inflammatory processes (Massa et al., 2015). For example, PRR activation of the NFκB transcription factor cascade by NTHi increased surfactant protein A (SP-A) expression in SP-A knockout mice, causing more severe inflammation. In contrast, wild-type mice showed improved bacterial aggregation, killing and macrophage clearance of the middle ear due to SP-A modulation of the host inflammatory response (Abdel-Razek et al., 2019).

Thus, differences in host PRR expression and subsequent signalling pathway activation can lead to differences in how the

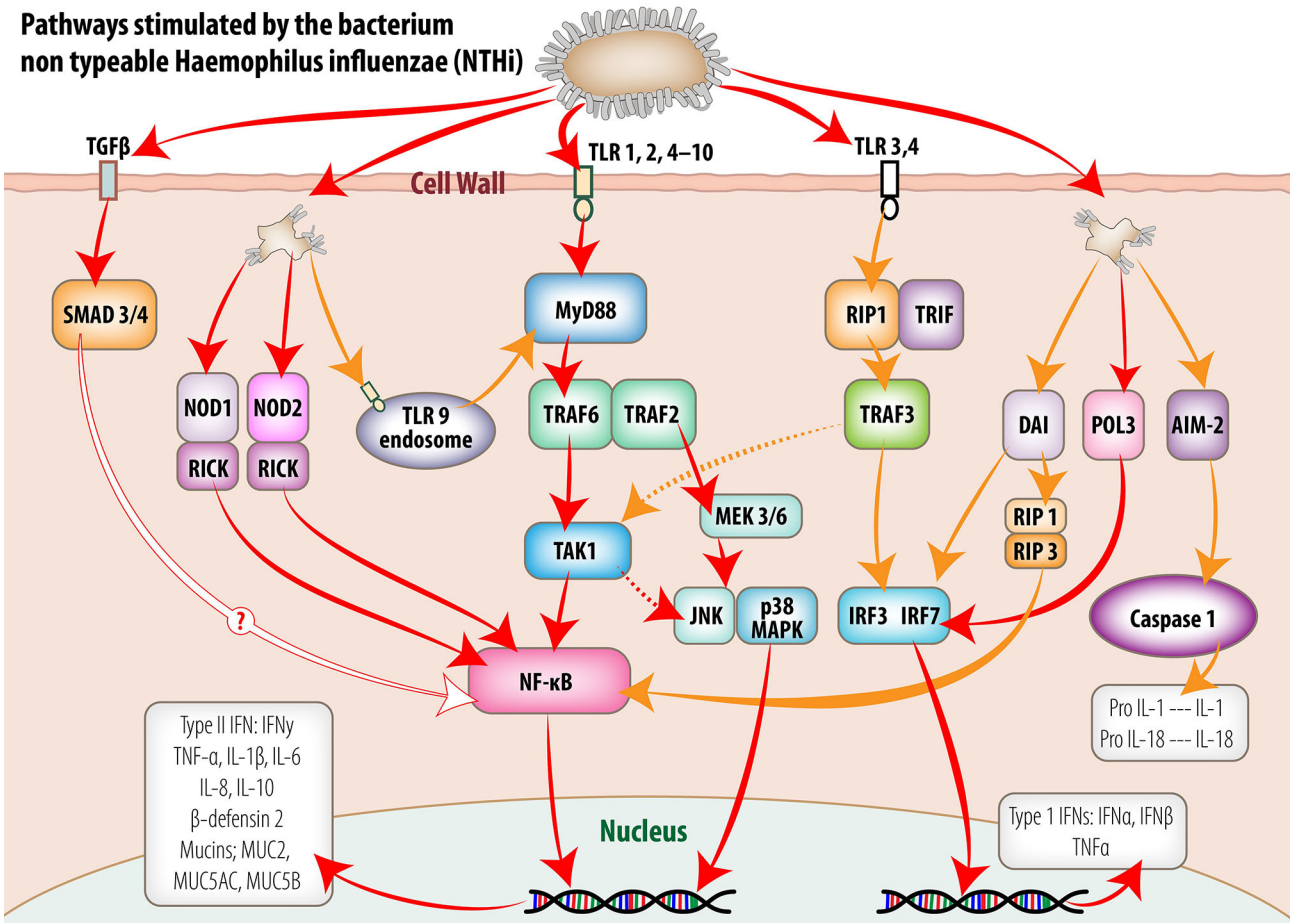

FIGURE 1 | Intracellular signaling pathways utilized by middle ear mucosa in response to stimulation by non-typeable *Haemophilus influenzae* (NTHi). Pattern recognition receptors (PRR) detect conserved molecular signatures of invading microbes and facilitate activation of synthesis and secretion of downstream cytokine cascades. This figure schematically represents the signaling pathways activated by NTHi both at the cell membrane and within the cell cytoplasm of the middle ear mucosal cells. The information portrayed was extracted from published reports of experiments utilizing middle ear cell lines and middle ear epithelium and mucosa from animal models and human biopsy material. (Reproduced, with permission, Massa et al., 2015).

innate immune response is regulated and how OM disease progresses. There is growing evidence that host genetic polymorphisms in PRR genes and differences in PRR expression can dysregulate innate immune signalling pathways in response to otopathogens and therefore play a significant role in the induction and pathogenesis of OM (Lin et al., 2017). Furthermore, NTHi is recently reported to utilise phase variable epigenetic regulation to modify and adapt its phenotype but also modify host immune responses (Robledo-Avila et al., 2020), creating a shifting immunological target for the host (Parrish et al., 2019).

PRR ACTIVATION AND INNATE IMMUNE SIGNALLING IN RESPONSE TO BACTERIAL OTOPATHOGENS

Toll-like receptor (TLR)-dependent activation of innate immune responses within the middle ear are currently best described from animal, particularly murine models. In these models, the cell surface-expressed TLRs, TLR-2 and TLR-4, play an important role in sensing and responding to bacterial otopathogens (Kweon et al., 2006; Moon et al., 2007; Trune and Zheng, 2009; Kim et al., 2010; Leichtle et al., 2011; Mittal et al., 2014b; Zhang et al., 2015). The overall effect TLR activation is to induce NF-κB signalling, resulting in mucosal hyperplasia and upregulation of pro-inflammatory cytokines (Lim et al., 2007) (see **Figure 1**). Thus, TLRs initiate and mediate expression of a variety of molecules from the middle ear mucosa including inflammatory cytokines (IL-1α, IL-1ß, IL-6, IL-10, TNFα, vascular endothelial growth factor (VEGF), chemokines Ccl3 (macrophage inflammatory protein 1a or MIP1a), Cxcl2 (macrophage inflammatory protein-2 or MIP2), keratinocyte-derived chemokine (KC or Cxcl1; recruits and activates macrophages) and antimicrobial peptides such as mouse ß-defensin 2 and mucin genes (MacArthur et al., 2011; Trune et al., 2015; Kurabi et al., 2016). Interestingly, in

association with changes in epithelial cell structure from pseudostratified ciliated columnar epithelium within the eustachian tube to a more squamous epithelium within the middle ear of the rat (Massa et al., 2015), more TLR2 and TLR4 receptor mRNA is expressed within the middle ear compared to other upper respiratory tract locations including the Eustachian tube, nasopharynx and oral cavity (Song et al., 2009). The essential role of TLR2 and TLR4 receptors and signalling molecules, MyD88 and TRIF in the activation of innate immune responses has also been demonstrated using knock-out murine models (Hernandez et al., 2008; Leichtle et al., 2009). Gene deletion of these receptors and signalling molecules results in persistent OM, in the form of thickened mucosa, delayed neutrophil and macrophage recruitment and reduced efficiency of bacterial killing and clearance from the middle ear (Leichtle et al., 2009; Leichtle et al., 2011; Underwood and Bakaletz, 2011; Kurabi et al., 2016).

The importance of cell surface TLR signalling in the innate immune response in OM is best demonstrated in a murine model in which heat-killed NTHi was used for trans-tympanic inoculation (MacArthur et al., 2006; Trune et al., 2015). In these mice, there was no active infection within the middle ear, although an inflammatory response was still induced in response to endotoxins, which act as PAMPs. Within 6 hrs, genes for MIP-1α, MIP-2α, IL-6 and Cxcl1 demonstrated significantly elevated expression (>50->1000 fold) whilst IL-1α (Ccl3), IL-1ß, IL-10, TNFα expression was more moderately upregulated (>4->36 fold). Expression of each of these genes returned to non-stimulated levels by 72 hr in the absence of active infection. Cytokine and chemokine production was increased in parallel with the gene upregulation, however the production of these proteins lagged behind upregulated gene expression by peaking 24hr after inoculation. In this model, TLR4 and TLR9 expression were also significantly increased, while TLR2 expression was only slightly increased (Trune et al., 2015). Interestingly, NTHi infection also increased Chemokine CXC receptor 4 expression in the mouse middle ear model, signalling the inflammatory response through IKKα and p38MAPK pathway activation (Ma et al., 2018). Studies using bacterial otopathogens other than NTHi are limited, however, it has been demonstrated in a mouse pneumococcal OM model that TLR2 expression and NF-κB signalling in the middle ear mucosa, is critical for the recruitment of macrophages. The absence of TLR2 expression resulted in impaired *S. pneumoniae* clearance from the middle ear and a prolonged inflammatory response (Komori et al., 2011; Huang et al., 2016).

Observations regarding the importance of TLR2 in the innate immune response in murine models of OM is supported by clinical studies. In children, TLR2 is a predominant NTHi receptor within middle ear epithelial cells and activation of this receptor leads to the induction of ß-defensin 2 through activation of the MyD88-IRAKI-TRAF6-MKK3/6-p38 MAP kinase signal transduction pathway (Lee et al., 2008). Increased mRNA expression of TLR4 and TLR2 has also been reported within the middle ear fluid (MEF) of children experiencing AOM, with TLR9 expression remaining unchanged. Coincident with the TLR upregulation, mRNA expression of cytokines including, pro-inflammatory TNFα, IL-6, IL-8, IL-10, IL-1ß and chemokines CCL2, CCL3, CCL4, CCR5 and CXCR3 were significantly higher (8-330 fold) in bacterial culture positive MEF samples, and increased mRNA expression of these molecules was associated with increased numbers of bacterial species identified within the sample (Kaur et al., 2015).

In addition to cell surface expressed TLR signalling, endosomal TLR9 was shown to be important in sensing NTHi when inoculated into the murine middle ear. Deletion of TLR9 from mice prolonged middle ear inflammation and bacterial clearance (Leichtle et al., 2012). In this same study, Leichtle et al, also identified non-TLR pathogen DNA sensing genes to be upregulated in response to infection by NTHi. These included DNA-dependent activator of IFN regulator factor (DAI), absent in melanoma-2 (AIM2) and Pol-III in addition to other genes encoding proteins that mediate downstream signalling pathways (Leichtle et al., 2012).

Several other families of PRRs contribute to PAMP recognition and activation of the signalling pathways to activate and modulate innate immune responses, including retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLR's), cytoplasmic nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs) and DNA sensing receptors (Kurabi et al., 2016). Compared to surface expressed TLR receptors, less is known concerning the importance of cytoplasmic PRRs in sensing and responding to bacterial infections in the middle ear (Kumar et al., 2011; Kurabi et al., 2016). However cytoplasmic NOD-like receptors and RIG-1 receptors exhibit reduced expression in patients with OM which may be associated with the development of recurrent OME (Kim et al., 2014). NOD1/NOD 2 receptors activate innate immune responses during OM to reduce infection (Lee et al., 2019). More specifically, NOD2 mediated β-defensin 2 regulation is activated after NTHi penetration of the cell membrane and helps to prevent OM development (Woo J. I. et al., 2014). Activin-like cell surface receptor kinases and serine/threonine kinases also activate important signalling pathways in the pathogenesis of OM. These receptors lead to the activation of TGF-β, which is a pleiotropic cytokine and a key regulator of tissue remodelling.

Although several murine models of bacterial OM have demonstrated an upregulation of pro-inflammatory responses to infection within the mucosa, the entire process of immunoregulation during OM may be more complex. A report on the transcriptome signature elicited from PBMCs at the onset of AOM in children caused by NTHi reported that genes associated with antibacterial activity and cell-mediated immunity were predominantly affected. Importantly, the study suggested that NTHi infection suppressed more immune responses than were activated. More specifically, 90% of genes associated with pro-inflammatory cytokine responses were down-regulated, as was classic complement pathway activation (Liu et al., 2013). Furthermore, the transcriptome of a complete episode of NTHi-induced AOM in a mouse model was examined *via* expression profiling utilising whole genome microarrays in

the murine model. Sets of genes involved in activation of the innate immune response, negative regulation of that response, epithelial and stromal cell marker changes and neutrophil and macrophage recruitment and function were identified. Overall, positive and negative regulation of inflammatory processes were recognised, and the importance of anti-inflammatory responses in control of OM pathogenesis were highlighted (Hernandez et al., 2015). Importantly, regulation of NTHi triggered activation of inflammatory responses by natural products such as the plant pigment Quercetin may provide potential therapeutic approaches to reduce OM (Ma et al., 2018).

VIRUS-INDUCED INNATE IMMUNITY

Respiratory viruses alone can induce AOM and lead to chronic presentations of OM (Heikkinen and Chonmaitree, 2000). A causal relationship between viral URTI and eustachian tube obstruction, middle ear pressure and bacterial colonisation has been demonstrated in both human challenge studies using rhinovirus (McBride et al., 1989; Buchman et al., 1994) and IAV (Doyle et al., 1994) and in both chinchilla and mouse models of AOM induced by IAV (Giebink et al., 1987; Short et al., 2011). In addition, viral-bacterial coinfections have demonstrated more severe outcomes for AOM in humans (Moore et al., 2010; Binks et al., 2011; Pettigrew et al., 2011; Chonmaitree et al., 2016; Schilder et al., 2016) and animal models (Giebink and Wright, 1983; Patel et al., 1992; Brockson et al., 2012), compared to bacterial colonisation alone. However, despite this clear link between respiratory virus infections and OM, the exact mechanisms by which viruses promote the development of AOM and subsequent chronic and recurrent OM is not well known due to a paucity of studies on the immune response during virus-only AOM. Therefore, our understanding of the role of respiratory viruses in the immune response during OM is based largely on our general understanding of the immunopathology of URTIs, which affect the nasopharynx foremost with AOM considered a secondary complication (Heikkinen, 2000; Chonmaitree et al., 2008; Bakaletz, 2010; Kalu et al., 2011; Chonmaitree et al., 2016).

There are few human clinical studies that have sought to characterise the immune response to respiratory viruses during OM and understand how this contributes to pathogenesis of disease. One study collected sera from 145 children with AOM and found that RSV-associated AOM correlated with elevated serum concentrations of proinflammatory cytokines (Patel et al., 2009a). These included G-CSF, MCP-1, IL-10, IL-6, IFN-γ and IL-8, with G-CSF concentrations predicting RSV-associated OM with 87% accuracy. In this study, local mucosal responses were not investigated, although in a related study, the cytokines detected in nasopharyngeal secretions of 326 children with virus-positive URTIs were identified and correlated with virus identification and the onset of AOM (Patel et al., 2009b). All viruses detected (adeno-, entero- rhino- viruses, RSV, Parainfluenza viruses, Influenza viruses) induced significant quantities of IL-1β, IL-6 and TNFα. However only IL-1β was significantly associated with the onset of AOM and was not correlated with any particular virus.

Some studies have identified innate immune responses of cultured human middle ear epithelial (MEE) cells when infected with IAV. A transcriptional study of cells up to 24h post infection revealed upregulation of genes encoding an array of type I interferon inducible signalling pathways, transcription factors, cytokine and chemokine genes, as expected in response to viral infection (Tong et al., 2004). MEE cells also secrete elevated levels of MIP-1α and MIP-1β, TNF-α, IL-6, IL-8 and IL-10 in response IAV infection (Tong et al., 2003; Tong et al., 2004). Interestingly, when IAV strains were compared, H3N2 IAV induced a stronger IL-6 and IL-8 response by MEE cells in culture than H1N1 (Short et al., 2013).

These human studies are informative regarding antiviral responses, although do not provide mechanistic detail regarding how the immune response to viral infections drives the development of OM. Animal models have been used to understand the role of viral URTIs in the development of OM. However, very few have focused on the induction of AOM by viruses alone, without bacterial involvement. Early chinchilla models demonstrated clinical signs and symptoms of OM induced by viruses alone (Giebink et al., 1987), including general observations of tympanic membrane inflammation (Giebink and Wright, 1983). Short et al. (2011) developed the first infant murine model of IAV-induced AOM (Short et al., 2011), where IAV replicated in the middle ear epithelium, and caused submucosal edema and an influx of immune cells, predominantly neutrophils, into the middle ear cavity. These mice also displayed hearing loss indicative of clinical AOM. In a follow up study, inoculation of mice with recombinant IAVs identified a pro-inflammatory response in the middle ear, specific to replicating viruses with a H3-type HA attachment viral protein. This response was dominated by upregulation of genes expressing IL-1β, IL-1α and CXCL2 in the middle ear (Short et al., 2013).

INNATE IMMUNE RESPONSES TO POLYMICROBIAL INFECTIONS

Adding to the complexity of the host innate immune responses, microbe to microbe interactions are being recognised within the upper respiratory tract microbiome (Schenck et al., 2016) which may enhance or impair bacterial competition and the host innate immune response. For example, peptidoglycan secretion by *H. influenzae* can activate neutrophils to enhance complement-dependent killing of *S. pneumoniae* (Lysenko et al., 2005), whilst *S. pneumoniae* can impair NTHi evasion of host immune responses (Shakhnovich et al., 2002). Furthermore, *S. pneumoniae* and NTHi can synergistically upregulate TLR2 expression, increasing inflammation (Ratner et al., 2005).

Most mechanistic studies in animal models of OM have used single infections to understand cause and effect. However, polymicrobial animal models for OM more closely replicate the complex microbial environment of the nasopharynx and

infected and/or inflamed middle ear (Bakaletz, 2010), and may better reflect COM pathogenesis (Holder et al., 2012) and immunisation responses (Novotny et al., 2017). A rat model of OM involving dual infection of the middle ear with NTHi and Pneumococcus type 6A (Pn6A) demonstrated upregulation of genes encoding inflammatory Th2 cytokines and effectors of the TGF-β signalling pathway, which resulted in pathogenic changes and thickening of the mucosa and submucosal layers of the middle ear during OM (Lee et al., 2011). It has been demonstrated in chinchilla that middle ear infection with NTHi promotes the persistence of *M. catarrhalis via* the formation of polymicrobial biofilms (Armbruster et al., 2010). In a mouse model, *M. catarrhalis* impacted pneumococcal OM more than NTHi regarding bacterial load, incidence rate and persistence of infection. Nitric oxide was measured as an indication of inflammation and was elevated in polymicrobial infections significantly more than single infections. Interestingly, pre-infections with the respiratory virus, Sendai Virus, enhanced bacterial OM for all three otopathogens, demonstrating the importance of viral infection in the development of bacterial OM (Krishnamurthy et al., 2009).

Most animal models of virus-induced OM have investigated the role of viruses in supporting subsequent bacterial colonisation of the eustachian tube and middle ear. The chinchilla model has been the most utilised to study the pathogenesis of viral-bacterial co-infection in OM (Giebink and Wright, 1983; Suzuki and Bakaletz, 1994). However, it does have limitations, in that the immune responses and the presentation of AOM in these animals can vary depending on the order and timing of infection and partnering of viruses and bacteria. These variables have made it difficult to build an accurate picture of the role of antiviral innate immune responses in AOM. It has also been difficult to identify specific mechanisms by which viral dysregulation of the innate immune response promotes bacterial colonisation. Therefore, most studies report general inflammation and clinical signs of OM on tympanometric investigation of animals.

Murine models of IAV and *S. pneumonia* co-infection have demonstrated more severe AOM than single infections of either virus or bacteria alone, including greater hearing loss, and middle ear inflammation (Short et al., 2013), in addition to reduced ciliation, hyperplasia of the mucosal epithelium and increased goblet cells (Tong et al., 2014). Tong et al. (2104) also identified that the anaphylatoxins C3a and C5a were expressed in both serum and middle ear lavage from IAV-infected mice indicating that induction of the complement alternative pathway reduced bacterial clearance and enhanced the severity of acute pneumococcal OM. When paired with *M. catarrhalis* and NTHi in experimental Chinchilla infections, RSV was associated with clinical signs of inflammation and haemorrhagic foci in the middle ear mucosa (Brockson et al., 2012), thus compromising the ability of the mucosa to combat ascending bacterial infections and biofilm formation in the middle ear. The chinchilla model has also been useful in demonstrating the ability of different viruses in enhancing bacterial OM. Type 5 adenovirus (Ad5) has been shown to promote infection of the middle ear by *S. pneumoniae* (Murrah et al., 2015), while type 1 adenovirus (Ad1) has been shown to promote ascension of NTHi (Suzuki and Bakaletz, 1994) although not *S. pneumoniae* (Tong et al., 2000) or *M. catarrhalis* (Bakaletz et al., 1995). It is likely that the role of viruses in OM is complex and involves not only inflammation and innate immune dysregulation, but also other factors that enhance bacterial colonisation such as epithelial damage, mucus production and modulation of antimicrobial peptides, as has been demonstrated within other compartments of the respiratory mucosa (Melvin and Bomberger, 2016).

ANTIMICROBIAL FACTORS RELEASED BY THE MUCOSAL EPITHELIUM

In addition to classical innate and adaptive immune responses, the production of anti-microbial molecules, mucus secretion, and mucociliary clearance within the middle ear and eustachian tubes, work in combination to maintain the relative sterility of the middle ear cavity (Lim et al., 2000; Massa et al., 2015). Morphologically, the middle ear region adjacent to the eustachian tube and the eustachian tube itself, exhibit cellular characteristics shared by other mucosal surfaces within the upper respiratory tract, such as a secretory, pseudostratified, and ciliated columnar epithelium (Lim, 1976; Lim, 1979; Bluestone and Doyle, 1988; Martin et al., 2017). The physical resilience of the epithelium is reinforced *via* tight junctions (Tsukita et al., 2008; Yonemura, 2011) and goblet cells that secrete mucus to provide a barrier to adherence and colonisation by bacteria, (Linden et al., 2008), and contribute to the mucociliary clearance of the tympanic cavity and eustachian tube (Evans and Koo, 2009; Martin et al., 2017).

Most importantly, the mucosal response includes activation of antimicrobial molecules, such as lysozyme, ß-defensins and lactoferrin which either independently or together, act to inhibit bacterial colonisation and activate the adaptive immune response (Underwood and Bakaletz, 2011). The significance of antimicrobial molecules has been demonstrated using animal models. For example, lysozyme knockout mice exhibit increased susceptibility to bacterial colonisation of the middle ear and enhanced inflammatory response to *S. pneumoniae* 6B infection (Shimada et al., 2008). Furthermore, in humans, lysozyme and ß-defensin 2 can synergistically partner to directly kill invading *S. pneumoniae* 6B (Lee et al., 2004) and protect against NTHi induced OM (Woo J. I. et al., 2014). Most recently, a study examining middle ear effusate from children experiencing recurrent AOM confirmed the importance of elevated antimicrobial protein (AP) and cytokines as potential markers for bacterial persistence and inflammation (Seppanen et al., 2019). Antimicrobial proteins or host defence peptides may offer future treatment options against polymicrobial infections (Bergenfelz and Hakansson, 2017; Batoni et al., 2021).

Viruses, conversely, have been shown to enhance bacterial infection of the middle ear by suppressing antimicrobial factors and enhancing bacterial adhesion molecules. McGillivary et al. (2007)

demonstrated that both viruses and bacteria can alter the expression of cationic APs in the upper airways, thus enabling an expansion of otopathogens. This elegant study involved the expression of recombinant cCRAMP, a cathelicidin homolog from the upper respiratory tract of the chinchilla in cultured chinchilla middle ear epithelial cells (CMEEs). CMEEs were infected with IAV, RSV or Ad1, and the effect on cCRAMP and also cBD-1, which is the murine ortholog of the human AP β-defensin, was investigated (McGillivary et al., 2007). IAV reduced cCRAMP mRNA expression by 50%, while RSV and Ad1 only had a minimal effect. In contract RSV reduced the expression of cBD-1 mRNA by 40%, while IAV and Ad1 did not. Thus, highly otopathic viruses, such as IAV and RSV appear adept at differentially reducing key antimicrobial defence molecules in airway epithelial cells using different mechanisms. The ability of RSV to suppress cBD-1 expression has also been demonstrated directly in a chinchilla RSV/NTHi co-infection model. In this model, RSV intranasal challenge diminished both cBD-1 mRNA and protein expression in the upper airway epithelium. Consequently, when chinchilla were inoculated with NTHi, there was a marked increase in both the quantity and duration of NTHi recovered from the nasopharynx (McGillivary et al., 2009). These studies provide evidence that virus-induced dysregulation of AP expression contributes to the elevated bacterial colonisation to precedes AOM (Bakaletz, 2010).

The net effect of viral URTI is a reduction in the protective function of the mucosal epithelium, which extends from the nasopharynx through the eustachian tubes and into the middle ear. This compromise in protection renders the middle ear susceptible to ascending bacterial infection, and can last from 2-10 weeks in children before homeostatic conditions are returned (Carson et al., 1985).

IMMUNOCOMPETENT CELL TYPES WITHIN THE MUCOSAL EPITHELIUM

The healthy middle ear of both humans and animal models of OM is host to immunocompetent cell types, although not in significantly high numbers until infiltration occurs during AOM (Jecker et al., 1996; Jecker et al., 2001; Suenaga et al., 2001) or COME (Enoksson et al., 2020). Mast cells and macrophages are the predominant cell types in non-inflamed middle ear, with dendritic cells and macrophages present in the tympanic membrane (Ichimiya et al., 1990; Mittal et al., 2014b) These antigen-presenting cell phenotypes provide a mechanism for interconnection of the local innate mucosal immune response with the more specific adaptive immune system (Ichimiya et al., 1997). Enhanced inflammation within the middle ear mucosa, stimulated through pro-inflammatory cytokine secretion including ß-defensin 2 and other defensins (Underwood and Bakaletz, 2011) and pre-inflammatory molecules including TNFα, IL-1β and CCL3, attract other immunocompetent cells *via* chemotaxis, including dendritic cells, memory T-cells, neutrophils and mast cells within the epithelium (Yang et al., 2007). In addition to direct bacterial killing, ß-defensin 2 contributes to the mediation of adaptive immunity (Yang et al., 2007; Underwood and Bakaletz, 2011).

Upregulation of the innate immune response, as evidenced by immunocompetent cell infiltration, has long been demonstrated in COM patients (Palva et al., 1981; Enoksson et al., 2020), whilst mouse models of NTHi infection of the middle ear have also demonstrated leukocyte infiltration (Hernandez et al., 2008; Hernandez et al., 2015; Trune et al., 2015). Furthermore, the acute inflammatory response during AOM in the rat model shows time-dependent increases in identification of macrophages, dendritic cells, polymorphs and natural killer cells (Jecker et al., 1996; Forseni et al., 1999), with T-cells present 3 days after perforation of the tympanic membrane (Tahar Aissa and Hultcrantz, 2009).

The activation and rapid response of the innate immune response within the middle ear mucosa is well evidenced from animal studies, particularly mouse models (Bhutta, 2012). Evidence of the precise timing and duration of immunocompetent cell changes occurring in the murine middle ear demonstrates that neutrophil recruitment is evident within 6 hrs of inoculation of middle ear and peaks 24 hours post inoculation at level ~10-fold greater than the peak recruitment of macrophages observed at 48 hours after inoculation (Leichtle et al., 2011). The number of neutrophils within the ME epithelium returned to control levels after 72h, whereas macrophages were still observed 120hr post inoculation. Epithelial effusion within the middle ear is present 24-48hr post inoculation, consistent with peak neutrophil and macrophage (Hernandez et al., 2015) recruitment. Unfortunately, increased neutrophil recruitment can be utilised by NTHi to avoid phagocytosis through host formation of neutrophil extracellular traps (Juneau et al., 2011; Schachern et al., 2017) which have been identified within the MEF of children with OM (Thornton et al., 2013).

Neutrophil infiltration of the middle ear is also a feature of IAV-induced OM in a murine model (Short et al., 2011). IAV also depressed polymorphonuclear leukocyte chemiluminescence activity in a chinchilla model (Giebink and Wright, 1983). Interestingly H3N1 had a more significant effect on this response than H1N1, similar to the findings of Short et al. (2013).

INNATE IMMUNE RESPONSE ACTIVATION OF THE ADAPTIVE IMMUNE RESPONSE

Overall, there is a paucity of human studies, however, many murine studies have explored the interconnection of the innate immune responses occurring within the middle ear and their role in activation of the adaptive immune response. Within 3 days post inoculation with type B *Haemophilus influenzae*, macrophages and Mac-1+ neutrophils were present in the middle ear of mice whilst within the middle ear mucosa, Lyt-1+ T cells and Lyt-2+ T suppressor/cytotoxic cells were present 7 and 14 days post inoculation. The majority of mucosal immunoglobulin-bearing cells at Day 14, were IgA+ lymphocytes although IgG+ and IgM+ T –cells were present 3, 7 and 14 days post-inoculation. Lyt-1/

L3T4+ T lymphocytes were present in larger numbers than B lymphocytes thus helper T cells appear to have significant involvement in AOM (Krekorian et al., 1991). Recent profiling of the cells within the murine middle ear using single-cell transcriptomics identified 17 different cell clusters, reflecting different cell types within the normal middle ear however tissue monocytes appear to have a primary role in regulation of acute middle ear infection response in the complex innate defence (Ryan et al., 2020). Interestingly, adaptive immune system factors such as TBX21, a transcription factor, may also participate in innate immune response regulation during *S. pneumoniae* infection, through modifying TLR2 expression (Woo C. H. et al., 2014). Whilst these model studies establish a link between innate and adaptive immune responses in the middle ear, additional studies are needed to confirm such links in the human middle ear.

Recent evidence highlights the complexity of the upregulation of specific inflammatory cells and mediator secretion varies in response to the microbial otopathogens involved and the child's gender, with *Moraxella* and *Haemophilus* species tending to stimulate more inflammatory mediators in the middle ear (Enoksson et al., 2020). Interestingly, within the few human studies available, reports of relationships between IgG, IgG1, IgG2 and IgA are complicated by case-definitions of the sampled population (Corscadden et al., 2013). A well-designed cross-sectional study of serum and middle ear levels of IgG, IgG1 and IgG2 in children under 3 years of age, with and without a history of frequent AOM reported that of 11 different pneumococcal serotypes present, only serotype 5 induced elevated serotype specific IgG and IgG1. This study evidenced that frequent AOM is not the result of deficiency in IgG, IgG1 or IgG2 response (Corscadden et al., 2013). Further research, focussed on both viral and bacterial otopathogens are needed to the confirm the innate activation of the adaptive immune responses of children experiencing OM.

HOST FACTORS THAT MODULATE THE MUCOSAL RESPONSE IN OM

In this review we have explored the innate immune response to predominant otopathogens in the context of OM pathogenesis. Induction of a productive innate immune response that will clear otopathogen infection, whilst minimising inflammation is ideal for AOM resolution, while a hyperinflammatory response and upregulation of bacterial adhesion mechanisms is deleterious to this process and may result in recurrent or chronic forms of OM. Otitis media susceptibility is associated with a number of host genes, identified in previous reviews (Kurabi et al., 2016; Bhutta et al., 2017; Geng et al., 2019). Genetic factors that influence the heritability of susceptibility to OM are reported to range between 40%-70%, based on several prospective, longitudinal prospective and retrospective twin studies (Mittal et al., 2014a). In humans, reduced expression of key PRRs TLR9, NOD1, NOD2 and RIG-I has been observed in the MEF of OM-prone compared to non-OM-prone children (Kim et al., 2010). In addition, TLR2, IL-1, IL-6 and TNFα gene and protein production were reduced in children aged 2-7 years compared to 0-2 and over 7-year olds, and were also lower in culture-positive OM (Kim et al., 2015). Interestingly, in contrast, one report identified no significant differences in mRNA and protein levels of TLR2, TLR4 and TLR5 between non-OM and chronic OM patients, however the levels fell for CSOM patients. These studies indicate a correlation between reduced PRR function and severity of disease (Si et al., 2014).

This lack of functionality in PRR signalling and subsequent protective innate immune responses in OM-prone individuals is likely due to polymorphisms in candidate genes related to innate and adaptive immunity. Children identified with TLR2 and TLR4 polymorphisms exhibit disrupted innate immune responses that increase their susceptibility to OM (Hafren et al., 2015; Toivonen et al., 2017). Genetic polymorphisms in Mannose-binding lectin and TLR2,3,4,7 and 8 can promote or protect children from the risk of respiratory infections and AOM (Toivonen et al., 2017) indicating that we do not fully understand the mechanisms of innate immunity influenced by gene polymorphisms.

Allelic association studies have identified polymorphisms related to the development of OM in genes encoding TLR4 receptors, IL-6, IL-10 and TNF-α (Emonts et al., 2007), mannose-binding lectins (Wiertsema et al., 2006), surfactant (Ramet et al., 2001), and Mucin gene MUC5AC (Ubell et al., 2010) [for review see (Mittal et al., 2014a; Lin et al., 2017; Geng et al., 2019)]. TLR4 locus polymorphisms have also been examined in patients with RAOM or COME and demonstrated a role for TLR4 in the regulation of the innate immune response. The inability of this study to replicate the association of a previously unrecognised TLR4 haplotype in two independent Finnish cohorts with UK or 2 US cohorts emphasises the potentially heterogenous nature of OM and the complexity of environmental and host factors that may modulate overall susceptibility to OM (Hafren et al., 2015; Einarsdottir et al., 2016). Polymorphism IL-1β+3953 is associated with more severe presentations of AOM and has been associated with higher risk of severe inflammation post-AOM infection (McCormick et al., 2011). Recently, the potential impact of cohort heterogeneity and the complexity of environmental and host factors on OM development has been reviewed for a unique "stringently-defined otitis-prone" population, where microbial confirmation of AOM through tympanocentesis was undertaken. The children in this cohort demonstrate a wide range of dysfunctional innate and adaptive immune responses that increase their vulnerability to upper respiratory tract infections and OM (Pichichero, 2020).

CONCLUSION

The middle ear clearly demonstrates the structural and functional features of a mucosal immune site, that for some children, results in increased susceptibility to recurrent or chronic OM. Investigations using a range of animal models (chinchilla, rat, mouse) and particularly genetically modified knockout mice, continue to improve our insight and

understanding of activation and regulation of the host-microbial interactions within the middle ear in response to a variety of pathogens. The cellular immune apparatus within the middle ear can mount a rapid innate immune response to invading pathogens. Ongoing investigation and characterisation of the innate and adaptive immune responses of the middle ear mucosal tissues using clinical studies and animal models will elucidate this region's response to individual and multiple otopathogen infections, and the host factors that influence OM disease progression or clearance and response to vaccination. Some pathogens, such as NTHi can activate multiple, overlapping induction pathways resulting in innate immune system upregulation. Co-infection with multiple pathogens can also influence the host response. It is increasingly recognised that genetic polymorphisms, the level of expression of key regulatory molecules for the innate immune response and environmental factors such as pathogen load may impair innate immune system function and substantially increase the risk of predisposition of some children to more frequent or severe OM. Future studies need to explore the genetic and environmental interrelationships of these polymorphisms and their impact on predisposition or susceptibility to OM and its severity. Next generation sequencing and large, well-phenotyped populations from multiple regions will expand the applicability of these data to support development of new treatment strategies that may enhance innate immunity such as monophosphoryl lipid A (MPL) studies in mice (Iwasaki et al., 2017), β-defensin 2 and probiotics or alter the activation of the innate immune responses to reduce inflammation and chronic OM pathogenesis.

AUTHOR CONTRIBUTIONS

All authors contributed to the concept and writing of this review and agree to publication.

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A Strong Decline in the Incidence of Childhood Otitis Media During the COVID-19 Pandemic in the Netherlands

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Introduction: Recent reports have highlighted the impact of the COVID-19 pandemic on the incidence of infectious disease illnesses and antibiotic use. This study investigates the effect of the pandemic on childhood incidence of otitis media (OM) and associated antibiotic prescribing in a large primary care-based cohort in the Netherlands.

Material and Methods: Retrospective observational cohort study using routine health care data from the Julius General Practitioners' Network (JGPN). All children aged 0-12 registered in 62 practices before the COVID-19 pandemic (1 March 2019 - 29 February 2020) and/or during the pandemic (1 March 2020 - 28 February 2021) were included. Data on acute otitis media (AOM), otitis media with effusion (OME), ear discharge episodes and associated antibiotic prescriptions were extracted. Incidence rates per 1,000 child years (IR), incidence rate ratios (IRR) and incidence rate differences (IRD) were compared between the two study periods.

Results: OM episodes declined considerably during the COVID-19 pandemic: IR pre-COVID-19 vs COVID-19 for AOM 73.7 vs 27.1 [IRR 0.37]; for OME 9.6 vs 4.1 [IRR 0.43]; and for ear discharge 12.6 vs 5.8 [IRR 0.46]. The absolute number of AOM episodes in

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which oral antibiotics were prescribed declined accordingly (IRD pre-COVID-19 vs COVID-19: -22.4 per 1,000 child years), but the proportion of AOM episodes with antibiotic prescription was similar in both periods (47% vs 46%, respectively).

Discussion: GP consultation for AOM, OME and ear discharge declined by 63%, 57% and 54% respectively in the Netherlands during the COVID-19 pandemic. Similar antibiotic prescription rates before and during the pandemic indicate that the case-mix presenting to primary care did not considerably change. Our data therefore suggest a true decline as a consequence of infection control measures introduced during the pandemic.

Keywords: otitis media, incidence, antibiotic, COVID-19 pandemic, children

INTRODUCTION

On 11 March 2020, the WHO (World Health Organization, 2020) declared a global pandemic of COVID-19 which enforced many countries to introduce generic infection control measures such as wearing face masks, hand washing, social distancing, working from home and closure of schools/daycare centers. Other than reducing SARS-CoV-2 transmission, these generic measures have likely affected transmission of other respiratory viruses (Fricke et al., 2021; Tang et al., 2021). Since otitis media (OM) is generally preceded by a viral upper respiratory tract infection (URTI) (Schilder et al., 2016), changes in transmission dynamics of these viruses may have had an impact on the incidence of OM. OM, one of the commonest conditions during early childhood and a prime reason for antibiotic prescriptions (Klein, 2000; Monasta et al., 2012; van den Broek d'Obrenan et al., 2014; Tong et al., 2018), consists of a spectrum of diseases, including acute otitis media (AOM), otitis media with effusion (OME), and chronic suppurative otitis media (CSOM). AOM is characterized by the presence of middle ear effusion (MEE) with rapid onset of signs and symptoms of an acute infection such as fever and ear pain (Lieberthal et al., 2013). Approximately 15%-20% of children with AOM present with ear discharge due to a spontaneous tear or perforation of the eardrum (Rovers et al., 2006; Smith et al., 2010). OME is defined by the presence MEE, without signs and symptoms of an acute infection (Rosenfeld et al., 2016). Hearing loss is the most common symptom of OME. CSOM is characterized by chronic inflammation of the middle ear and mastoid mucosa together with a non-intact tympanic membrane and persisting ear discharge (Verhoeff et al., 2006). Several reports from the early phase of the COVID-19 pandemic have suggested a decline in doctor consultations for OM in children (Hatoun et al., 2020; Aldè et al., 2021; Angoulvant et al., 2021; Iannella et al., 2021; Kaur et al., 2021; Torretta et al., 2021; van de Pol et al., 2021). The question is whether the infection control measures or the sudden COVID-19 related changes in health care access and delivery are responsible for these changes. We will address this question by investigating the effect of the COVID-19 pandemic on OM consultations and associated antibiotic prescribing in children in a large primary care cohort in the Netherlands where the general practitioner (GP) is the first point of call (Grobbee et al., 2005) for the management of OM for all children and practices could be contacted for medical advice throughout the pandemic.

MATERIAL AND METHODS

Design and Study Population

In this retrospective observational cohort study, data were obtained from the Julius General Practitioners' Network (JGPN). Its database contains anonymously extracted routine health care data from electronic records from 62 general practices in the Utrecht area (Smeets et al., 2018). All children aged 0-12 registered 1 March 2019 - 29 February 2020 (pre-COVID-19 pandemic) and/or 1 March 2020 - 28 February 2021 (COVID-19 pandemic) were included. The Medical Research Ethics Committee Utrecht has reviewed the study protocol and declared that official ethical approval is not required since this research is outside the scope of the Dutch Medical Research Involving Human Subjects Act (protocol no 21-562/C).

Data Extraction

From the electronic health records, GP consultations - both face-to-face as well as telephone consultations - of OM (International Classification of Primary Care [ICPC] code H04 (ear discharge); H71 (acute otitis media) H72 (otitis media with effusion; all episodes, irrespective of preceding GP consultation of AOM) and H01 (ear pain) were extracted. A new OM episode started if there was no OM-related GP consultation for 28 days. For each episode, the start date, the child's age at the start of the episode, the number and type of consultations, antibiotic prescriptions and complications (mastoiditis, ICPC code H74.02) were extracted. OM treated with antibiotics was defined as an OM episode with an oral or topical antibiotic prescription according to the Anatomical Therapeutical Chemical (ATC) classification. Since episodes and antibiotic prescriptions are not directly linked in the JGPN database, antibiotic prescriptions within two days before and after the start and stop date of the episode were captured. The full list of ATC codes used in this study can be found in **Supplementary Table 1**. Additionally, data on acute upper respiratory tract infections (URTI, ICPC code R74) were extracted.

Implementation of Infection Control Measures in the Netherlands

On March 15th 2020, the prime minister of the Netherlands introduced social distancing, working from home, and the closure of restaurants/bars, sport facilitates and schools/daycare. Primary schools and daycare centres reopened 11th of May 2020 (Rijksoverheid, 2020), but a 1.5 meter distance rule remained in place and wearing non-medical face-masks was introduced (both not obligatory for children up to 12 year of age). The second lockdown, including closure of schools and daycare centres, started the December 14th, 2020 and ended 9 February 9th, 2021.

Analysis

We calculated the total number of OM episodes pre-COVID-19 and during the COVID-19 pandemic. Incidence rates (IR) were calculated per 1000 person-years by dividing the number of OM episodes by the total number of person-years in that specific time period. In stratified analyses, children were split into the following age groups: <2 year, 2-6 year and ≥ 6-12 years. Differences in overall OM episodes and those treated with antibiotics between the two time periods were expressed as rate ratios (IRR) and rate differences (IRD) with accompanying 95% confidence intervals (CI). All statistical analyses were performed with SPSS (version 26.0, Chicago, IL, USA) and MedCalc for Windows, version 19.4 (MedCalc Software, Ostend, Belgium). The p-values for IRD were obtained using the Chi-square statistic, while the Exact Mid-P test was used to obtain the p-values for IRR.

RESULTS

Study Population

In the pre-COVID-19 period, electronic health record data of 67,245 children aged 0-12 years were available (time point: 1 September 2019) whereas data of 67,134 children were available during the pandemic (time point: 1 September 2020). Sex and age distribution were similar across periods: 51% male, 16% aged <2 years, 33% 2-6 years and 51% ≥ 6-12 years.

Overall OM Episodes

OM episodes declined considerably during the COVID-19 pandemic (**Table 1**).The IR per 1,000 child-years pre-COVID-19 vs COVID-19 for AOM were 73.7 vs 27.1 [IRR 0.37, 95% CI 0.35-0.39], for OME 9.6 vs 4.1 [IRR 0.43, 95% CI 0.37-0.49], for ear discharge 12.6 vs IR 5.8 [IRR 0.46, 95% CI 0.41-0.52] and for ear pain 18.1 vs 11.8 [IRR 0.65, 95% CI 0.60-0.71]. Gender (**Supplementary Figure 1**) and age-specific analyses revealed similar results, except for a less pronounced decline in OME episodes in children aged 0-2 years [IRD -0.66, 95% CI -2.50-1.18].

Figure 1 shows the monthly incidences of AOM episodes per 1,000 child months for various age groups before and during the pandemic and illustrates the absence of the usual winter peak in AOM incidence during the COVID-19 pandemic, especially in young children. **Supplementary Figure 2** shows the monthly incidence of OME episodes per 1,000 child months for various age before and during the pandemic.

Figure 2 illustrates the timing of implementation of generic infection control measures together with the monthly incidences of AOM and OME per 1,000 child months from March 2019 to March 2021. AOM and OME incidences decrease sharply during the COVID-19 peaks as well as during closure of schools and daycare centers. Acute upper respiratory tract infections show a similar pattern (**Supplementary Figure 3**).

OM Episodes Treated With Antibiotics

Similar to the overall OM episodes, the absolute number of AOM episodes in which oral antibiotics were prescribed declined accordingly (IRD pre-COVID-19 vs COVID-19: -22.4 per 1,000 child years), but the proportion of AOM episodes with antibiotic prescription was similar in both periods (47% vs 46%, respectively) (**Table 1**).

Type of Consultation

The numbers of OM-episodes are based on ICPC-codes, which consist of both face-to-face and telephone GP consultations. The proportion of OM episodes (AOM, OME, ear discharge combined) which were coded based on only telephone consultation(s) only, increased over time [pre-COVID-19 vs COVID-19: 7.4% vs 22.3%].

Complications

The incidence of acute mastoiditis remained low throughout the study period; IR per 1,000 child year pre-COVID-19 vs COVID-19: 0.15 vs 0.10 [RR 0.70, 95% 0.23-2.04].

DISCUSSION

This large retrospective cohort study showed that GP consultation for AOM, OME and ear discharge declined by 63%, 57% and 54% respectively in the Netherlands during the COVID-19 pandemic.

Previous studies in other countries have reported a similar trend in childhood OM incidence during the first COVID-19 peak (Hatoun et al., 2020; Aldè et al., 2021; Angoulvant et al., 2021; Iannella et al., 2021; Torretta et al., 2021). Under normal circumstances, OM typically shows a seasonal pattern with a winter peak coinciding with the increase in URTI incidence (Castagno and Lavinsky, 2002). Our study demonstrates the absence of the usual winter peak in AOM and OME during COVID-19 which is comparable with the reports of bronchiolitis from Belgium (Van Brusselen et al., 2021). The observed reduction in childhood OM might be attributed to the generic infection control measures, or changes in health care access and delivery. Although primary care services in the Netherlands remained accessible during the pandemic, the measures could

TABLE 1 | Number of Otitis media episodes and episodes with antibiotic prescription pre-COVID-19 era and COVID-19 era, including rate ratios and rate difference.

Episodes of	Pre-COVID-19 era[a]					COVID-19 era[a]					Rate Ratios and Rate Differences			
	N *patients*	N *episodes*	IR *episodes*[b]	% *AB oral*[c]	% *AB top*[d]	N *patients*	N *episodes*	IR *episodes*[b]	% AB oral[c]	% AB top[d]	IRR *episodes*[e]	IRD *episodes*[e]	IRD *episodes with AB oral*[f]	IRD *episodes with AB top*[g]
AOM	67245	4959	73,7	47,4	10,7	67134	1822	27,1	46,2	14,5	0,37 (0,35;0.39) *p<0,0001*	-46,6 *(-49,0;-44,2)* *p<0,0001*	-22,4 *(-24,1;-20,8)* *p<0,0001*	-3,9 *(-4,7; -3,1)* *p<0,0001*
Age 0 - 2	10896	1574	144,5	61,6	6,7	10243	681	66,5	62,1	4,1	0,46 *(0,42; 0,50)* *p<0,0001*	-78,0 *(-86,8; -69,2)* *p<0,0001*	-47,6*(-54,6; -40,7)* *p<0,0001*	-7,0 *(-9,1; -4,8)* *p<0,0001*
Age 2 - 6	21731	2354	108,3	44,0	11,5	21859	743	34,0	41,0	15,5	0,31 *(0,29; 0,34)* *p<0,0001*	-74,3 *(-79,3; -69,3)* *p<0,0001*	-33,7*(-37,0; -30,4)* *p<0,0001*	-7,2*(-8,9; -5,4)* *p<0,0001*
Age 6 - 12	34618	1031	29,8	33,8	14,8	35032	398	11,4	28,4	30,7	0,38 *(0,34; 0,43)* *p<0,0001*	-18,4 *(-20,6; -16,3)* *p<0,0001*	-6,8 *(-8,0; -5,6)* *p<0,0001*	-0,9 *(1,9; -0,0)* *p=0,0491*
OME	67245	648	9,6	8,5	10,2	67134	277	4,1	7,2	12,6	0,43 *(0,37; 0,49)* *p<0,0001*	-5,5 *(-6,4; -4,6)* *p<0,0001*	-0,5 *(-0,8; -0,27)* *p=0,0001*	-0,5 *(-0,75; -0,17)* *p=0,0021*
Age 0 - 2	10896	54	5,0	16,7	7,4	10243	44	4,3	18,2	4,5	0,87*(0,57; 1,31)* *p=0,4837*	-0,7 *(-2,5; 1,2)* *p=0,4819*	-0,04*(-0,81; 0,72)* *p=0,9083*	-0,17*(-0,63; 0,28)* *p=0,4586*
Age 2 - 6	21731	297	13,7	9,4	10,1	21859	110	5,0	7,3	7,3	0,37 *(0,29; 0,46)* *p<0,0001*	-8,6*(-10,5; -6,8)* *p<0,0001*	-0,9*(-1,5; -0,4)* *p=0,0008*	-1,0 *(-1,6; -0,5)* *p=0,0003*
Age 6 - 12	34618	297	8,6	6,1	10,8	35032	123	3,5	3,3	20,3	0,41*(0,33; 0,51)* *p<0,0001*	-5,07 *(-6,2; -3,9)* *p<0,0001*	-0,4 *(-0,7; -0,1)* *p=0,0026*	-0,2 *(-0,6; 0,2)* *p=0,3310*
Ear discharge	67245	847	12,6	25,4	40,3	67134	388	5,8	14,2	48,2	0,46 *(0,41; 0,52)* *p<0,0001*	-6,82 *(-7,84;-5,79)* *p<0,0001*	-2,34 *(-2,9; -1,9)* *p<0,0001*	-2,3 *(-3,0: -1,6)* *p<0,0001*
Age 0 - 2	10896	222	20,4	36,9	27,0	10243	78	7,6	28,2	19,2	0,37 *(0,29; 0,49)* *p<0,0001*	-12,8 *(-16,0; -9,6)* *p<0,0001*	-5,4*(-7,3; -3,5)* *p<0,0001*	-4,0 *(-5,7; -2,4)* *p<0,0001*
Age 2 - 6	21731	388	17,9	27,1	41,0	21859	158	7,2	15,8	46,8	0,40 *(0,33; 0,49)* *p<0,0001*	-10,6 *(-12,7; -8,5)* *p<0,0001*	-3,7*(-4,7; -2,7)* *p<0,0001*	-3,9*(-5,3; -2,6)* *p<0,0001*
Age 6 - 12	34618	237	6,8	11,8	51,5	35032	152	4,3	5,3	64,5	0,63 *(0,51; 0,78)* *p<0,0001*	-2,5 *(-3,6; -1,4)* *p<0,0001*	-0,6 *(-0,9; -0,2)* *p=0,0008*	-0,7 *(-1,6; 0,11)* *P=0,088-*

[a]*Pre COVID-19 era = March 2019 – 29 February 2020, COVID-19 era = 1 march 2020 - 28 February 2021.*

[b]*Incidence rate = total episodes per 1000 persons years.*

[c]*Percentage of episodes with an oral antibiotic prescriptions, including amoxicillin, amoxicillin/clavulanic-acid, co-trimoxazole, clarithromycin, azithromycin.*

[d]*Percentage of episodes with a topical antibiotic prescriptions, including tobramycin, ofloxacin, dexamethasone/tobramycin, dexamethasone/framycetine/gramicidin, hydrocortisone/colistin/bacitracin.*

[e]*Incidence Rate ratios (IRR) and Incidence Rate differences (IRD) per episode (Pre COVID-19 era vs COVID-19 era), including 95% confidence interval.*

[f]*IRD episodes with an oral antibiotic prescription per 1000 persons years (Pre COVID-19 era vs COVID-19 era), including 95% confidence interval.*

[g]*IRD episodes with a topical antibiotic prescription per 1000 persons years (Pre COVID-19 era vs COVID-19 era), including 95% confidence interval.*

have led to a higher threshold for consulting the GP, particularly early in the pandemic.

We found no evidence of an increase in the proportion of childhood OM episodes treated with antibiotics despite a substantial reduction in GP consultations for OM. This suggests that the observed decline in doctor consultations for OM was not related to OM severity and therefore not primarily attributed to a higher threshold to consultation. In line with our

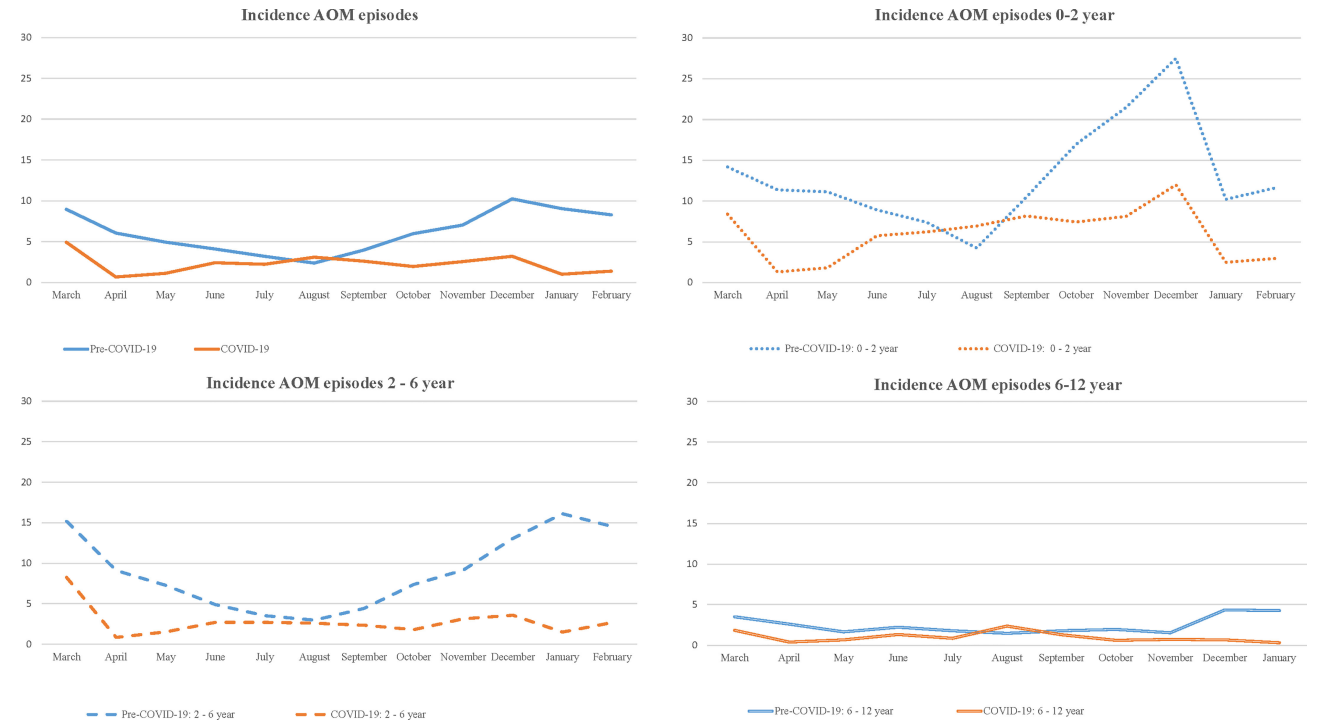

FIGURE 1 | Incidence of AOM episodes per 1,000 childmonths (total and according to age) pre-COVID-19 era and COVID-19 era.

findings, a previous study in Scotland reported that the COVID-19 lockdown led to a decline in pediatric emergency care consultations without an associated increase in severity (Williams et al., 2021). Another recent study in the United States has also found a lower rate in respiratory infection visits around September 2020 likely attributed to the infection control measures.

The major strengths of our study are its large sample size using well-documented electronic routine primary care-based health care data. The longitudinal nature of our study allowed us to compare the same study population within the same practices during two full years, i.e. one full year pre-COVID-19 and a complete year during the COVID-19 pandemic. Some methodological limitations need to be considered. First, misclassification might have occurred. Particularly during the COVID-19 pandemic, a substantial proportion of OM diagnoses were based on telephone consultation only which could have led to misclassification in OM diagnosis. A previous study from our group found that only 50% of parent-reported OM (fever and ear pain) episodes led to a GP diagnosis of OM (Fortanier et al., 2015).However, despite the increase in telephone consultations, we found a decline in the ICPC code 'ear pain' as well. This suggests that our observations reflect a true decline of OM during the pandemic. Moreover, misclassification might have occurred for the ICPC code 'ear discharge' since we were unable to determine whether this related to an acute onset of ear discharge or chronic suppurative otitis media. Furthermore, some children presenting with AOM and ear discharge will likely be classified by their GP as 'AOM' [ICPC code H71], i.e. those with AOM who were prescribed topical antibiotics. Second, we were unable to reliably extract data on specialist referrals and data about out of hours primary care were not available. Therefore, our results regarding complications should be interpreted with caution. Reassuringly, a previous study from Italy did not find a significant difference in OM-related complications during the first COVID-19 wave (Torretta et al., 2021) Finally, we were not able to link reductions in childhood AOM episodes to changes in causative viruses and bacteria over time. Such data would have allowed us to better explain our observations.

Implications

The observed decline in OM incidence similar to those observed in other common respiratory infections during the COVID-19 pandemic (Hatoun et al., 2020; Angoulvant et al., 2021; Kaur et al., 2021; Barschkett et al., 2021) providing further evidence for social interactions an hygiene as risk factors for OM. Continuing infection control measures like frequent handwashing (Little et al., 2015) may have a lasting effect on OM incidence beyond COVID-19 pandemic.

Although we should keep in mind the importance of social contacts in the development of children when considering implementing these infection control measures.

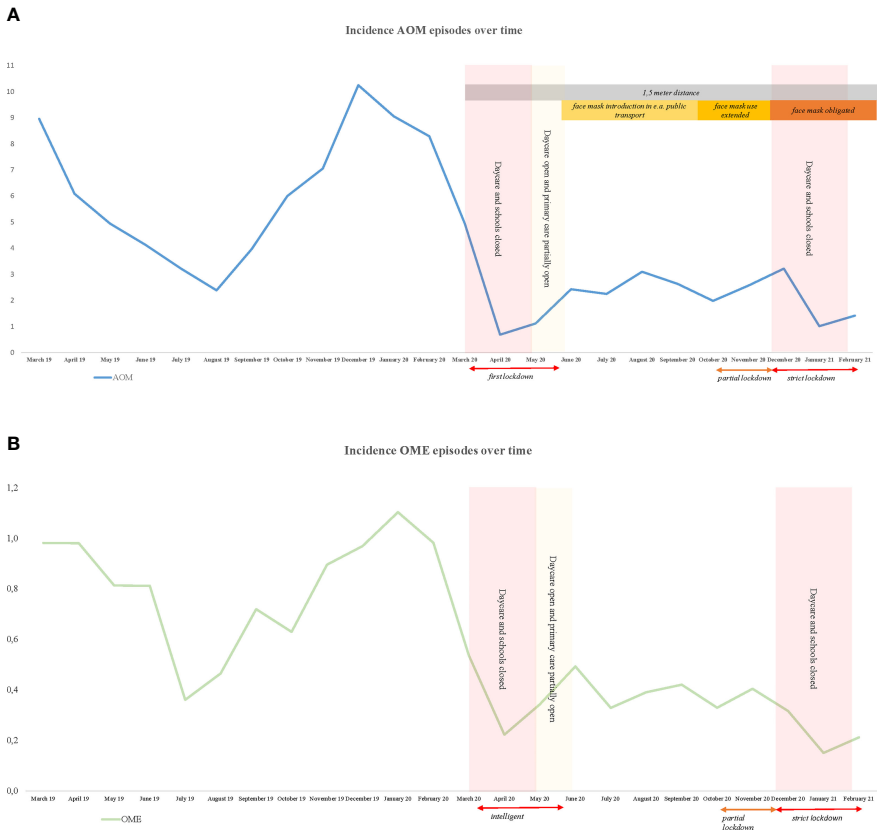

FIGURE 2 | Incidence AOM and OME episodes per 1000 childmonths and government restrictions. **(A)** AOM, **(B)** OME.

CONCLUSION

GP consultation for AOM, OME and ear discharge declined by 63%, 57% and 54% respectively in the Netherlands during the COVID-19 pandemic. Similar antibiotic prescription rates before and during the pandemic indicate that the case-mix presenting to primary care did not change considerably. Our data therefore suggests a true decline as a consequence of infection control measures introduced during the pandemic.

DATA AVAILABILITY STATEMENT

The data analyzed in this study is subject to the following licenses/restrictions: Data are not publicly available due to ethical and legal restrictions. Requests to access these datasets should be directed to not applicable.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the local legislation and institutional requirements. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Conceptualization AS, RV, and SH. Methodology, RV, SH, AP, and AV. Formal analysis, SH. Resources, RD. Writing— original draft preparation, all authors. Writing—review and editing, all authors. Visualization, SH. All authors have read and agreed to the published version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.768377/full#supplementary-material

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Leukotriene B4 Is a Major Determinant of Leukocyte Recruitment During Otitis Media

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Background: Pathogens of otitis media (OM) induce inflammatory responses in the middle ear (ME), characterized by mucosal hyperplasia, leukocyte infiltration, and inflammatory mediators, including arachidonic acid metabolites. We studied the role of the eicosanoid leukotriene B4 (LTB4) in OM.

Methods: Expression of LTB4-related genes was evaluated by gene array and single-cell RNA-Seq in MEs infected with nontypeable *Haemophilus influenzae* (NTHi). An inhibitor of LTB4 receptor 1 (i.e. U75302) was also used to block LTB4 responses.

Results: ME expression of LTB4-related genes was observed by gene arrays and scRNA-Seq. However, not all genes involved in LTB4 generation occurred in any one specific cell type. Moreover, LTB4 receptor inhibition significantly reduced mucosal hyperplasia and virtually eliminated leukocyte infiltration.

Conclusions: ME expression of LTB4-related genes suggest a functional role in OM disease. The fact that LTB4-generation is spread across different cell types is consistent with a transcellular pathway of eicosanoid biosynthesis involving cell-to-cell signaling as well as transfer of biosynthetic intermediates between cells. The dramatic reduction in ME leukocyte infiltration caused by U75302 indicates that LTB4 plays a major role in ME inflammatory cell recruitment, acting *via* the LTB4R1 receptor. Given that there are many other chemotactic factors that occur in the ME during OM, the ability of LTB4 to activate leukocytes and stimulate their extravasation may explain the effects of inhibition. Reduction in mucosal hyperplasia due to U75302 administration may be secondary to the reduction in leukocytes since LTB4R1 is not expressed by mucosal epithelial or stromal cells. The results suggest that LTB4 receptor antagonists could be useful in treating OM.

Keywords: leukocytes, otitis media (OM), leukotriene B4 (LTB4), mucosa, non-typeable Haemophilus influenzae (NTHi), arachidonic acid (AA), inflammation

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INTRODUCTION

Otitis media (OM) is the most prevalent disease of childhood (Casselbrant et al., 1993). More than 90% of children experience at least one episode of OM before the age of five. While the majority of acute OM is self-limiting and resolves within a few days even without treatment, 10-15% of children suffer from chronic or recurrent OM (Teele et al., 1989; Kaur et al., 2017). OM peaks between the ages of 6 months and two years (Pichichero, 2016), which is a critical period for the development of speech and language.

In developed countries, OM causes significant health and financial burdens. In the US, OM causes more pediatrician visits, antibiotic prescriptions, and surgeries than any other condition for children under 5 years of age (Rovers et al., 2008), at a cost estimated at $5 billion per year (Rosenfeld and Bluestone, 2003; Klein, 2000; Tong et al., 2018). Hearing loss due to chronic OM has been linked to delays in speech (Friel-Patti and Finitzo, 1993), language acquisition (Klausen et al., 2000), deficits in learning (Williams and Jacobs, 2009), and disorders of central auditory processing (Klein et al., 1988). However, in parts of the developing world, OM is a much more serious public health problem. The WHO estimates that undertreated OM, leading to chronic suppurative OM (CSOM), leads to 30,000 deaths per year due to meningitis. WHO also estimates that CSOM is responsible for half of the world's burden of handicapping hearing loss, more than 275 million cases (WHO, 2004; Leach et al., 2020; WHO, 2020). It has even been suggested that CSOM represents a neglected tropical disease (Li et al., 2015).

OM is a multifactorial disease, with contributions from Eustachian tube dysfunction, prior upper respiratory viral infection, genetics, and environmental factors (Schilder et al., 2016). However, these factors often lead to a final common pathway of bacterial infection, primarily by nontypeable *Haemophilus influenzae* (NTHi), *Streptococcus pneumoniae* and/or *Moraxcela cattharalis*, with some viral co-infection (Ruohola et al., 2006). While streptococcal vaccines have reduced ME infections by covered strains, OM is an opportunistic infection and the incidence of ME infection by other species and streptococcal strains has increased (Casey et al., 2010). The result is that OM incidence has declined only modestly due to vaccinations (Vojtek et al., 2017).

Despite many years of research, treatments for OM have seen little improvement. Antibiotics remain the most common therapy for OM. Although antibiotics have been shown to be of little benefit for children over two, antibiotic prescriptions remain very common due largely to parental demand for treatment (McGrath et al., 2013). Pressure equalization tubes are recommended for chronic/recurrent OM, but they require surgery and general anesthesia for children, and can lead to scarring of the tympanic membrane (Yaman et al., 2010). Clearly, new therapies are desirable. Recent research on OM has illuminated pathways that contribute to OM pathogenesis and recovery (Kurabi et al., 2016). These pathways offer potential opportunities for alternative interventions.

The invading pathogens that are the hallmark of OM trigger innate and, eventually, adaptive immunity. Acute, uncomplicated OM typically resolves in a few days even without treatment (Rosenfeld and Kay, 2003). Because this is too soon for adaptive immune activation, it is clear that innate immunity is the default process for OM resolution. Many studies in animals and patients (see Kurabi et al., 2016 for review) have confirmed that innate immune receptors and effectors contribute to OM resolution. However, innate immune activation of inflammation also appears to be responsible for many of the pathogenic features of OM, including fluid infiltration of the ME producing serous OM, mucus generation leading to glue ear, and mucosal hyperplasia (Mittal et al., 2014). Understanding the roles of innate immune processes in OM may lead to the development of novel therapies.

A major component of inflammation is the recruitment of pro-inflammatory leukocytes, which invade the ME in large numbers in response to infection (Lim and Birck, 1971). Key among these pro-inflammatory cells are polymorphonuclear cells (PMNs) and monocytes (Qvarnberg et al., 1984), which enter the ME from the circulation in response to chemotactic factors. These can be secreted by macrophages that reside in the ME, or by non-immune ME cell types (Ryan et al., 2020). Monocytes that enter the ME typically differentiate into macrophages. PMNs and macrophages phagocytose and kill pathogens, but they also secrete pro-inflammatory substances that can harm middle ear tissues. Moreover, because of their relatively short life span in tissue (Colotta et al., 1992), dying PMNs release toxic substances such as the reactive oxygen species resident in their numerous intracellular granules as well as cellular debris that can impede clearance of ME fluid. The resultant inflammation induces fluid extravasation into the ME and hyperplasia of the ME mucosa, along with mucosal secretion of mucus and other factors.

Inflammation also induces arachidonic acid (AA) metabolism (Hanna and Hafez, 2018). As shown in **Figure 1**, the cytosolic enzyme phospholipase A2 group IV-A (PLA2G4A) converts cell membrane lipids into AA. AA can be metabolized by cyclo-oxygenases into prostaglandins, or by arachidonate-5-lipoxygenase (ALOX5) into the eicosanoid leukotriene A4 (LTA4). The latter process requires ALOX5 activating protein (ALOX5AP), which anchors ALOX5 to the membrane and plays an essential role in the transfer of AA to ALOX5. LTA4 in turn is converted by ALOX12 or ALOX15 into lipoxins. Alternatively, it can be converted by leukotriene A4 hydrolase (LTA4H) into leukotriene B4 (LTB4). The biological effects of leukotrienes and lipoxins often oppose each other, with LTB4 generally being pro-inflammatory and lipoxins being anti-inflammatory. LTB4 is a major chemoattractant of inflammatory leukocytes. LTB4 signals primarily *via* two LTB4 receptors, LTB4R1 and LTB4R2, which are expressed by leukocytes. LTB4R1 is a high-affinity receptor specific for LTB4, while LTB4R2 has a lower affinity and can also respond to other eicosanoids (Sheppe and Edelmann, 2021). Alternative, eicosanoid pathways lead to the production of other leukotrienes or lipoxygenases, while the cyclo-oxygenase pathway can generate lipoxygenases, prostaglandins, and thromboxane.

The role of LTB4 in OM is largely unexplored. The purpose of the present study was to evaluate the expression of genes associated with LTB4 generation as well as those encoding its receptors, during an acute episode of OM. Gene expression data

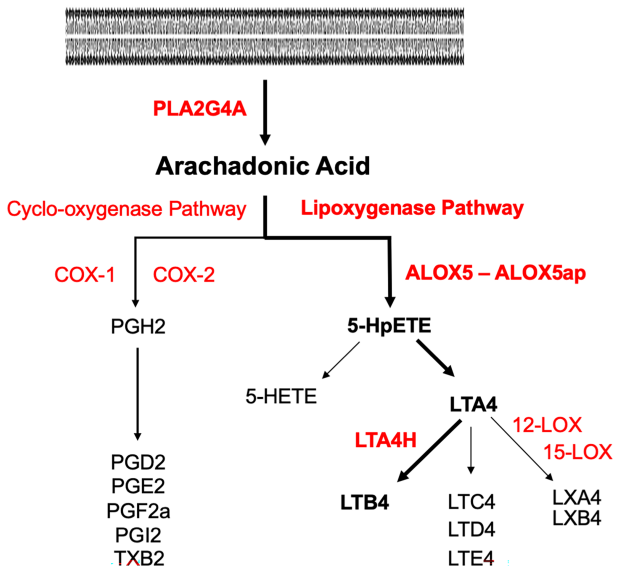

FIGURE 1 | Pathways of arachidonic acid metabolism. Enzymes are in red, metabolites are in black. The pathway to LTB4 *via* the lipoxygenase pathway is delineated in bold. Phospholipase A2 (PLA2G4A) generates AA from cell membranes. Lipoxygenase A5 (ALOX5) and its activating protein (ALOX5AP) convert AA to 5HpETE, which then partitions between reduction to the 5-HETE and conversion to leukotriene A4 (LTA4). LTA4 hydrolase (LTA4H) converts LTA4 to LTB4.

are used because LTB4 is a lipid moiety with a half-life in minutes, and therefore difficult to quantify. To explore the functional role of LTB4 in OM, inhibition of the LTB4 receptor LTB4R1 was employed.

METHODS

Animals

Mice used for gene array analysis were 60-90 day old C57BL/6:CB F1 hybrids, while those for single-cell RNA-Seq were C57BL/6 (Jackson Labs, Bar Harbor, ME, USA). Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN, USA) were used for inhibitor studies. All experiments were performed to National Institutes of Health guidelines and approved by the VA San Diego Medical Center IACUC.

OM Generation

Mice or rats were deeply anesthetized with rodent cocktail (ketamine 50 mg/kg, xylazine 1 mg/kg, acepromazine 5 mg/kg in 50 µl, i.p.) and the ME bulla surgically exposed. A small opening was created in each bulla and *Haemophilus influenzae* strain 3655 (nontypeable, biotype II; NTHi), was infected in 5 µl PBS for mice and 50 µl for rats, at a titer of 10^4.

Gene Array

Gene arrays were used to provide quantitative information on gene expression levels within the ME, since mRNA is extracted in a uniform manner from all cells in a tissue. Forty mice per time point were inoculated in the ME bilaterally with NTHi. Uninoculated animals served as controls. Mucosal tissue and exudate were harvested from 20 mice at each of the following intervals: 0 hours (0h, no treatment), 3h, 6h, 24h, 2 days (2d), 3d, 5d and 7d after inoculation, and pooled. The tissue was homogenized in TRIzol (Life Technologies, Carlsbad, CA) and total RNA extracted, reverse transcribed, amplified and transcribed *in vitro* to generate biotinylated cRNA probes that were hybridized to 2 Affymetrix (Agilent Technologies, Santa Clara, CA) MU430 2.0 microarrays, according to the manufacturer's protocol. This procedure was duplicated for each time point to obtain a second, independent biological replicate. Thus each data point represents 2 separate samples consisting of 20 mice each, and 4 Affymetrix arrays. Specific genes were assessed at individual time points using Genespring GX 7.3 (Agilent Technologies, Santa Clara, CA). To identify changes in gene expression, the data were first analyzed using a variance modeling approach. The raw MAS5 expression values were imported into the VAMPIRE software without prior normalization. This program uses a Bayesian approach to identify significantly altered genes (Hsiao et al., 2005). Hybridization to probes for genes involved in AA metabolism and signaling were assessed at individual time points for difference from control (uninfected) samples, after Bonferonni correction for multiple tests and using ANOVA in Genespring GX 7.3 (Agilent Technologies, Santa Clara, CA). Additional details of methods are provided in our previous publication (Hernandez et al., 2015) in which the genes evaluated here were not included.

Single-Cell RNA-Seq

Gene arrays generated from bulk tissue cannot identify which cells are expressing a given gene. We used single-cell RNA-Seq (scRNA-Seq) to provide precise, cell-level data. However, because the isolation of different cell types will vary in efficiency depending upon fragility and strength of bonding to other cells, this method is less able to determine overall ME levels of gene expression. Groups of six C57BL/6 mice each were untreated as controls. Additional groups were inoculated in the ME with NTHi and mucosal tissue and exudate harvested 24 hours later, when the gene array data indicated maximum regulation of genes related to LTB4. The pooled tissue for each sample was digested with thermolysin (0.5 mg/ml, Sigma-Aldrich, #T7902) followed by FACSMax cell dissociation solution (Genlantis, #T200100), and triturated into single cells. Dissociated cells were diluted to 700 cells/µL. Three replicates were performed to obtain independent biological samples of control MEs, while four replicates were performed 24 hours after NTHi inoculation. Single-cell libraries were prepared using the Chromium Controller (10X Genomics, Pleasanton, CA) according to the manufacturers' instructions. The libraries were sequenced on an Illumina HiSeq 2500 (Illumina, San Diego) and yielded approximately 200 million reads per sample.

Bar-coded reads were demultiplexed using Cellranger 2.0.2 (10X Genomics) and mkfastq in conjunction with bcl2fastq

2.17.1.14 (Illumina) and aligned to a murine reference genome mm10 (Ensembl 93) provided by 10X Genomics. Reads were filtered to remove short reads and reference genome mismatches to improve library quality, quantified and subjected to principal component analysis (PCA) clustering. For the three samples from normal MEs, the average number of analyzed cells/sample was 2,257, and the number of genes detected/sample averaged 17,323. For the four samples at 24 hours the number of cells/sample averaged 3,978 and genes detected averaged 17,413. Identification of cells in each cluster was based on the following marker genes: epithelial cells, high expression of *Epcam and Krt18*; ciliated epithelial cells, *Epcam* and *Hydin*; immature epithelial cells, low *Epcam and Krt18*; stromal cells, *Col1a2*; vascular endothelial cells, *Egfl7* and *Flt4*; lymphatic endothelial cells *Egfl7* and *Flt1*; pericytes, *Rgs5*; monocytes, *Csf1r*; lymphocytes, *Ptprcap*; and melanocytes, *Mlana*. After infection, PMNs were identified by expression of *Il1f9* and *Stfa2l1* and RBCs by *Hba-a1*. Graph-based and K-means analysis of gene expression was then performed for genes related to LTB4 generation and sensing. Expression of genes by ME cells was visualized using 10X Genomics cLoupe. A cluster plot for each gene was generated using log2 data, for optimal visualization of expressing cells. Violin plots of expression levels for each cluster were log-normalized for optimal comparison across the cluster cell population. Additional details of methods are available in our previous publication on normal ME scRNA-Seq (Ryan et al., 2020), in which no genes related to AA metabolism were reported. The gene data is available at the Science Data Bank (https://www.scidb.cn/en) under doi:10.11922/sciencedb.01353.

Inhibitor Studies

Because of the small size (5 μL) of the mouse ME, inhibitor studies were performed in rats. Rats and mice are closely related and have highly similar responses to ME NTHi inoculation (Melhus et al., 1994; Melhus and Ryan, 2003). For the inhibitor study, 4 rat MEs per treatment were inoculated with NTHi as above. Control rats received NTHi alone in PBS plus 0.1% DMSO. Inhibitor groups received NTHi with different doses of the specific BLT1 inhibitor U75302 [(6-(6-(3R-hydroxy-1E,5Z-undecadien-1-yl)-2-pyridinyl)-1,5S-hexanediol; refs; Cayman Chemical Company, Ann Arbor, MI, USA] plus DMSO. Three U75302 dosages were used: 1.5 μM, 15 μM, 150 μM. 150 μM U75302 only into the ME served as an additional control. Rats were sacrificed for ME harvest 48 hours after inoculation.

Histology

Rats were sacrificed by decapitation under deep anesthesia. ME bullae were extracted, post-fixed with 4% PFA overnight, and decalcified in an 8% EDTA/4% PFA solution over a 14-day period. The bullae were embedded in paraffin and sectioned at 7 μm. Sections were stained with hematoxylin-eosin and mounted. As described previously (Leichtle et al., 2010), from four sections at the midpoint of each ME, mucosal thickness was measured at six standard locations and averaged to obtain a measure for that ME. From the same sections, the total area of the ME lumen and the percent of the lumen area occupied by leukocytes were digitally determined using NIH Image software. A 300X image of the ME infiltrate was captured for each section, and the number of neutrophils and macrophages quantified to capture the proportions of each cell type in the infiltrate. Leukocyte data were not normally distributed and were analyzed using the Kruskal-Wallace nonparametric ANOVA.

RESULTS

Gene Array

As assessed by cRNA probe hybridization to gene arrays, several genes involved in LTB4 production and sensing were significantly upregulated following NTHi inoculation of the ME (**Figure 2A**). The *Pla2g4a* gene was only slightly (1.5-fold) upregulated during OM. The *Alox5* gene increased expression 3-fold at 1 day, while *Alox5ap* increased expression 5-fold at 3 days after inoculation. The *Lta4h* gene for the LTB4 generating LTA4 hydrolase enzyme increased 2-fold at 1 day. Most dramatically, the LTB4 receptor gene *Ltb4r1* increased by 170-fold at 1 day after NTHi inoculation of the ME, while *Ltb4r2* was not significantly regulated. Some other enzymes of AA metabolism were also significantly regulated, especially *Cox2* but also *Alox12* and *Alox15* (**Figure 2B**). Detailed data on fold change ranges and statistics are presented in the **Supplementary Table**.

Single-Cell Gene Expression

ScRNA-Seq was used to map the expression of LTB4 synthesis and sensing genes to uninfected ME cells, and cells harvested at 24 hours after NTHi inoculation when expression of most genes peaked (**Figure 2A**). PCA generated 10-15 clusters for each condition, which corresponded to different ME cell types. Some cell types, such as epithelial cells, consisted of multiple PCA clusters that corresponded to recognized subtypes. Other cell types, such as PMNs, consisted of several clusters not easily defined. After identification of the cell types in each cluster, genes related to LTB4 generation and sensing were visualized using the 10X Genomics cLoupe function.

Figure 3 presents gene expression by cells in the uninfected ME. The expression by cell types is illustrated in the cLoupe PCA projection of **Figure 3A**, which shows the results in a representative control sample. As can be seen in the figure, *Pla2g4a* mRNA was expressed by a small number of most ME cell types, with the exception of ciliated epithelial cells, where most cells were positive, and endothelial cells, melanocytes and lymphocytes, which were essentially negative. *Alox5* was observed in small subsets of monocytes and lymphocytes. *Alox5ap* was expressed in most monocytes and approximately half of the stromal cells, as well as a few epithelial cells and lymphocytes. *Lta4h* was observed in small subsets of all ME cell types, while *Ltb4r1* was prevalent only in subsets of monocytes and lymphocytes. *Ltb4r2* was not expressed by any ME cells. **Figure 3B** shows violin plots of gene expression by PCA cluster, from the same control sample. The plots provide a relative

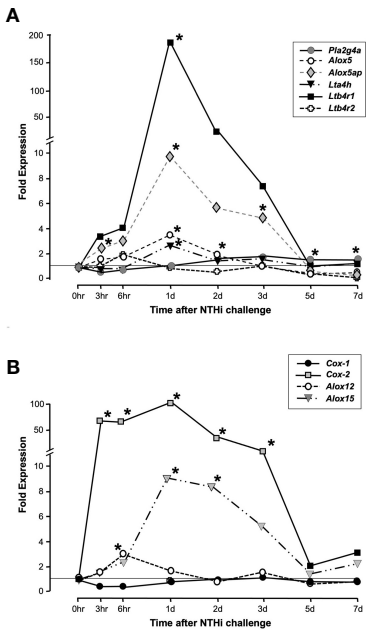

FIGURE 2 | Expression of genes involved in LTB4 generation and sensing during a complete episode of acute NTHi-induced OM in the mouse, from initiation to recovery, evaluated by gene arrays. Panel **(A)** shows AA Lipoxygenase pathway related genes. *Ltb4r1* is very strongly and significantly upregulated during OM, with lesser but significant upregulation of *Alox5*, *Alox5ap* and *Lta4h*. *Ltb4r2* was not significantly regulated. Panel **(B)** shows related enzymes of AA metabolism. *and gray color = p <.05, ANOVA. Detailed data on fold change ranges and p values are presented in the **Supplementary Table**.

quantitative analysis of gene expression. They confirm that only a small number of cells expressed LTB4-related genes at high levels, with the majority of cells not expressing. The exceptions were monocytes and stromal cells, of which many cells expressed *Alox5ap* at appreciable levels.

Figure 4 presents results from a representative ME sample collected 24 hours after NTHi infection. All of the cell types observed in control MEs were present, except for melanocytes. In addition, a new category of immature epithelial cells was present, and large numbers of neutrophils and a few erythrocytes had infiltrated the ME. In agreement with our array data, substantially more expression of LTB4-related genes was observed 1 day after infection. *Pla2g4a* mRNA was expressed in virtually all monocytes and variable subsets of other cell types. *Alox5* mRNA was observed primarily in a subset of infiltrating neutrophils, and a few monocytes. *Alox5ap* was expressed by

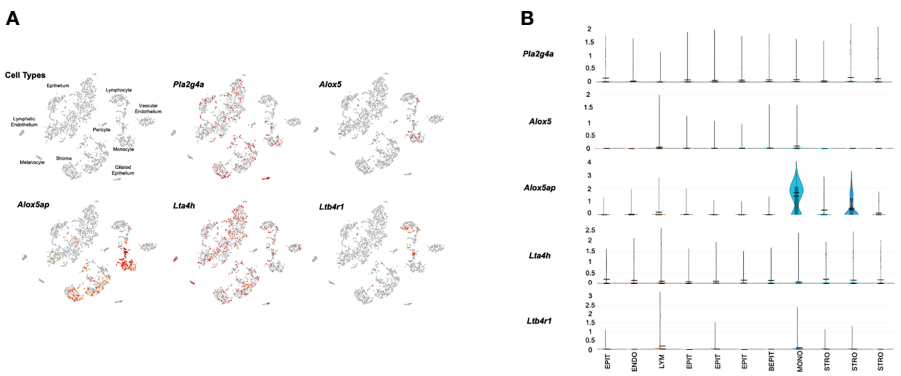

FIGURE 3 | Expression of LTB4-related genes by individual ME cell types, before inoculation of the murine ME with NTHi. For each sample, twelve MEs were pooled and digested into a single-cell suspension for single-cell RNA-Seq. Panel **(A)** represents 2,858 cells recovered from the normal ME, clustered by principal component analysis, and identified by recognized cell-type marker genes. Expression of each gene by cell is indicated in red. Panel **(B)** presents violin plots for individual PCA clusters, labeled by cell type, with some cell types represented by multiple clusters. The width of each plot represents the frequency of cells expressing the gene at that log normalized level. The upper horizontal line in each plot represents the cluster mean, and the lower line the median. The height of the vertical line represents the highest expressing cell.

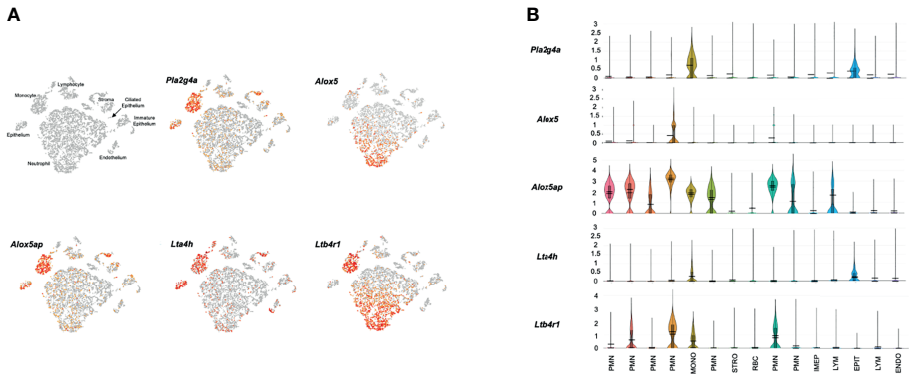

FIGURE 4 | Expression of LTB4-related genes by individual ME cell types, 24 hours after inoculation of the murine ME with NTHi. **(A)** PCA clusters of 9,604 cells, with cell types identified and cells expressing each gene indicted in red. **(B)** Violin plots demonstrating cell type expression patterns as well as the much higher level expression of LTB4-related genes.

almost all monocytes, the majority of neutrophils and small subsets of other cell types. *Lta4h* was present in most epithelial cells and monocytes, but also by small subsets of other cell types. *Ltb4r1* was produced exclusively by monocytes and a subset of neutrophils. *Ltb4r2* expression again was not observed. The violin plots of **Figure 4B** confirm the distribution of LTB4-related gene expression by cell type, and provide relative quantitative expression levels which can be compared to those for the cell types in **Figure 3B**. Each of the genes was differentially expressed at a significant level (p<.05, ANOVA) between control and infected MEs, for the cell types with major differences in violin plots. PMNs, which were not present in the control sample, could not be thus analyzed.

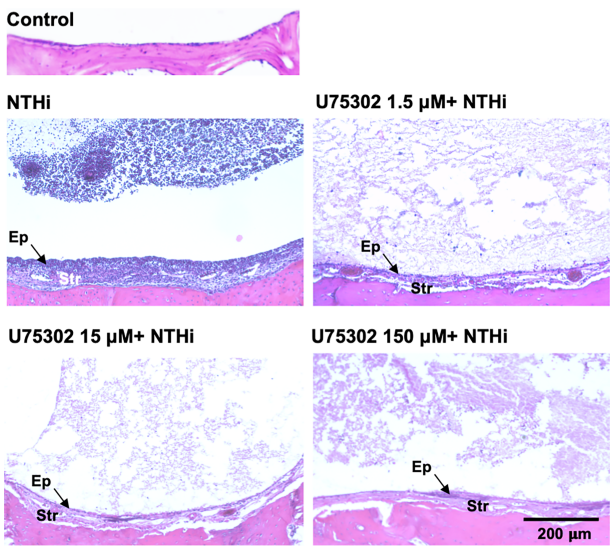

FIGURE 5 | The MEs of a control, untreated mouse and of mice 2 days after infection with NTHi, or NTHi plus different doses of the specific LTB4R1 inhibitor U75302. At all doses, hyperplasia of both the mucosal epithelium (Ep) and stromal (Str) was reduced. In addition, infiltration of the ME lumen by leukocytes was virtually eliminated. A luminal precipitate was observed in all treated ears, which presumably represents an acellular exudate.

Mucosal Hyperplasia

Figures 5 shows representative examples of ME histology for control MEs, before infection, for MEs 48 hours after inoculation with NTHi alone, for MEs after 48 hours after inoculation with NTHi plus one of three dosages of U75302, and in MEs 72 hours after injection of the highest dosage of U75302 alone. Quantitative analysis of mucosal thickness is provided in **Figure 6A**. ME infection with NTHi substantially increased mucosal thickness when compared to control MEs. All three dosages of U75302 significantly reduced mucosal thickness (p <.05; ANOVA). Thickness after injection of U75302 alone was not significantly different from control.

Leukocyte Infiltration of the ME

Figure 5 also illustrates the leukocyte response of the ME to NTHI inoculation, with and without the LTB4R1 inhibitor U75302. It is clear from **Figure 5** that leukocyte infiltration of the ME lumen was dramatically reduced by all three dosages of inhibitor. This is confirmed by the quantitative analysis presented in **Figure 6B** (p <.001; Kruskal Wallis ANOVA). However, the presence of substantial amounts of precipitate in the ME suggests that LTB4 inhibition did not reduce infiltration of the ME by fluid from the vasculature. U75302 alone did not induce significant cellular or fluid infiltration of the ME.

Leukocyte Cell Types in the ME

Infection by NTHi typically produces ME infiltration by large numbers of PMNs, and a lower number of monocytes/macrophages. This was also observed in the control animals of the present study. **Figures 6C, D** represent each cell type as counted in 300X micrographs of infiltrate in the ME lumen. Not surprisingly given the data shown in **Figure 6B**, treatment with U75302 virtually eliminated both cell types from the ME (p <.001; Kruskal Wallis ANOVA).

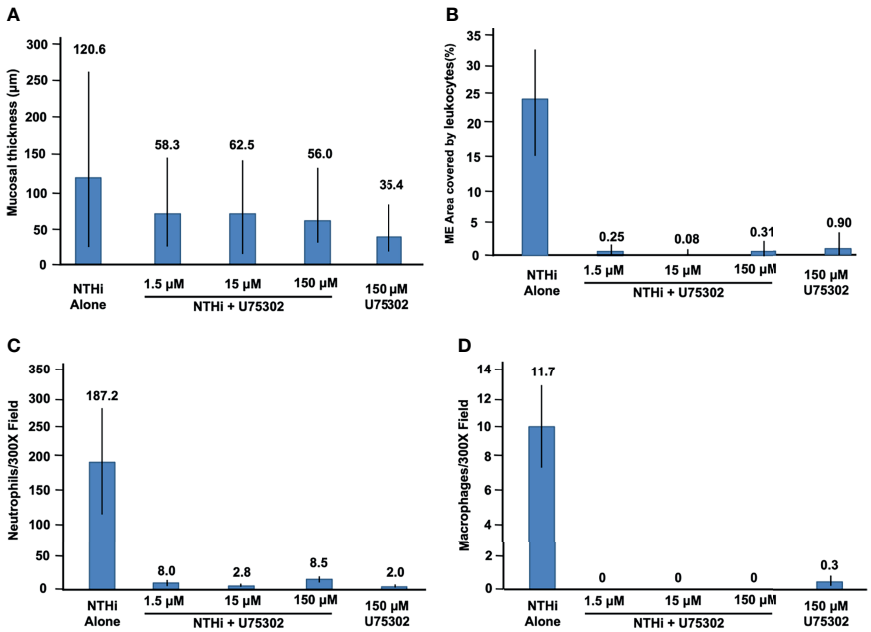

FIGURE 6 | Quantitative evaluation of the effects of U75302 on OM. **(A)** ME mucosal thickness (mean, range: max and min). **(B)** Percent of the ME lumen occupied by leukocytes. **(C)** Number of neutrophils counted in high-power images of ME exudate. **(D)** Number of macrophages counted in high-power images of ME exudate.

DISCUSSION

Significant increases in the expression of genes related to LTB4 generation and receptor sensing after ME inoculation with NTHi provide evidence that this AA metabolite could play a role in OM. In particular, the dramatic rise in *Ltb4r1* mRNA observed early in OM suggests that either ME cells generated large amounts of this receptor, or that cells expressing these receptors entered the ME in large numbers. Conversely there were no significant changes in the expression of *Ltb4r2 mRNA*. Genes involved in the generation of LTB4 also peaked at 24 hours, with *Alox5ap* also showing a significant increase earlier. Infection also increased the expression of other genes of the AA metabolic pathway, in both the cyclo-oxygenase and lipoxygenase pathways. These data suggest that other AA metabolites participate in OM, and their production has been documented in experimental OM (Goldie et al., 1993). However, responses to any of these would not be expected to be inhibited by U75302.

Single-cell RNA-Seq confirms and validates the expression of mRNA encoding components of the LTB4 synthetic pathway, and its specific receptor LTB4R1. Interestingly, all of the synthetic components were not present in any one ME cell type, but rather were distributed across a variety of cells. This is consistent with other cellular systems, where eicosanoids are often the result of intercellular interactions involving cell-to-cell signaling and the transfer of biosynthetic intermediates, such as LTA4, between cells (e.g. Fradin et al., 1989). This process is known as the transcellular pathway of eicosanoid biosynthesis (Sala et al., 2010). It requires one cell to synthesize and release a component of the biosynthetic pathway, and another cell to take up that intermediate and process it into a final active molecule. In the case of eicosanoids, this is aided by their lipid solubility.

This intercellular process appears to occur in the ME as well. From the pattern of *Pla2g4a* expression, it can be inferred that most AA is produced by ME epithelial cells, with a contribution from some resident leukocytes. The enzymes that produce LTB4 are primarily expressed by leukocytes, but the production of its precursor, LTA4H, is mostly by epithelial and stromal cells. The exchange of AA and LTA4H between epithelial and leukocytic cell types, and potentially signaling between the cell classes, appears to be necessary for the ME production of LTB4. The expression of LTB4R1 only in leukocytes was expected. The absence of cells expressing mRNA encoding the specific LTB4 receptor LTB4R2 indicates that inhibition of LTB4R1 would completely block LTB4 signaling in the ME.

The final step in LTB4 production involves the conversion of LTA4 to LTB4 by LTA4H (see **Figure 1**). *Lta4h* mRNA is highly expressed in epithelial cells and monocytes, and some stromal cells, vascular endothelial cells, and PMNs. These cells are likely to be the primary sources of direct LTB4 production. However, PMNs produce the most ALOX5 and are likely the primary source of the precursor molecules 5-HpETE and LTA4.

The virtual elimination of leukocytic infiltration of the ME caused by the LTB4R1 inhibitor U75302 indicates that LTB4 activation of this receptor is required for the recruitment of PMNs and monocytes/macrophages to the tympanic cavity. Given that several other chemoattractants that target these two cell types are also expressed in the ME (Hernandez et al., 2015; Dennifel et al., 2017; Hur et al., 2021), the dominant role of LTB4

was unexpected. It seems likely that LTB4's role in the activation of leukocytes, which is required for extravasation, is responsible for its ME effects since this could disable recruitment by all chemoattractants.

The results of our animal study have potential clinical implications. A prior study found that LTB4 is present in ME fluid from children, and that high levels are associated with treatment failure (Chonmaitree et al., 1996). The authors concluded that LTB4 may play role in delaying acute OM recovery and recurrence. They suggested that more effective treatment of OM would require the combined use of antibiotics that reduce inflammatory leukocyte function. Another study from the same group found that treatment with steroids did not decrease LTB4 in the ME fluid of children (McCormick et al., 2003). Our results indicate that LTB4 receptor antagonists, or perhaps natural pro-resolution compounds that oppose the effects of LTB4, such as resolvin E1 (Arita et al., 2005), may be useful in treating OM.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Science Data Bank repository, accession number 31253.11.sciencedb.01353 and can be viewed at https://www.scidb.cn/en/s/BJJBBv.

ETHICS STATEMENT

The animal study was reviewed and approved by VA Health IACUC.

AUTHOR CONTRIBUTIONS

KH and KP performed experiments and data collection. AR, KH, and AK discussed the study idea, analyzed the data and generated the figures. KH wrote the initial manuscript draft. All authors contributed to final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.768815/full#supplementary-material

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REVIEW
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Otitis Media Practice During the COVID-19 Pandemic

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The global coronavirus disease-2019 (COVID-19) pandemic has changed the prevalence and management of many pediatric infectious diseases, including acute otitis media (AOM). Coronaviruses are a group of RNA viruses that cause respiratory tract infections in humans. Before the COVID-19 pandemic, coronavirus serotypes OC43, 229E, HKU1, and NL63 were infrequently detected in middle ear fluid (MEF) specimens and nasopharyngeal aspirates in children with AOM during the 1990s and 2000s and were associated with a mild course of the disease. At times when CoV was detected in OM cases, the overall viral load was relatively low. The new severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) is the causative pathogen responsible for the eruption of the COVID-19 global pandemic. Following the pandemic declaration in many countries and by the World Health Organization in March 2020, preventive proactive measures were imposed to limit COVID-19. These included social distancing; lockdowns; closure of workplaces; kindergartens and schools; increased hygiene; use of antiseptics and alcohol-based gels; frequent temperature measurements and wearing masks. These measures were not the only ones taken, as hospitals and clinics tried to minimize treating non-urgent medical referrals such as OM, and elective surgical procedures were canceled, such as ventilating tube insertion (VTI). These changes and regulations altered the way OM is practiced during the COVID-19 pandemic. Advents in technology allowed a vast use of telemedicine technologies for OM, however, the accuracy of AOM diagnosis in those encounters was in doubt, and antibiotic prescription rates were still reported to be high. There was an overall decrease in AOM episodes and admissions rates and with high spontaneous resolution rates of MEF in children, and a reduction in VTI surgeries. Despite an initial fear regarding viral shedding during myringotomy, the procedure was shown to be safe. Special draping techniques for

otologic surgery were suggested. Other aspects of OM practice included the presentation of adult patients with AOM who tested positive for SARS-2-CoV and its detection in MEF samples in living patients and in the mucosa of the middle ear and mastoid in post-mortem specimens.

Keywords: otitis media, COVID-19, coronavirus infection, admission, burden analysis, acute otitis media, otitis media with effusion, mastoiditis

CORONAVIRUS FAMILY

The coronavirus (CoV) family is a group of enveloped, positive-sense, single-stranded, highly diverse RNA viruses, with sizes ranging from 60-140 nm in diameter, having crown shape projections on their surface, hence their name: coronaviruses. Four main serogroups exist HKU1, NL63, 229E, and OC43, which have previously been shown to affect humans, and usually caused mild upper respiratory tract infections (URTIs) (Zumla et al., 2016). CoV was also detected from nasopharyngeal aspirates (NPAs) in infants during asymptomatic health visits (Chonmaitree et al., 2015). Research interest in the CoV family has been minimal over the past few decades because RNA viruses were hard to study, and large sero-epidemiolgical surveys conducted during the 1970s and 1980s showed high antibody titers in both children and adults, assuming that CoV infections were common and self-limiting, like any other respiratory viruses (Wenzel et al., 1974; Sarateanu and Ehrengut, 1980; Schmidt et al., 1986).

Over the last two decades, 3 new CoV members emerged: 1) Severe Acute Respiratory Syndrome-associated coronavirus (*SARS-CoV*), identified in 2002 in China, which caused a limited-scale epidemic involving 2 dozen countries with ~8000 cases and ~800 deaths (fatality rate: 9.6%) (Drosten et al., 2003); 2) Middle East Respiratory Syndrome-associated coronavirus (*MERS-CoV*), identified in 2012 in Saudi Arabia, which affected ~2,500 patients and caused ~800 deaths (fatality rate: 35%) (Rasmussen et al., 2016), and 3) *SARS-CoV-2*, identified in China in 2019, which caused a global pandemic, commonly known as coronavirus disease-2019 (*COVID-19*), currently affecting >191 millions of patients and causing >4.1 million deaths worldwide (source: Johns Hopkins University Center for Systems Science and Engineering, https://github.com/CSSEGISandData/COVID-19, accessed July 20, 2021).

EVIDENCE FOR COV DETECTION IN OTITIS MEDIA CASES

Old CoV Serotypes

Table 1 shows published reports on CoV detection in pediatric AOM cases. Using traditional polymerase chain reaction (PCR) and microplate hybridization assays, CoV detection rate in middle ear fluid (MEF) and NPAs in AOM cases was anecdotal. However, when newer real-time PCR assays became available and in widespread use, CoV detection rates increased up to 50% (Chonmaitree et al., 2008). A recent review showed that CoV load in the middle ear in various OM cases was overall very low (Liaw et al., 2021). There were no reports on AOM cases during the SARS-CoV and MERS outbreaks.

SARS-2-CoV

The basis for the hypothesis that suggested SARS-2-CoV infection can spread from the nasopharynx to the middle ear can be explained by the abundant expression of angiotensin converting enzyme (ACE)-2 receptors by goblet cells lining the Eustachian tube mucosa, which are critical for the intracellular entry of SARS-CoV-2 (Matusiak and Schurch, 2020; McMillan et al., 2021).

Albeit the abundance of ACE-2 receptors in the Eustachian tube, only several reports were published linking SARS-2-CoV infection to AOM. The first description was a 35-year-old Turkish female who presented with otalgia and tinnitus.

TABLE 1 | CoV detection in AOM cases.

Country	Year	Age	No. of Children	Main Findings
Finland (Pitkaranta et al., 1998)	1998	3m-7y	69	CoV RNA was detected in both MEF and NPA in 5 (5%), in MEF alone in 2 (2%), and in NPA alone in 9 (10%). RSV and HCV were detected in 1 NPA sample.
Finland (Nokso-Koivisto et al., 2000)	2000	2-24m	329	In confirmed AOM cases: 13 NPA CoV+, but no CoV+ MEF specimens.
France (Vabret et al., 2005)	2005	<20y	300	Of the 28/300 patients that had NPA CoV+, 28% had AOM.
Turkey (Bulut et al., 2007)	2006	6-144m	120	5/42 pure viral cases had MEF CoV+.
Finland (Ruohola et al., 2006)	2006	7-71m	79	MEF collected from children with otorrhea from TTs: 1 *Moraxella catarrhalis* + rhinovirus + CoV and 1 *Streptococcus pneumoniae* + *Haemophilus influenzae* + CoV.
USA (Chonmaitree et al., 2008)	2008	6m-3y	294	50% CoV+ detection rate in 440 AOM episodes.
Australia (Wiertsema et al., 2011)	2011	6-36m	180	14.4% NPA and 4.9% MEF samples were CoV+.
The Netherlands (Stol et al., 2012)	2012	<5y	116	MEF collected from children during TT surgery: 4/116 (3%) were CoV+.

AOM, acute otitis media; MEF, middle ear fluid; NPA, nasopharyngeal aspirate; CoV, coronavirus; RSV, respiratory syncytial virus, TT, tympanostomy tube.

Otoscopy, audiometry, and tympanometry confirmed AOM. Her NPA was positive for SARS-CoV-2, but she had no typical respiratory symptoms (Fidan, 2020). Because myringotomy was not performed, it was impossible to determine SARS-CoV-2 presence in the MEF. A case-series publication described 8 Iranian adult COVID-19 patients with AOM (Raad et al., 2021). Interestingly, 1 patient tested negative for SARS-CoV-2 from the oropharynx, but his MEF sample, obtained *via* myringotomy, tested positive. A 23-year-old American COVID-19 patient presented with complicated AOM and facial palsy, although his MEF sample tested negative for SARS-CoV-2 (Mohan et al., 2021). SARS-CoV-2 was detected in the mastoid and middle ear mucosa in autopsies performed 14-46h post-mortem in 2/3 COVID-19 deceased adult patients who did not have a preceding AOM episode (Frazier et al., 2020).

REDUCTION IN OTITIS MEDIA BURDEN

Following the declaration of the COVID-19 pandemic by the World Health Organization in early March 2020 (Jee, 2020), preventive proactive measures were imposed to limit SARS-CoV-2 transmission and infection, including social distancing, recommendations to stay at home, lockdowns in varying severities, closure of workplaces, kindergartens, and schools, increased hygiene measures, increased use of antiseptics and alcohol-based gels, frequent temperature measurements, and mask-wearing.

Reduction in Overall Pediatric Emergency Department Visits

The COVID-19 pandemic and national and international interventions aimed at limiting its spread deeply changed "traditional" healthcare habits, limiting people's mobility and access to medical facilities. Some Health and Welfare policy measures even discouraged non-urgent access to (pediatric) emergency departments (PED), and more generally any frontal visits to family physicians/pediatricians, which were not considered urgent. A substantial reduction in the number of pediatric referrals/admissions to PEDs has been globally reported in Singapore, USA, Italy, and Argentina (Chong et al., 2020; McBride et al., 2020; Ferrero et al., 2021; Finkelstein et al., 2021; Pepper et al., 2021).

Reduction in Pediatric AOM/OME Cases

The measures adopted to contain the COVID-19 pandemic also resulted in a decrease of airborne-mediated respiratory infections other than COVID-19, including URTI, bronchiolitis, and AOM (Kuitunen et al., 2020; Lin et al., 2020; Torretta et al., 2020; Alde et al., 2021; Angoulvant et al., 2021; Torretta et al., 2021). In addition, the number of children presenting to healthcare facilities for non-urgent complaints, such as AOM or otitis media with effusion (OME), was also affected by the fear of contracting COVID-19 at the hospital or the outpatient clinics. Thus, there was a decrease in healthcare utilization for pediatric AOM/OME.

Reports from Milan, the largest and the most densely populated city in Lombardia, the Northern Italian region placed at the epicenter of the Italian epidemic, where all elective medical activities were discontinued between March-May 2020, showed a substantial reduction in pediatric OM burden (Alde et al., 2021; Torretta et al., 2021). All outpatient periodic visits for otitis-prone children regularly followed at a tertiary outpatient clinic were replaced with telephone call contacts during the lockdown, and parents were asked to give a subjective assessment about the child's condition and describe ear-related complaints (ongoing AOM episodes, with/without tympanic membrane perforation, antibiotic treatments). The results of this survey were compared with the corresponding assessments reported 1 year prior. A statistically significant reduction in the mean number of AOM episodes and systemic antibiotic treatments during the COVID-19 first lockdown period compared with the previous year was reported, and 82% of parents had an impression of clinical improvement during the lockdown (Torretta et al., 2021).

Furthermore, a positive effect was reported on OME prevalence among Milanese children who attended an outpatient clinic during two pre-lockdown periods (May-June 2019 and January-February 2020) vs. the first post-lockdown period (May-June 2020) (Alde et al., 2021). It was found that OME prevalence dramatically decreased after the lockdown: 41%, 52%, and 2%, during the 1st, 2nd and 3rd periods, respectively. The resolution rate of OME was significantly higher in May-June 2020 when compared with the corresponding period of May-June 2019 (93% vs. 21%).

These findings were in line with results from a study conducted in *De Marchi*, Milan's largest PED, aiming to measure the children's flow, in terms of diagnosis related to any ear, nose, and throat (ENT) diseases during the lockdown (February-May 2020), compared with the corresponding previous period (February-May 2019) (Torretta et al., 2021). They found a substantial regional decrease in children's attendance to that PED; this effect was particularly noticeable when the analysis was restricted to ENT diagnoses (80.4% vs. 19.5%, February-May 2019 and February-May 2020, respectively; p-value < 0.001), including middle ear infections (92.8% vs. 7.2%). In addition, non-complicated AOM episodes more frequently occurred in February-May 2019 (92.0% vs. 8.0%), but no significant differences were found between the number of patients with complicated middle ear diseases (95.8% vs. 4.2%).

Table 2 summarizes current worldwide data on AOM burden reduction. A reduction in AOM burden was observed in many countries. In France, Angoulvant et al. (2021) conducted a time-series analysis for PED visits and related hospital admissions in the greater Paris area, from January 2017 to mid-April 2020. A global reduction in post-lockdown PED visits and hospital admissions was found (-68% and -45%, respectively), with a significant decrease (>70%) in the observed over expected rates of several infectious diseases, including AOM. These results are in line with the findings by Kuitunen et al. (2020), who evaluated the immediate effect of Finnish national lockdown on PED visits.

TABLE 2 | Reduction in otitis media burden during COVID-19 pandemic.

Country	Setting	COVID-19 Period	Comparative Period(s)	Variable	Rate Difference
Finland (Kuitunen, 2021)	Two hospitals	16/3/2020-12/4/2020	17/2/2020-15/3/2020	No. of AOM visits	-1%, -30%
USA (DeLaroche et al., 2021)	27 pediatric hospitals	15/3/2020-31/8/2020	15/3/2017-31/8/2017; 15/4/2018-31/8/2018; 15/4/2019-31/8/2019	Diseases of the ear and mastoid process	-68%
France (Angoulvant et al., 2021)	6 Paris area hospitals	18/3/2020-19/4/2020	1/1/2017-17/3/2020 (Time interrupted)	No. AOM referrals	-70%
Spain (Enrique et al., 2021)	Tertiary pediatric hospital	1/1/2020-30/6/2020	1/1/2010-31/12/2019 (Time interrupted)	No. of mastoiditis cases	+45%
Italy (Iannella et al., 2021)	Telephone/telemedicine contact with families of children with OM	1/2/2020-30/4/2020	1/2/2019-30/4/2019	No of AOM episodes, No. of otorrhea episodes, No. of ABx/month	-81% -97% -89%
USA (DeLaroche et al., 2021)	Tertiary and community health providers	1/1/2020-13/3/2020; 1/6/2020-13/12/2020 (post-lockdown)	1/1/2019-13/3/2019; 1/6/2019-13/12/2019	No. of tympanostomy tube procedures	-64%
Italy (Alde et al., 2021)	Tertiary pediatric hospital	1/5/2020-30/6/2020 (post-lockdown)	1/5/2019-30/6/2019; 1/1/2020-29/2/2020;	No. of children with OME, No. of children with type B tympanometry	-40% -95%
The Netherlands (van de Pol et al., 2021)	Tertiary hospital and clinics network	1/3/2020-31/5/2020	1/3/2019-31/5/2019	No. of AOM episodes	-10%
USA (Ramgopal et al., 2021)	37 Children's hospitals	1/1/20-31/12/20	1/1 to 31/12 for each year: 2010-2019	No. of AOM visits	-55%
Italy (Torretta et al., 2021)	Pediatric emergency Department	21/2/2020-4/5/2020	1/2/2019-21/2/2020	No. of AOM visits	-92%
Germany (Rohe et al., 2021)	146 ENT practice centers	Q2-Q3 2020	Q2-Q3 2019	No. of AOM visits	-43%
UK (Stansfield et al., 2021)	One large center	17/3/2020-17/6/2020	17/3/2019-17/3/2019	No. of emergency department OM visits	-86%
Italy (Torretta et al., 2020)	Telephone/telemedicine contact with families of children with OM	9/3/2020-19/5/2020	1/2/2020-30/4/2020	No. of AOM episodes	-90%
UK (Quraishi et al., 2021)	3 secondary care ENT departments	1/3/2020-28/2/2021	1/3/2019-29/2/2020	No. of AOM visits No. of acute mastoiditis cases	-26.9% -14.3%
The Netherlands (Hullegie et al., 2021)	Julius General Practitioners' Network	1/3/2020-28/2/2021	1/3/2019-29/2/2020	AOM, 0-2 years AOM, 2-6 years AOM, 6-12 years	-47.6% -33.7% -6.8%
Switzerland (Bucher et al., 2021)	Tertiary referral center	16/3/2020-26/4/2020	16/3/2019-26/4/2019	AOM, all ages	-79.5%

AOM, acute otitis media; MEF, middle ear fluid, ABx, antibiotics; OM, otitis media.

A major decrease in the daily rate of PED visits was reported, along with an overall decrease in the number of hospitalized patients for any respiratory disease during the lockdown, compared with the previous period. A study from 27 PED across the USA has demonstrated that visit numbers decreased by 45.7% (range: 36.1%-96.9%) during the COVID-19 pandemic in 2020 when compared with the same period during 2017-2019. The largest decrease was for respiratory disorder visits (DeLaroche et al., 2021). A sharp decline was observed for both AOM and URTI: 75.1% and 69.9%, respectively.

However, not all studies reported a downward trend in OM burden. A Spanish study showed that although there was no increase in the total ENT infections during January-April 2020 when compared with the same period in 2010-2019, there was a significant increase in complicated cases of mastoiditis (e.g., subperiosteal abscess, facial nerve paralysis, intracranial abscess). Although 54% of these patients were exposed to COVID-19 patients, only 15.4% had IgG antibodies (Enrique et al., 2021). The authors explained the increased complication rates might be explained due to lack of staff during the initial pandemic, and that public fear of getting infected may have prevented patients and parents from seeking medical treatment earlier.

Based on these reports, we speculate that vigorous lockdown measures also had a critical impact on the spread of many infectious diseases other than COVID-19 in children. Data are still accumulating.

REDUCTION IN ANTIBIOTICS PRESCRIPTION RATES FOR AOM

Data regarding antibiotics prescription rates for AOM during the COVID-19 pandemic are limited. The COVID-19 era provided a unique opportunity for healthcare providers to utilize treatment guidelines for common childhood infections, such as AOM. The "Choosing Wisely" campaigns, presented in >20 countries, provide statements regarding when to use antibiotics, to avoid unnecessary treatment of viral infections (Leis et al., 2020). New

toolkits, such as the "Cold Standard toolkit", published by the Canadian College of Family Physicians, as part of the "Choosing Wisely" campaign, provide information on how to use antibiotics in virtual care visits during the COVID-19 era (https://choosingwiselycanada.org/perspective/the-cold-standard/). This campaign encourages a conversation between clinicians and patients to avoid antibiotic overuse.

In general, a delayed prescription is accepted for AOM cases; the prescription is filled and purchased only if symptoms persist (Spurling et al., 2017). As described by Leis et al. (2020), infants >6 months with otalgia should be seen in person only when symptoms persist >48h, fever >39°C despite antipyretic medications, or in ill-looking children. Otherwise, a virtual visit can take place, and the child can be treated with oral pain analgesics, according to the Canadian Pediatric Society guidelines on AOM management (Le Saux et al., 2016). However, virtual visits are limited in their diagnostic accuracy, and therefore may eventually lead to over-prescription of antibiotics (Leis et al., 2020).

A Scottish study examined the pandemic's impact on outpatient antibiotics prescriptions rates, in comparison with the preceding year. It was shown that initially, there was a sharp increase in the numbers of prescriptions used for respiratory infections, followed by a decrease for all age groups, which was more pronounced in children aged 0-4 years. They suggested that the initial peak was due to "just-in-case" prescriptions. The decrease may be due to fewer URTI episodes and increased self-care practice and a reduced number of patients with bacterial infections presenting to their general practitioners (GPs) (Malcolm et al., 2020).

A Dutch study compared the number of GPs' consultations and antibiotic prescription rates during March-May 2019, with the same period in 2020. The number of respiratory/ear infections decreased during the pandemic period from 16,672 to 15,580 and the antibiotics prescription rate decreased from 21% to 13%, respectively, without an increase in acute mastoiditis cases (van de Pol et al., 2021).

A web-based survey among 169 Israeli pediatricians circulated during the pandemic aimed to evaluate the frequency of telemedicine use, as well as its influence on decision-making in clinical scenarios, such as AOM. They reported an increase in daily use of text messages, pictures, and videoconference from 24%, 15%, and 1% before the pandemic to 40%, 40%, and 12% during the lockdown, respectively. Interestingly, there was a high likelihood of prescribing antibiotics for suspected AOM *via* telemedicine contacts (Grossman et al., 2020).

OTOLOGIC SURGERY

Changes in Concept

COVID-19 emergence required otologists to adopt a changed mindset for otologic office procedures and ear surgery. Otologic surgery, including drilling of the mastoid, is known to cause a substantial dispersion of small and large aerosols (Anschuetz et al., 2021; Chari et al., 2021; Hajiyev and Vilela, 2021; Merven and Loock, 2021; Sharma et al., 2021) and droplets (Sharma et al., 2020; Mohan et al., 2021; Sharma et al., 2021), and is a cause of concern regarding contamination in the operating room (OR). To prevent unnecessary risk of infection, otologic procedures were categorized as urgent versus elective, according to different authors and otolaryngological societies (Kozin et al., 2020; Leboulanger et al., 2020; Pattisapu et al., 2020; Saadi et al., 2020), suggesting that urgent operations should be performed, while all other operations should be postponed, depending on the pathology and the patient's preference.

To overcome the obstacle of personal contamination through patient care, several methods have been studied/suggested (Cottrell et al., 2020; Ally et al., 2021; Chari et al., 2021): personal protective equipment (PPE) for clinics and the ORs, a shift to telehealth communication, environmental protection (microscope draping, tents), modification of surgical settings and alteration of surgical techniques (actively preferring endoscopic approaches, use of exoscopes, use of enhanced smoke/suction devices, povidone-iodine irrigation of mastoid). SARS-CoV-2 testing is pre-operatively advised, and if the test is negative, additional precautions may not be necessary. When SARS-CoV-2 testing is not possible, or when testing positive, extra care should be taken as listed below.

Personal Protective Equipment

PPE can be divided into two categories: 1) respiratory protection (N95 respirator, powered air-purifying respirator [PAPR]), and 2) body protection, including eye protection, sterile and waterproof clothes around the neck, and disposable cap, gown, overshoes, and gloves. PPE is advised for any surgery performed, and especially for procedures with a high aerosol dispersion potential, such as mastoidectomy (Ayache and Schmerber, 2020; Gordon et al., 2020; Kozin et al., 2020; Leboulanger et al., 2020; Sharma et al., 2021).

Surgical Setting

It was advised that the minimal number of staff enter the OR, and only the most skilled surgeon operating (Kozin et al., 2020). It is advised that for adults, VTI should be performed under local anesthesia. For pediatric cases, bag ventilation is not advised, and all procedures should be performed with endotracheal intubation. Operating rooms with negative pressure ventilation should be used as with a designed filtration system (Leboulanger et al., 2020).

Surgical Technique

Regarding surgical techniques, the endoscopic approach is preferred, if possible (Ayache et al., 2021). When a mastoidectomy is unavoidable, it should be performed without drilling, if possible, and by using instruments such as a hammer, gouge, and/or curette, together with a continuous high-powered suction that should be placed next to the surgical field. The use of monopolar cautery and laser may result in an increased risk of viral dissemination, and thus should be avoided (Ayache and Schmerber, 2020; Kozin et al., 2020; Lavinsky et al., 2020; Leboulanger et al., 2020; Saadi et al., 2020). Exoscopes should be

chosen in place of microscopes, as exoscopes place the surgeon and staff a more secure distance from the surgical field (Gordon et al., 2020; Kozin et al., 2020; Ally et al., 2021; Ridge et al., 2021; Tu et al., 2021).

Environmental Protection

Various methods of surgical field isolation for better staff protection have been proposed (Cottrell et al., 2020; Panda et al., 2020; Chari et al., 2021; Hajiyev and Vilela, 2021). **Table 3** describes the proposed materials. All these methods aim to isolate the surgical field to prevent aerosol and particle dispersion. Environmental protection uses commonly available surgical drapes and other equipment traditionally present in the OR. It is important to note that most of the proposed methods are prototypes (McCarty et al., 2021), and have not yet been rigorously proven.

Changes in Ventilating Tube Surgery

Mohan et al. examined the risk of aerosol-generating procedures, such as suctioning of MEF during ventilating tube insertion (VTI), by performing a cadaveric simulation of bedside myringotomy and fluorescein-labeled fluid injection into the middle ear to examine the potential risk (Mohan et al., 2021). Image analysis showed no fluid in the proximal external auditory canal nor the ear speculum following the procedure and suctioning. Unlike first speculations, there was no measured increase in aerosol particle numbers during VTI (Campiti et al., 2021).

Another report from Milan addressed the clinical activities pertinent to pediatric OM and modifications of surgical waiting lists during the COVID-19 pandemic, with patient selection based upon the priority of certain conditions, as defined by the Italian Society of Otorhinolaryngology-Head and Neck Surgery (Torretta et al., 2020). Priority for VTI surgery was granted to candidates with persistent OME, causing a negative impact on language development. During the pandemic, VTI rates significantly decreased among Bostonian children: the age of patients undergoing surgery increased, and more children were sent for surgery in a tertiary setting (Diercks and Cohen, 2021). It was also reported that in young Floridian children, the prevalence of intraoperative OME during the COVID-19 pandemic was significantly lower compared with pre-COVID-19 as assessed during VTI surgery (65% vs. 83%, $p < 0.001$) (Nguyen et al., 2021).

TELEMEDICINE

Telemedicine is the branch of telehealth that connects patients-to-providers and providers-to-providers, for the delivery of healthcare at a distance. Although this development offered greater healthcare access, telemedicine is limited in accuracy and general acceptance by a lack of physical examination and real-time intervention, remaining as a service for special circumstances, or as an adjunct to in-office and in-hospital visits. Telemedicine has been well-received as a modality for patient visits. Caregivers and care providers alike report high satisfaction in both the convenience and the care provided by the service (McIntosh et al., 2014).

The COVID-19 pandemic significantly expanded the usage of telemedicine. The call for strict socially distancing, and patients' fears of any medical environment, meant that any means for assessment was preferable to the risk of contracting the virus. The result has been more widespread use of newly developing

TABLE 3 | Draping techniques during otologic surgery.

Author	Drape "tent" (Other than Otological Surgical Drape/4K 3D Exoscope Drape)	Accessories	A place for Surgical Assistant and Instrument Table	Ease of Construction (Yes/No)	Cost	Name of Installation
Panda et al. (2020)	Steri-Drape (3M)	Gottingen laser support table	+	Y	+	
Cottrell et al. (2020)	C-arm draping	–	–	N	++	
(Hajiyev and Vilela, 2021)	Modified chair drape	–	–	Y	+	
Chari et al. (2021)	Steri-Drape (3M)	–	–	Y	+	Ototent 1
Chari et al. (2021)	Modified Zeiss OPMI microscope drape (Carl Zeiss, Meditec AG, Germany)	–	–	Y	++	Ototent 2
Tolisano et al. (2020)	Modified Zeiss OPMI microscope drape (Carl Zeiss, Meditec AG, Germany)	PVC pipes as a specialized frame, Sterile bags	–	N	++	**Covid-19 Airway Management Isolation Chamber with otologic modification (CAMIC-Ear)**
Ally et al. (2021)	A plastic sterile drape (3M Steri-Drape 1015)	–	–	Y	+	
Lawrence et al. (2020)	*CE-marked* sterile polyethylene drape with a custom-made hole over the lens cap	'L' support	–	Y	+	

+, not expensive; ++, expensive.

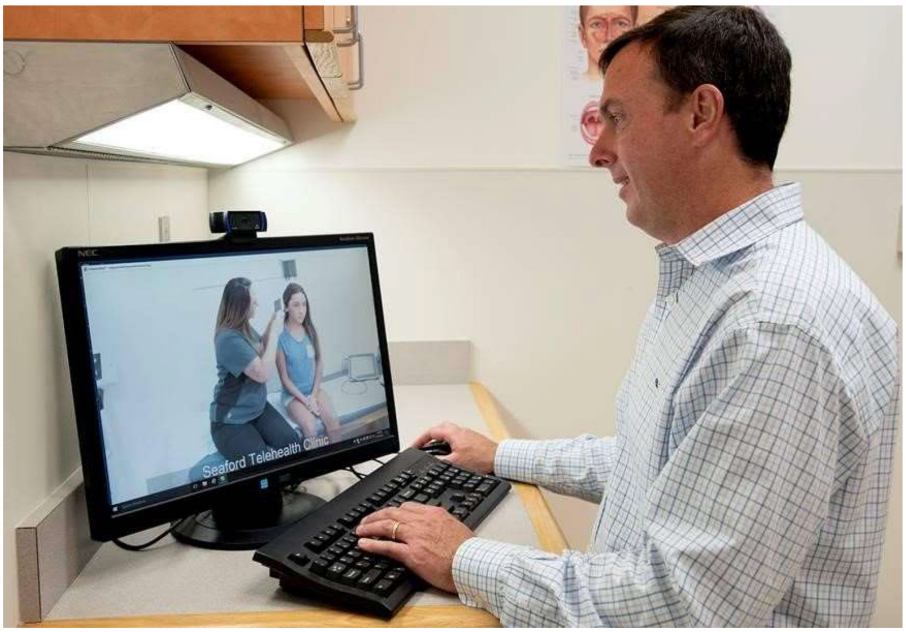

FIGURE 1 | Medical assistant telemedicine in use. A physician provider to the far right is watching the medical assistant at the far left on the screen using a device to examine the ear of a patient. The device will relay images of the patient's ear exam to the physician (Photo used with permission of A. Saporito. Image courtesy of Dr. Patrick Barth).

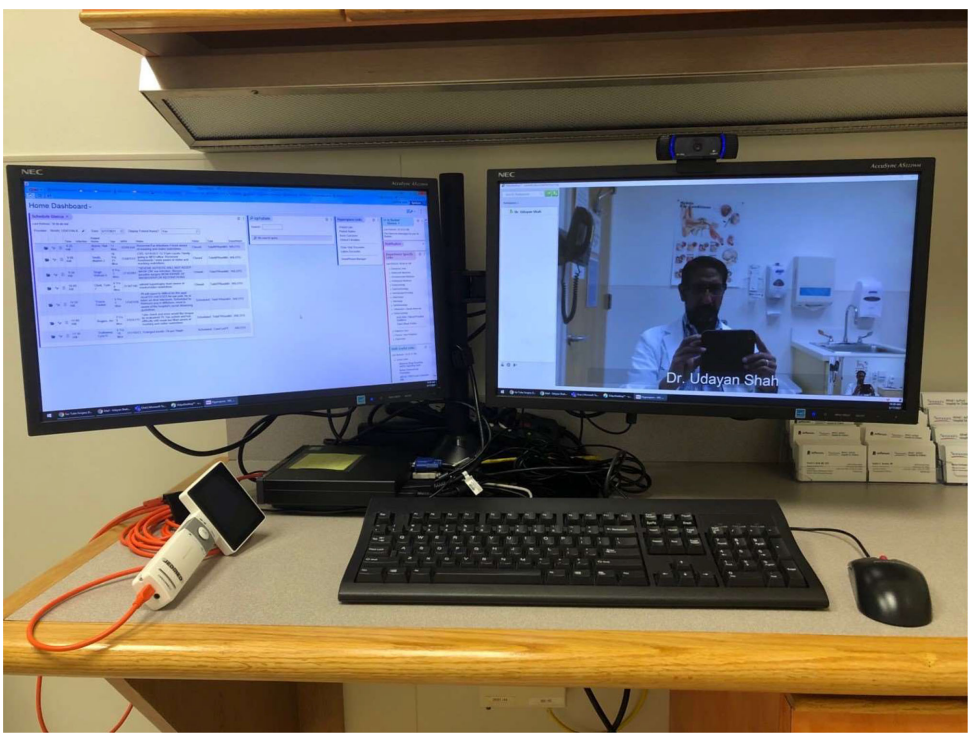

FIGURE 2 | Possible telemedicine setup. A two-screen setup allows the medical record to be seen on the left, while the examination can be seen on the right. A white hand-held video-otoscope is seen to the left of the keyboard (Image courtesy of Dr. Udayan Shah).

technologies, permitting triage and assessment of patients remotely. For OM, evaluation of the tympanic membrane and the middle ear by a tele-provider is conducted in one of two ways: video microscopy or video otoscopy, the latter of which also often permits pneumatic otoscopy. The exam is carried out by the patient's family or a telemedicine facilitator, and findings may be transmitted to a provider in real-time, or recorded and delivered for interval review (Metcalfe et al., 2021).

Types of Telemedicine

1. Home-based telemedicine (HBT) starts with a smartphone. Instrumentation in the home setting may be owned by the patient or may be sent to the family, in anticipation of a telemedicine visit. A hybrid model for telemedicine care, blending telemedicine with an in-person office visit, can be offered when further needs arise for better information or from the opportunity for a different interaction with the provider.
2. Facility-based telemedicine (FBT) requires that the patient be brought to a clinical setting with visualization technology available for use. Information is sent to a physician or advanced-practice provider located at a distance.

Two temporal modes of telemedicine exist:

1. *Synchronous telemedicine* involves real-time telephone or audiovisual interaction *via* smartphone, tablet, or computer with patients and families. This may occur in the patient's own home in a direct-to-consumer model or an external facility.
2. *Asynchronous telemedicine* includes "store and forward" technology in which messages, images, and data are securely sent and subsequently reviewed and responded to within a certain time interval. The utilization of patient portals is often utilized.

Telemedicine for AOM

Because AOM is the most common reason for pediatric outpatient visits, HBT can offer earlier, more accurate diagnoses for primary care providers, meaning more judicious

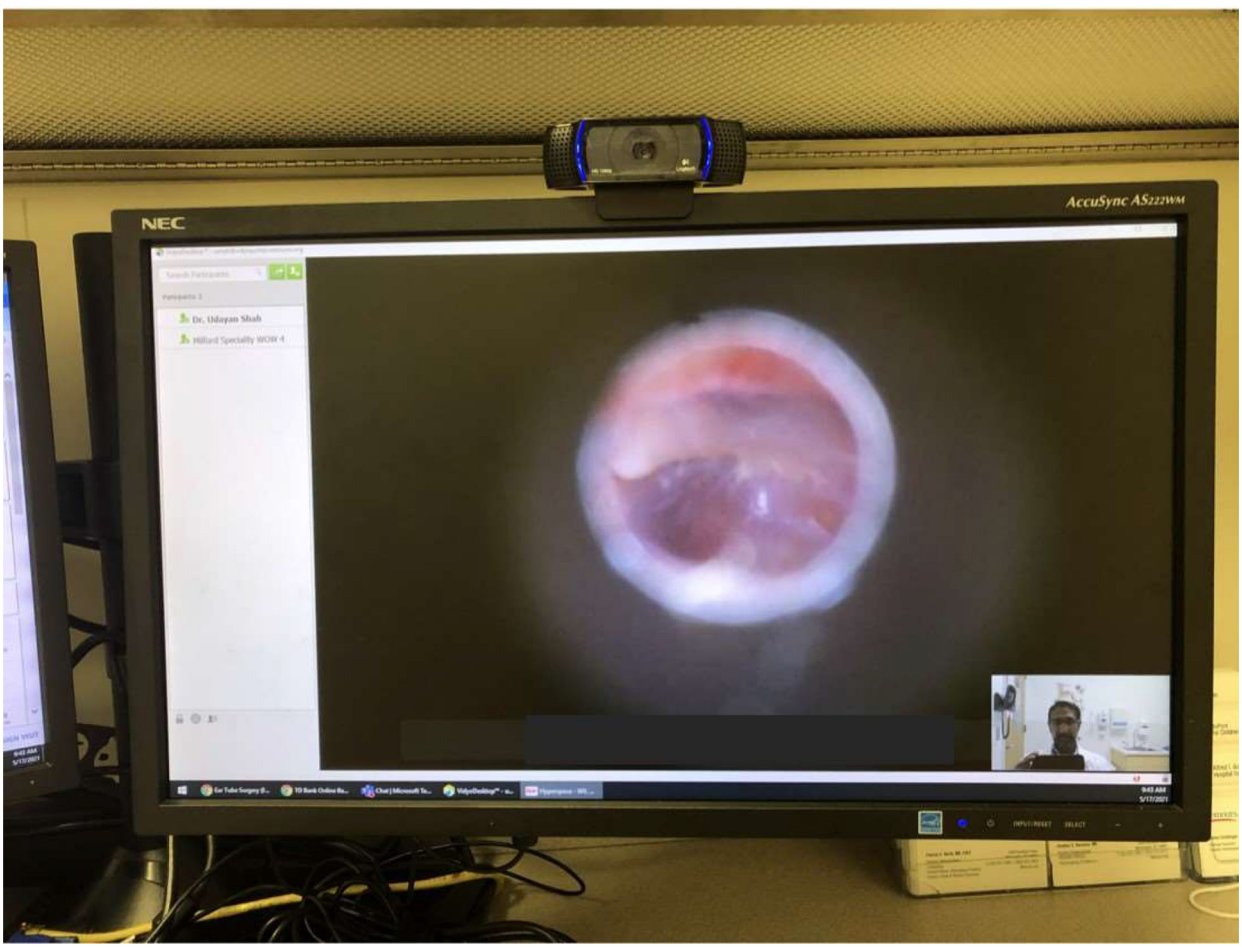

FIGURE 3 | Otoscopy *via* telemedicine. The camera is at top of the monitor.

antibiotic usage, and better anticipation of impending complications. Telemedicine in AOM is equivalent to office-based visits regarding recommendations for surgery, additional testing, or routine follow-up (Kolb et al., 2021). The challenge of patient assessment in the setting of potential OM is in direct visualization of middle ear structures. Another important factor for this process is the attainment of satisfactory image quality. Regulatory restrictions are an additional limitation at present. Licensure, and credentialing providers, limit the extent of care that can be provided. In some organizations, the provider is a trained medical assistant. Because any healthcare provider can be trained, this approach to a hybrid model of telemedicine and in-person care is termed "Provider Assisted Telemedicine" (PAT). Additionally, tympanometry can be performed when performing FBT for AOM: the delivering provider obtains a tympanogram which is then messaged to the remote, directing provider (**Figures 1**–**3**).

Interestingly, studies on telemedicine and antibiotic prescription before the COVID-19 era have shown that children seen by telemedicine visits received less diagnostic testing and would not seek antibiotic treatment if they have received a clear explanation on diagnosis, safety, treatment plan, and reassurance (Mangione-Smith et al., 1999).

Telemedicine was found to help guide decision-making regarding medical and surgical management for AOM, though a consensus in the literature on the benefits of telemedicine in pediatrics for triage and assessment of otitis media has not yet been established. Telemedicine continues today to demonstrate its utility during the COVID-19 pandemic.

CONCLUSION

The paucity of reports of living COVID-19 patients with AOM, particularly for children, can be explained by the variability in clinical severity and viral load (with more severe clinical course portending a higher viral titer), very low incidence of myringotomies to aspirate MEF, low levels of passive reflux from the nasopharynx, a low predilection for SARS-CoV-2 to invade the middle ear mucosa and inadequate detection technique. In addition, hospital admission reports and insurance-claims data showed a substantial decrease in OM visit rates and even related complications during the COVID-19 pandemic periods, compared with previous years. Otologic surgeons identified several methods to minimize the spread of the COVID-19 virus. These methods include PPE, environmental protection, and alteration of surgical methods and techniques. The combination of these methods may best protect the surgeon and the staff against this ever-changing disease.

Future research should focus on OM burden following the introduction of SARS-2-CoV vaccines (currently approved only for children >12 years), relaxation of lockdown measures, and re-emergence of viral URTIs other than SARS-CoV-2, and appearance of new SARS-CoV-2 variants.

AUTHOR CONTRIBUTIONS

TM, JP, and STa contributed to conception and design of the study. TM and STa identified and assigned areas of review. TM, JP, and STa wrote the first draft of the manuscript. US, STa, STo, PM, AK, and PB wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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The *FUT2* Variant c.461G>A (p.Trp154*) Is Associated With Differentially Expressed Genes and Nasopharyngeal Microbiota Shifts in Patients With Otitis Media

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Otitis media (OM) is a leading cause of childhood hearing loss. Variants in *FUT2*, which encodes alpha-(1,2)-fucosyltransferase, were identified to increase susceptibility to OM, potentially through shifts in the middle ear (ME) or nasopharyngeal (NP) microbiotas as mediated by transcriptional changes. Greater knowledge of differences in relative abundance of otopathogens in carriers of pathogenic variants can help determine risk for OM in patients. In order to determine the downstream effects of *FUT2* variation, we examined gene expression in relation to carriage of a common pathogenic *FUT2* c.461G>A (p.Trp154*) variant using RNA-sequence data from saliva samples from 28 patients with OM. Differential gene expression was also examined in bulk mRNA and single-cell RNA-sequence data from wildtype mouse ME mucosa after inoculation with non-typeable *Haemophilus influenzae* (NTHi). In addition, microbiotas were profiled from ME and NP samples of 65 OM patients using 16S rRNA gene sequencing. In human carriers of the *FUT2* variant, *FN1, KMT2D, MUC16* and *NBPF20* were downregulated while *MTAP* was upregulated. Post-infectious expression in the mouse ME recapitulated these transcriptional differences, with the exception of *Fn1* upregulation after NTHi-inoculation. In the NP, Candidate Division TM7 was associated with wildtype genotype (FDR-adj-p=0.009). Overall, the *FUT2* c.461G>A variant was associated with transcriptional changes in processes related to response to infection and with

increased load of potential otopathogens in the ME and decreased commensals in the NP. These findings provide increased understanding of how *FUT2* variants influence gene transcription and the mucosal microbiota, and thus contribute to the pathology of OM.

Keywords: *FUT2*, microbiota, otitis media, p.Trp154*, RNA-sequencing, rs601338

INTRODUCTION

Infection and inflammation of the middle ear (ME), known as otitis media (OM), is the most frequently diagnosed disease in infants and young children in the United States and is globally a leading cause of hearing loss (Monasta et al., 2012; GBD, 2021). In children, an estimated 60% of hearing loss is due to preventable causes, and infections and chronic OM account for around 31% of pediatric hearing loss (Schilder et al., 2016; GBD, 2021). In the United States, treatment of OM costs over $5 billion annually and typically includes antibiotics and surgery such as tympanostomy tube insertion (Schilder et al., 2016; Suaya et al., 2018). OM risk and pathology are influenced by many factors including environmental factors such as age, sex, daycare attendance and breastfeeding as well as genetic factors (Zhang et al., 2014; Brennan-Jones et al., 2015). Heritability of OM is estimated to be as high as 74%; furthermore, genes related to OM predisposition are known to function in pathways that include innate immune response, cell-mediated immune dysfunction and pathogen-host-environment interactions (Casselbrant et al., 1999; Mittal et al., 2014).

OM is often bacterial or viral in origin, wherein pathogens in the nasopharynx (NP) migrate *via* the Eustachian tube to the ME. This creates an inflammatory cycle in the ME with an accumulation of mucus and fluid which can lead to permanent damage and hearing loss (Rosenfeld et al., 2013). It is important to note that prior to infection, the ME is essentially sterile as it is generally separated from the external environment by the tympanic membrane, whereas the NP has an established microbiota that can vary based on microbial exposure and host genetics, but these microbes in the NP do not become resident in the ME if the Eustachian tube is functioning well (Jervis-Bardy et al., 2019). Some NP commensals are potential opportunistic otopathogens of the ME (Yatsyshina et al., 2016). It is well-known that increased abundance of potential otopathogens in the NP is associated with higher risk for OM (Jervis-Bardy et al., 2017; Browne et al., 2021; Xu et al., 2021).

FUT2 (MIM 182100) encodes alpha-(1,2)-fucosyltransferase which is responsible for secretion and expression of ABO(H) antigens on mucosal epithelia (Kelly et al., 1995). Secretory status directly influences pathogen binding in mucosal epithelia in multiple organ systems. The *FUT2* stop variant c.461G>A (p.Trp154*; rs601338) has been associated with multiple mucosal phenotypes and is in strong linkage disequilibrium (LD) with a synonymous *FUT2* variant rs681343 that was previously associated with childhood ear infections in genome-wide association studies (GWAS) (Pickrell et al., 2016; Tian et al., 2017). This variant has also been confirmed to confer familial OM risk in multiple cohorts (Santos-Cortez et al., 2018). Additionally, *Fut2* expression transiently increased in the mouse ME after infection with non-typeable *Haemophilus influenzae* (NTHi), which is a common otopathogen in humans (Santos-Cortez et al., 2018).

Non-secretors, i.e., homozygous for *FUT2* c.461G>A, show higher rates of bacterial infections [e.g. with *Streptococcus pneumoniae*, NTHi in different organ systems], but decreased susceptibility to viral infection (i.e. viral diarrhea or HIV-1), possibly due to the effects of the glycan on the mucus barrier (Magalhaes et al., 2016; Azad et al., 2018; Santos-Cortez et al., 2018). Though *FUT2* is well-studied, to our knowledge there are no previous studies of transcriptome-wide differences in host gene expression based on carriage of the *FUT2* c.461G>A variant in humans. Furthermore, to date only seven studies investigated changes in the host microbiota that were associated with carriage of this variant (Rausch et al., 2011; Wacklin et al., 2011; Wacklin et al., 2014; Kumar et al., 2015; Davenport et al., 2016; Kumbhare et al., 2017; Turpin et al., 2018; Chen et al., 2021). These studies were limited to assessment of the gut microbiota according to variant carriage and identified associations seemed to be environment- or disease- specific. While some studies observed no associations between gut microbiome and *FUT2* c.461G>A genotype, others noted that *Bifidobacterium* levels, among other taxa, were significantly different between variant carriers and wildtype (Wacklin et al., 2011; Wacklin et al., 2014; Davenport et al., 2016; Turpin et al., 2018). Furthermore, in Crohn's Disease and throughout pregnancy, the *FUT2* c.461G>A variant was associated with differences in the gut microbiota diversity and abundance of individual taxa (Rausch et al., 2011; Kumar et al., 2015).

In order to further elucidate the role of *FUT2* in OM pathogenesis, the goal of this study was to investigate the potential downstream effects of the *FUT2* c.461G>A (p.Trp154*) variant on gene expression and site-specific colonization by commensals and known otopathogens. Characterization of this common variant and its role in the interplay between host genetics, host immune response, and mucosal microbiotas not only expands our general understanding of these complex relationships but also, within the context of OM, provides clinically relevant insight that can be used to better determine individual risk and inform treatment. In this study, we performed differential expression (DE) analysis on RNA-sequence data from saliva of OM-affected individuals and identified multiple differentially expressed genes based on carriage of the *FUT2* c.461G>A variant. These DE genes were replicated using genome-wide expression data from infected mouse ME. We also performed microbiota analysis using 16S rRNA sequence data from ME and NP samples of OM-affected individuals and identified bacterial taxa that were different in relative abundance according to genotype.

MATERIALS AND METHODS

Ethics Approval

Ethical approval was obtained from the COMIRB prior to the start of the study. Informed consent was obtained from study participants, including parents of children enrolled in the study. The IACUC of the Veterans Affairs Medical Center, San Diego, California granted approval for mouse studies.

Subject Ascertainment and Sample Collection

Clinical data were obtained from 91 pediatric patients undergoing surgery for OM, with information on age, sex, self-reported ethnicity, family history, breastfeeding history, history of exposure to smoking, OM diagnoses and surgical technique (**Table 1**). We also had clinical information and samples from 15 adult patients with OM, but these samples were removed from further analyses because of marked differences in expression and microbiota profiles due to age (**Figure 1**). DNA samples were collected from the 91 pediatric patients with OM using the Oragene-DNA OGR-500 or OGR-575 kits (DNA Genotek, Ottawa, Ontario, Canada).

Saliva samples were also collected from pediatric patients with OM using Oragene-RNA RE-100 kits and sufficient RNA was isolated from 30 samples using the manufacturer's protocol (**Figure 1**). A total of 296 microbial samples were obtained from the ME (n=171) and NP (n=125) of 86 individuals, including 74 ME swabs, 86 ME aspirates, and seven ME mucosal tissue samples. Four ME cholesteatoma/granuloma tissue samples and 125 NP swabs were also collected (**Figure 1**). Microbial DNA was isolated from 217 (73%) samples using the Epicentre Masterpure Complete DNA Purification Kit (Lucigen, Middleton, WI, USA); the rest of the samples from which no microbial DNA was isolated were excluded from further study (**Figure 1**).

Human DNA Sequencing for *FUT2* and *RASIP1* Variants

A variant in *RASIP1* c.1801C>T (p.Arg601Cys; rs2287922) is in moderate LD with the *FUT2* variant c.461G>A ($r^2 = 0.82$) and with the rs681343 variant ($r^2 = 0.65$) that was associated with childhood ear infections (Pickrell et al., 2016; Buniello et al., 2019). Sanger sequencing was performed for the *FUT2* NM_000511.6:c.461G>A and *RASIP1* NM_017805.3: c.1801C>T variants using DNA from saliva samples of pediatric patients with OM. Both variants were in Hardy-Weinberg equilibrium within the entire cohort and in each cohort used for RNA-seq and microbiota analyses (**Table 1**).

Human RNA-Sequencing and Analysis

Thirty salivary RNA samples (median RIN=7.1) were submitted for RNA-sequencing at the University of Colorado Denver Genomics and Microarray Core, as previously described (Larson et al., 2019). In summary, RNA samples were processed using the Nugen Trio RNA-Seq Kit (Tecan, Redwood City, CA, USA). Sequencing was performed on an Illumina HiSeq 4000 with an average of 31 million reads per

TABLE 1 | Characteristics of OM patients by dataset.

Cohort characteristics[a]	Entire pediatric cohort (n=91)	Microbiota (n=65)	RNA-seq (n=28)
Sample type	Saliva, middle ear swab/aspirate/mucosa, nasopharynx swab	Middle ear swab/aspirate/mucosa, nasopharynx swab	Saliva
Median age (years)	2.0	2.0	2.3
% Female	33.0%	32.3%	17.9%
% Self-reported ethnicity	74.7% White, 11.0% Hispanic, 1.1% Asian, 12.1% other or mixed	80.0% White, 9.2% Hispanic, 1.5% Asian, 9.3% other or mixed	85.7% White, 10.7% Hispanic, 3.6% Asian
FUT2 c.461G>A genotype	25.6% GG, 48.8% GA, 25.6% AA	21.5% GG, 52.3% GA, 26.2% AA	18.5% GG, 51.9% GA, 29.6% AA
Otitis media type			
- % Recurrent/acute[b]	74.7%	72.3%	78.6%
- % Chronic/effusive[b]	16.5%	12.3%	7.1%
- % Both/either	8.8%	15.4%	14.4%
Otitis media surgery			
- % Ventilation tubes	91.2%	93.8%	85.7%
- % Tympanoplasty	8.8%	6.2%	14.3%
% Breastfed	89.0%	89.2%	82.1%
% Smoking Exposure	13.2%	13.8%	25.0%
% (+) Family history	63.7%	49.2%	42.9%

[a]*Statistical tests for effect of FUT2 variant on distribution in overall cohort—*
Sex: Chi-squared test of independence p= 0.45.
Age: Wilcoxon Rank Sum p=0.02.
Ethnicity: Chi-squared test of independence (White vs non-White) p=0.63.
Hardy Weinberg Equilibrium p=0.94.
[b]*Recurrent/acute OM (RAOM), defined as ≥3 OM episodes in 6 months or ≥4 OM episodes in 12 months; Chronic/effusive OM (COME), defined as ME effusion persisting for ≥2 months (Rosenfeld et al., 2016).*

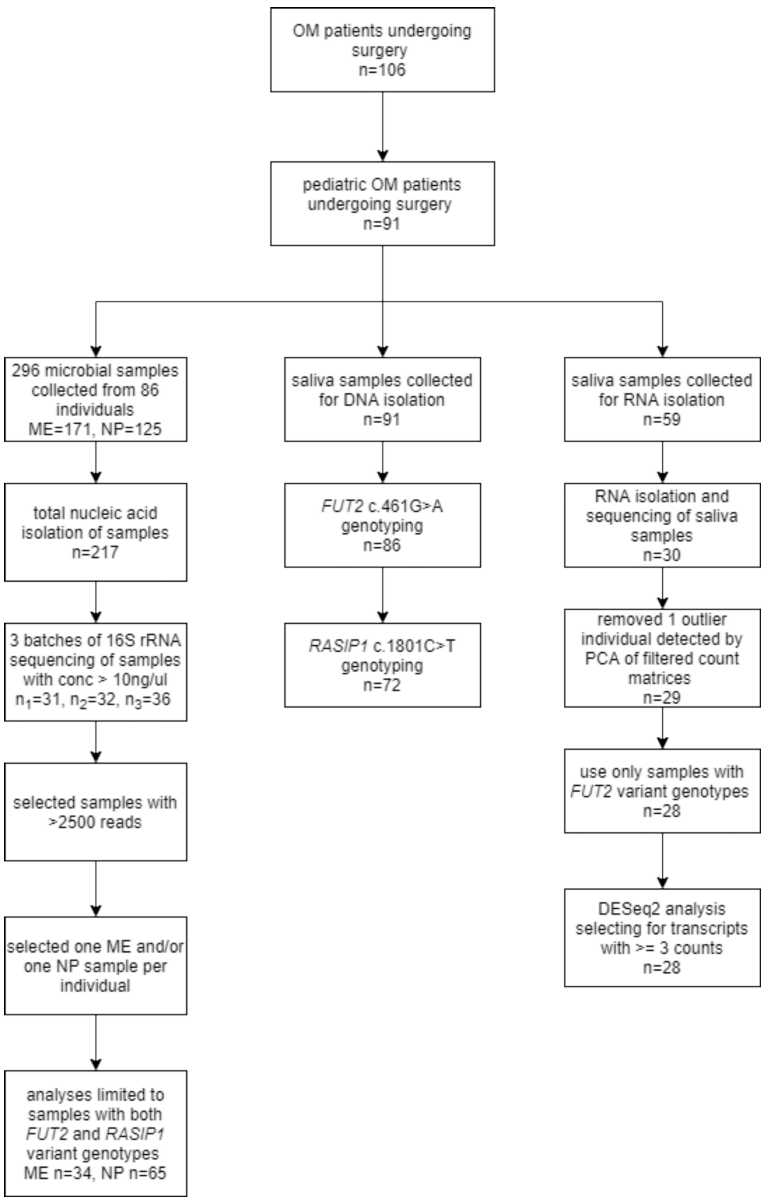

FIGURE 1 | Study flowchart. The flowchart shows the number of saliva and microbial samples included for genotyping, RNA-seq and microbiota analyses.

sample. Reads were trimmed using the FASTX-Toolkit v0.0.13 and aligned using STAR v2.5.3a (Dobin et al., 2013). Principal components analysis (PCA) was performed on this dataset and one outlier sample was removed from further analyses due to not clustering with other samples (**Supplementary Figure 1**). Transcript counts were summarized at the gene level and analyses included genes with an average read count >3. DE analysis was performed on 28 samples (**Figure 1**) according to carriage of the *FUT2* c.461G>A variant using the DESeq2 package in R (Love et al., 2014), with correction for age, sex and batch effects (**Supplementary Figure 1**). Results were considered significant for genes with $\log_2$-transformed fold change > ± 2 and false discovery rate (FDR)-adjusted p-value <0.05 using the Benjamini-Hochberg method.

Network and Pathway Analysis

FUT2, *RASIP1* and DE genes were used as input in NetworkAnalyst for construction of a protein-protein interaction network using the IMEx interactome database (Xia et al., 2014; Xia et al., 2015). Pathway enrichment analysis was performed on the resulting network using the KEGG and PANTHER GO-slim BP databases in NetworkAnalyst (Kanehisa and Goto, 2000; Kanehisa, 2019; Mi et al., 2019; Kanehisa et al., 2021). Pathways with an FDR-adjusted p<0.05 were deemed significantly enriched.

16S rRNA Sequencing and Microbiota Analysis

A total of 171 ME and 125 NP samples were obtained from 86 Coloradan pediatric patients with OM and submitted for 16S rRNA sequencing. Microbial DNA isolation was performed using the Epicentre MasterPure™ Kit. In order to test for contaminating bacterial DNA in reagents or plastics, every batch of samples that was submitted for 16S rRNA gene PCR and sequencing included ≥3 negative process controls. Bacterial profiles were determined by broad-range PCR amplification and sequence analysis of the 16S rRNA gene V1V2 regions, as previously described (Santos-Cortez et al., 2018; Frank et al., 2021). Illumina paired-end sequencing was performed on MiSeq using the 600 cycle version 3 kit. Assembled and quality-filtered sequences were aligned and classified with SINA (1.3.0-r23838) using the 418,497 bacterial sequences in Silva 115NR99 (Pruesse et al., 2012; Quast et al., 2013). Operational taxonomic units (OTUs) were produced by clustering sequences with identical taxonomic assignments (median: 115,176 sequences/sample; interquartile range: 46,274.5 – 170,300.0). Goods coverage scores were ≥99.7% for all samples, indicating adequate depth of sequence coverage for all samples. Of the 296 microbial samples submitted for sequencing, 79 did not pass quality control (DNA concentration ≥10 ng/ul; 2500 reads after sequencing; **Figure 1**). Because it was not possible to determine whether the lack of microbial DNA is due to a relatively sterile ME or from a sample collection issue, these 79 samples were excluded. Bacterial alpha-diversity indices (richness, diversity, and evenness; Robertson et al., 2013) were tested for association with carriage of each of the *FUT2* c.461G>A or *RASIP1* c.1801C>T variants independently *via* Wilcoxon test and adjusted for ethnicity (Robertson et al., 2013). Associations of individual OTUs with *FUT2* c.461G>A and *RASIP1* c.1801C>T variants were assessed using linear regression with sample batch as a covariate. To minimize multiple-comparisons, only taxa with a prevalence >10% and relative abundance >1% were included in the analysis. Beta-diversity was determined *via* PERMANOVA using the Morisita-Horn dissimilarity index and adjusted for age, sex and batch effects. R software was used for data analyses and figure generation.

Gene Expression in Infected Murine Middle Ear

All animal experiments were performed according to the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and carried out in strict accordance with an approved Institutional Animal Care and Use Committee (IACUC) protocol (A13-022) of the Veteran Affairs Medical Center (San Diego, CA). All animal experiments employed the best efforts for minimizing animal suffering under general anesthesia according to the NIH guidelines.

For gene array studies, wild-type (WT) C57/WB F1 hybrid mice were purchased from the Jackson Laboratory (Bar Harbor, ME USA). NTHi strain 3655 (non-typeable, biotype II, originally isolated from the ME of a child with OM in St Louis, MO USA) was cultured in defined liquid media (Coleman et al., 2003). To induce ME infection, mice were deeply anesthetized with an intraperitoneal injection of rodent cocktail (13.3 mg/ml ketamine hydrochloride, 1.3 mg/ml xylazine, 0.25 mg/ml acepromazine; at 0.1-0.2 ml per 25-30 g body weight of the mouse). The bullae were bilaterally exposed through soft tissue dissection *via* a ventral approach. A hole was made in the bulla with a 23 gauge needle, allowing approximately 5 µl of NTHi inoculum (~$5x10^4$ CFU/mL) to be injected using a Hamilton syringe with a 30-gauge needle. After the injection of NTHi inoculum, the tympanic membranes were visually inspected and confirmed to be intact. The incision was then stapled and the mice were given normal saline and analgesics (buprenorphine at 0.05mg/Kg) subcutaneously while recovering on the heated mat. Following recovery from anesthesia the mice appeared healthy, with a clinical activity index ≤ 3 throughout the duration of OM.

Gene array data were generated as previously described (Hernandez et al., 2015). In summary, forty mice per time point were inoculated bilaterally with NTHi. Mucosal tissue and exudate were harvested from 20 mice at each of the following intervals – 0 hours (0h, no treatment), 3h, 6h, 1 day (1d), 2d, 3d, 5d and 7d after inoculation – then pooled. The tissue was homogenized in TRIzol (Life Technologies, Carlsbad, CA) and total RNA extracted, reverse transcribed and re-transcribed *in vitro* to generate biotinylated cRNA probes that were hybridized to 2 Affymetrix MU430 2.0 microarrays. Hybridization intensity data were median-normalized and differences in gene transcript expression levels evaluated using variance-modeled posterior inference (VAMPIRE) (Hsiao et al., 2005). Bonferroni multiple testing correction (αBonf < 0.05) was applied to identify only those genes with the most robust changes. The data were duplicated at each time point to obtain a second, independent biological replicate. Thus each data point represents 2 separate samples consisting of 20 mice each, and 4 Affymetrix arrays. A total of 3,605 genes, approximately 14.4% of the mouse genome, defined the signature of acute, NTHi-induced OM across time. Hybridization of RNA to specific gene probes was assessed at individual time points by comparison to uninfected MEs, after Bonferroni correction for multiple tests, using Genespring GX 7.3 (Agilent Technologies, Santa Clara, CA).

For single-cell RNASeq, the same ME inoculation protocol was followed, except that C57BL/6J mice (Jackson Labs) were employed. Single-cell samples for RNA-sequencing were generated from the entire contents of the mouse ME (Ryan et al., 2020). For each of three independent samples, tissue was harvested from both ears of six young adult C57-BL6 mice 6 hours after inoculation of the ME with NTHi. Single-cell libraries were generated using the 10X Genomics (Pleasanton, CA, USA) Chromium Single Cell 3' Reagent Kit V2. cDNA synthesis, barcoding, and library preparation were then carried out on a 10X Genomics Chromium Controller according to the manufacturers' instructions. After validating quality of cDNA library, sequencing was performed on an Illumina HiSeq 2500 (Illumina, San Diego, CA USA). Reads were demultiplexed and aligned to the murine reference genome (mm10 with

annotations from Ensembl, release 84). 10X Genomics Cellranger aggr and Seurat were used to generate PCA clustering (Satija et al., 2015). The expression of well-recognized marker genes identified 24 distinct cell types (Ryan et al., 2020). Linearized relative expression levels of each gene examined in this study were log-transformed from single-cell mRNA copy numbers, normalized, and scaled for each cell type. Data were visualized in 10X Genomics cLoupe, with UMI numbers expressed colorimetrically for each cell.

RESULTS

Cohort Summary

Samples were collected from 91 pediatric patients with OM with ages ranging from 8.7 months to 14.9 years old (median 2.0 years; **Table 1**). Of these, 86 had sufficient DNA sample for Sanger sequencing of *FUT2* c.461G>A (**Figure 1**) and 83.3% are homozygous or heterozygous for the *FUT2* variant. Carriage of the *FUT2* variant was not associated with age, sex, ethnicity or OM diagnosis among children with OM (**Table 1**). In the entire cohort and in each subset analyses, males were predominant (≥81%), which is a known phenomenon for OM (Paradise et al., 1997).

Differentially Expressed Genes in OM Patients With the *FUT2* c.461G>A Variant

RNA-seq data from 28 pediatric patients (0.8 to 14.8 years old; **Table 1**) passed QC and were available for analysis according to *FUT2* genotype. DE analysis was performed using *FUT2* c.461G>A variant carriage as the classifier (5 wildtype and 23 variant carriers) and with adjustment for age, sex and batch effects (**Table 1** and **Supplementary Figure 1**). Five DE genes were significant, namely: *FN1* (log-fold change = -3.7, FDR-adj-*p*=0.006); *KMT2D/MLL2* (log-fold change = -3.8, FDR-adj-*p*=0.04); *MUC16* (log-fold change = -4.3, FDR-adj-*p*=0.04); *MTAP* (log-fold change = +5.4, FDR-adj-*p*=0.006); and *NBPF20* (log-fold change = -3.5, FDR-adj-*p*=0.04). In carriers of the *FUT2* c.461G>A variant, *FN1*, *KMT2D/MLL2*, *MUC16* and *NBPF20* were downregulated whereas *MTAP* was upregulated (**Figure 2**).

To further investigate how *FUT2, FN1*, *KMT2D/MLL2*, *MUC16, MTAP*, *NBPF20* and *RASIP1* are related, these genes were used as input for network analysis. *RASIP1, FN1*, *KMT2D/MLL2* and *MTAP* were connected in a single protein-protein interaction network (**Figure 3A**). Pathway enrichment analysis of this network revealed 27 significant pathways in KEGG and 21 significant processes in PANTHER GO-slim BP, many of which overlap (**Figures 3B, C** and **Table 2**). Among these are processes pertaining to viral and bacterial infection, cell cycle regulation, apoptosis, and endocytosis (**Table 2**).

Differentially Expressed Genes Were Also Significantly Regulated in Infected ME of Wildtype Mice

To further understand the role and interactions between *FUT2, RASIP1* and DE genes, expression of orthologs *Fut2, Fn1*, *Kmt2d*, *Muc16, Mtap* and *Rasip1* were measured by gene array in ME of wildtype mice at multiple time points (from 3 hours to 7 days) post-infection with NTHi (**Figure 4** and **Tables 3**, **4**). The *NBPF* gene family results from segmental duplication events in primate, thus an ortholog for *NBPF20* is not present in mice (Vandepoele et al., 2005). Expression of *Fut2, Rasip1* and *Mtap* were significantly increased after inoculation, with *Fut2* and *Mtap* peaking around one day post-inoculation, and *Rasip1*

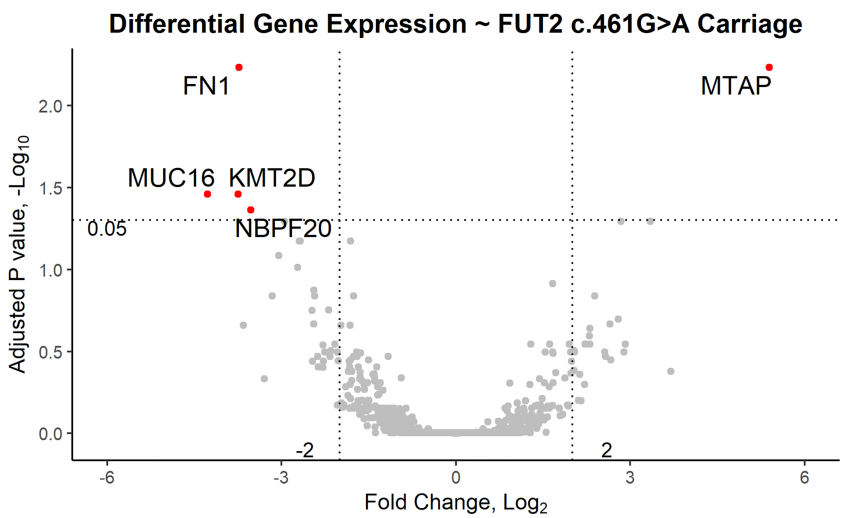

FIGURE 2 | Volcano plot of differentially expressed genes based on carriage of the *FUT2* c.461G>A variant in patients with OM. In variant carriers, *KMT2D/MLL2, MUC16, NBPF20* and *FN1* were downregulated (FDR-adjusted p < 0.05, log2 fold change < -2) and *MTAP* was upregulated (FDR-adjusted p < 0.05, log2 fold change > 2).

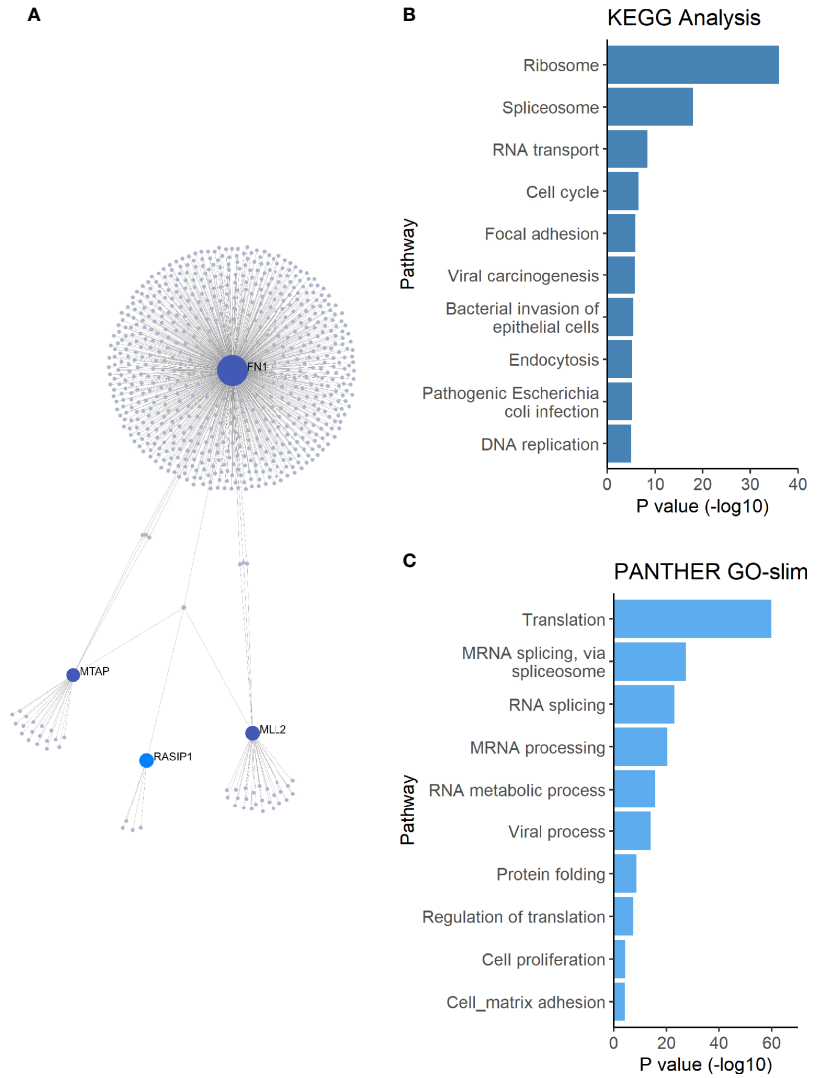

FIGURE 3 | Network and pathway enrichment analysis of differentially expressed genes. **(A)** A single PPI network was constructed using the *FUT2, RASIP1* and the DE genes as input. **(B)** KEGG and **(C)** PANTHER GO-slim:BP pathway enrichment analysis results, showing the top 10 pathways with the smallest *p*-values. *MUC16* and *FUT2* are not connected to this network, suggesting a different mechanism for the interaction of these two genes in relation to OM.

and *Fn1* at 3 hours post-inoculation (**Figure 4**). Additionally, expression of *Muc16* was significantly decreased one day post-inoculation. For *Fn1, Mtap* and *Muc16*, DE was sustained through days 2-7 post-inoculation, including when OM is supposedly in recovery phase (Hernandez et al., 2015). *Kmt2d* showed no significant changes in ME expression at any point during the 7 days when compared to control mice (**Figure 4**).

Single-cell RNA-sequence (scRNA-Seq) data were derived from the MEs of NTHi-infected mice six hours after inoculation (**Figure 5** and **Table 5**). In uninfected ME (time point 0h), *Fut2* was expressed primarily in ciliated epithelial cells (*Hydin*+). *Muc16* was expressed in most epithelial cells except basal epithelial cells (*Krt14*+). *Rasip1* was expressed in most endothelial cells (*Egfl7*+) and *Fn1* mostly in stromal cells (*Col1a2*+) and melanocytes (*Mlana*+). *Mtap* and *Kmt2d* were modestly expressed in all ME cell types. Six hours after ME inoculation with NTHi, when overall expression data was strongest (**Figure 5**), *Fut2* had increased expression in non-ciliated epithelial cells (*Krt18/19*+) and *Muc16* in all epithelial cell types. *Rasip1* continued to be expressed in endothelial cells, but was also observed in polymorphonuclear cells (PMNs) and monocytes (*Csf1r*+). *Fn1* increased expression in stromal cells and monocytes and some endothelial cells (**Figure 5**). *Mtap* and *Kmt2d* remained moderately expressed in all ME cell types except infiltrating PMNs and red blood cells. Level of gene expression per cell peaked at 1 day, and then declined (**Table 5**). Taken together, the mouse ME expression profiles for *Fut2, Rasip1* and DE genes support the findings of DE genes in OM patients using RNA-seq data from saliva (**Table 4**),

TABLE 2 | Significant pathways within network connecting DE genes.

KEGG		PantherBP : GO-slim	
Pathway	**FDR-adj-p**	**Pathway**	**FDR-adj-p**
Ribosome	2.82E-34	**Translation**	2.37E-58
Spliceosome	1.60E-16	**MRNA splicing, *via* spliceosome**	3.15E-26
RNA transport	4.05E-07	**RNA splicing**	6.20E-22
Cell cycle	2.09E-05	MRNA processing	2.46E-19
Focal adhesion	8.29E-05	**RNA metabolic process**	7.10E-15
Viral carcinogenesis	8.66E-05	**Viral process**	3.14E-13
Bacterial invasion of epithelial cells	0.0002	Protein folding	5.78E-08
Endocytosis	0.0002	**Regulation of translation**	1.14E-06
Pathogenic E. coli infection	0.0002	**Cell proliferation**	0.001
DNA replication	0.0003	**Cell_matrix adhesion**	0.001
Proteoglycans in cancer	0.001	**Rhythmic process**	0.001
Huntington's disease	0.001	**Negative regulation of apoptotic process**	0.002
Proteasome	0.002	MRNA 3'_end processing	0.002
Regulation of actin cytoskeleton	0.002	**Intracellular protein transport**	0.003
Carbon metabolism	0.003	**Vesicle_mediated transport**	0.003
Adherens junction	0.004	**Glycolytic process**	0.005
Endocrine and other factor-regulated calcium reabsorption	0.004	**DNA replication**	0.01
mRNA surveillance pathway	0.004	RNA splicing *via* transesterification reactions	0.02
Aminoacyl-tRNA biosynthesis	0.004	**Receptor_mediated endocytosis**	0.02
Estrogen signaling pathway	0.008	Protein transport	0.02
Leukocyte transendothelial migration	0.01	DNA recombination	0.03
Glycolysis/Gluconeogenesis	0.02		
Hepatitis B	0.03		
Shigellosis	0.04		
Pyruvate metabolism	0.04		
Salmonella infection	0.04		
Bladder cancer	0.049		

Overlap between databases in bold.

and also the overall expression of these genes in other human mucosal tissues (**Table 6**).

ME and NP Microbiota Profiles of Patients Carrying the *FUT2* c.461G>A Variant

A total of 296 microbial samples were collected from the NP and ME of 86 children (**Figure 1**). For microbiota analyses, samples were filtered for: (1) those with >2500 16S rRNA sequencing reads; (2) one ME and one NP sample per individual where bilateral samples were collected (if bilateral, right-sided sample was used); and (3) available genotypes for *FUT2* and *RASIP1* variants (**Figure 1**). No differences were identified between right and left NP or ME samples from the same individuals in PCA and PERMANOVA analyses (data not shown). After filtering, 16S rRNA sequence data from 34 ME and 65 NP samples were analyzed according to carriage of the *FUT2* c.461G>A (p.Trp154*) variant.

In the ME, based on carriage of the *FUT2* variant, Chao1 which denotes bacterial richness was significant when all ethnic groups were included (p=0.03); however, all alpha-diversity indices were not significant when only individuals of European descent were included in analyses (**Supplementary Table 1**). Overall microbiota composition (i.e., beta-diversity) did not differ significantly by *FUT2* variant according to PERMANOVA analysis with adjustment for age, sex, or batch effects (**Figure 6A**). Additionally, the relative abundances of *Haemophilus* (nominal p=0.03) and *Moraxella* (nominal p=0.02) were increased with wildtype *FUT2* genotype, whereas increased *Propionibacterium* (nominal p=0.04) and *Anoxybacillus* (nominal p=0.02) were associated with presence (homozygous or heterozygous genotypes combined) of the variant (**Figure 6C** and **Supplementary Table 2**). Performing these analyses by genotype had no overall effect on results (**Supplementary Figure 2**).

In the NP, there were also no significant differences in alpha- or beta-diversity (**Supplementary Table 1** and **Figure 6B**). Similar to ME, *Propionibacterium* had increased relative abundance in the NP (nominal p=0.01) among carriers of the *FUT2* variant. In addition, the relative abundances of *Actinobacillus* (nominal p=0.03), *Selenomonas* (nominal p=0.03) and Candidate Division TM7 (*Saccharibacteria*; nominal p=0.0002) were increased in wildtype individuals (**Figure 6D**, **Supplementary Table 2**). When individual taxa were tested for association by genotype, no taxa were significant (**Supplementary Figure 2**). Note however that these *FUT2*-microbiota associations were nominal and were non-significant after FDR correction, with the exception of Candidate Division TM7 in the NP (FDR-adjusted p=0.009).

RASIP1

Sanger sequencing of DNA samples confirmed that the *RASIP1* c.1801C>T and *FUT2* c.461G>A variants are in moderate LD in our cohort as the genotypes for 57 of 71 (80.3%) individuals were identical. In the ME, similar to findings with the *FUT2* variant, an increased relative abundance of *Haemophilus* (nominal

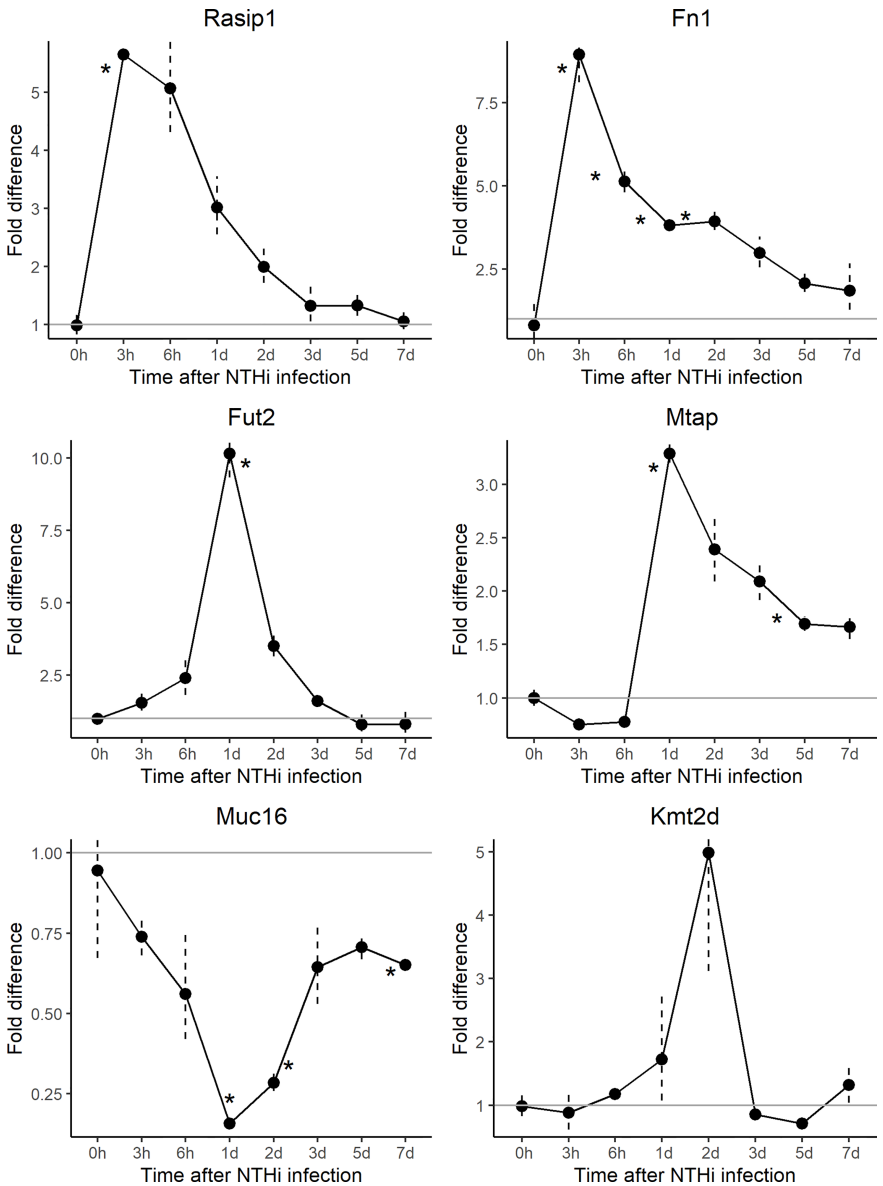

FIGURE 4 | Gene array expression data for select genes post-inoculation with non-typeable *Haemophilus influenzae (NTHi).* Mouse middle ear expression of select genes across different time points, shown as fold change in middle ears inoculated with NTHi as compared to placebo. *Fut2*, *Muc16* and *Mtap* reached peak change in expression at 24 hours post-inoculation while *Muc16* demonstrated sustained downregulation. On the other hand, *Rasip1* and *Fn1* reached peak upregulation at 3 hours post-inoculation. In this experiment, time point 0h represents uninfected middle ear. *$p < 0.05$; see **Table 3** for gene expression values by time point and gene.

p=0.04) was associated with wildtype genotype whereas increased *Propionibacterium* (nominal p=0.04) was associated with the *RASIP1* variant (**Figure 7C** and **Supplementary Table 3**). When analyzed by genotype, *Haemophilus* remained nominally associated with wildtype (**Supplementary Figure 3**). In the NP, increased abundance of *Propionibacterium* (nominal p=0.006), chloroplast (FDR-adjusted p=0.05), *Escherichia-Shigella* (nominal p=0.04) and *Staphylococcus* (nominal p=0.04) was associated with carriage of the *RASIP1* variant, whereas increased abundance of Candidate Division SR1 (FDR-adjusted p=0.05), Candidate Division TM7 (FDR-adjusted p=0.05), and *Actinobacillus* (nominal p=0.01) was associated with wildtype genotype (**Figure 7D** and **Supplementary Table 3**).

DISCUSSION

Variants in *FUT2*, including the c.461G>A (p.Trp154*) variant investigated here, have been associated with increased

TABLE 3 | Mouse ME gene expression values by time point.

Gene	Probe	Time	Fold diff.	lower	upper	p-value (*<0.05)
Fut2	143862_at	0h	0.99	0.86	1.14	0.96
		3h	1.53	1.27	1.85	0.27
		6h	2.39	1.81	3.16	0.20
		1d	10.14	9.32	11.03	0.02*
		2d	3.49	3.13	3.89	0.55
		3d	1.60	1.48	1.74	0.11
		5d	0.79	0.55	1.13	0.63
		7d	0.80	0.51	1.28	0.72
Rasip1	1428016_at	0h	0.99	0.83	1.17	0.95
		3h	5.65	5.55	5.74	0.006*
		6h	5.07	4.32	5.95	0.06
		1d	3.01	2.56	3.55	0.09
		2d	1.99	1.72	2.31	0.13
		3d	1.33	1.06	1.66	0.43
		5d	1.33	1.15	1.54	0.30
		7d	1.06	0.92	1.21	0.76
Fn1	1437218_at	0h	0.80	0.40	1.60	0.80
		3h	8.94	8.11	9.85	0.03*
		6h	5.13	4.80	5.48	0.03*
		1d	3.82	3.70	3.94	0.01*
		2d	3.93	3.67	4.21	0.03*
		3d	2.97	2.55	3.47	0.09
		5d	2.07	1.81	2.35	0.11
		7d	1.85	1.28	2.67	0.34
Mtap	1451345_at	0h	0.10	0.93	1.08	0.98
		3h	0.75	0.73	0.78	0.08
		6h	0.78	0.73	0.83	0.15
		1d	3.29	3.20	3.37	0.01*
		2d	2.39	2.09	2.73	0.10
		3d	2.09	1.92	2.28	0.07
		5d	1.69	1.63	1.76	0.047*
		7d	1.67	1.55	1.79	0.09
Muc16	1432358_at	0h	0.95	0.67	1.33	0.90
		3h	0.74	0.68	0.80	0.17
		6h	0.56	0.42	0.75	0.30
		1d	0.16	0.15	0.16	0.01*
		2d	0.28	0.26	0.31	0.047*
		3d	0.64	0.53	0.78	0.27
		5d	0.71	0.67	0.75	0.10
		7d	0.65	0.64	0.67	0.04*
Kmt2d	1427555_at	0h	0.99	0.83	1.17	0.95
		3h	0.88	0.62	1.26	0.79
		6h	1.18	1.09	1.27	0.28
		1d	1.73	1.08	2.77	0.46
		2d	4.98	3.12	7.97	0.18
		3d	0.85	0.80	0.91	0.25
		5d	0.71	0.62	0.81	0.24
		7d	1.32	1.04	1.67	0.45

**Denotes p-values < 0.05.*

TABLE 4 | Comparison of DE gene regulation in human saliva of *FUT2* c.461G>A variant carriers vs non-carriers and NTHi- vs placebo-inoculated mouse middle ear (ME).

Gene	FUT2 Variant Carrier vs Wildtype (human saliva expression)	NTHi- vs PBS-inoculated (mouse ortholog ME expression)
FUT2	*Genotype as classifier variable*	Upregulated in NTHi at 1 day
RASIP1	*Genotype as classifier variable*	Upregulated in NTHi at 3 hours
FN1	Downregulated in variant carriers	Upregulated in NTHi, peak at 3 hours
MTAP	Upregulated in variant carriers	Upregulated in NTHi, peak at 1 day
MUC16	Downregulated in variant carriers	Downregulated in NTHi, peak at 1 day
KMT2D/MLL2	Downregulated in variant carriers	Not significant
NBPF20	Downregulated in variant carriers	*Not applicable*

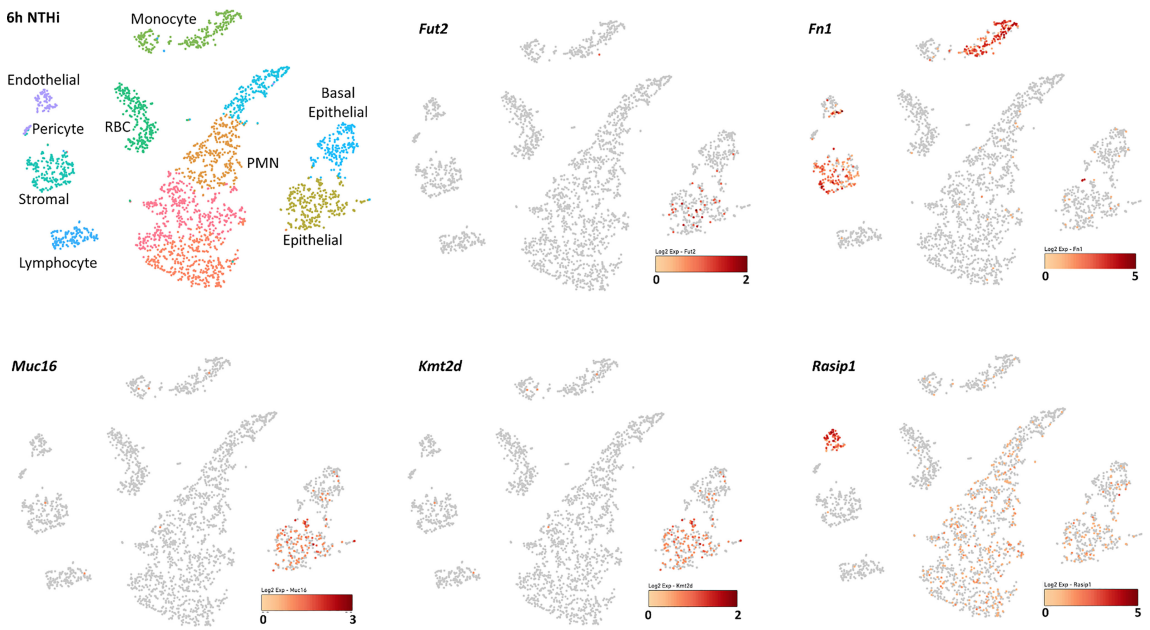

FIGURE 5 | Single-cell RNA-seq expression data from mouse middle ear 6 hours after inoculation with NTHi. Expression of *Fut2, Fn1, Muc16, Kmt2d* and *Rasip1* in wildtype mouse middle ear, 6 hours post-infection. Cell types of the various PCA clusters were identified by the expression of unique marker genes. Darkness of color (*red* for each gene) indicates level of UMI expression by each cell, according to the associated $\log_2$ scale. Epithelial cell clusters were identified based on the expression of *Krt18* and/or *Krt19*. Basal epithelial cells also express *Krt14*, while ciliated epithelial cells express *Hydin*. Stromal cells are identified by *Col1a2*. Vascular endothelial cells express *Egfl7* and *Flt4*, lymphatic endothelial cells *Egfl7* and *Flt1*. Melanocytes express *Mlana* and pericytes *Rgs5*. Monocytes express *Csf1r*, lymphocytes *Ptprcap*, polymorphonuclear cells *Il1f9* and *Stfa2l1*, and red blood cells *Hba-a1*.

TABLE 5 | Single-cell RNA-seq expression levels in mouse ME by time point after NTHi inoculation.

Gene	0 hour	6 hours	1 day	5 days	7 days
Fut2	modest in ~10% of ciliated epithelial cell and a few other non-basal epithelial cells	modest in ~10% of non-ciliated non-basal epithelial cells	moderate in ~30% of epithelial cells ~10% of vascular endothelial cells and a few PMNs	modest in only a few epithelial cells	modest in ~10% of ciliated and other non-basal epithelial cells
Rasip1	moderate in most endothelial cells, both vascular and lymphatic	very strong in most endothelial cells, modest in ~20% of PMNs and ~5% of monocytes	strong in vascular endothelial cells, modest in some PMNs, monocytes	strong in most vascular endothelial, modest in ~50% of other cells but stromal, lymphocytes	moderate in most endothelial cells
Fn1	strong in ~50% of stromal cells, melanocytes, a few endothelial cells and monocytes	strong in most stromal cells, moderate in most monocytes, a few endothelial cells	very strong in most monocytes, some stromal cells, and some vascular endothelial cells	very strong in all stromal cells; moderate in ~50% of monocytes and vascular endothelial cells	strong in all vascular endothelial cells, moderate in most stromal cells, modest in ~10% of monocytes
Mtap	modest in ~10% of all cell types	modest in ~10% of all cell types but PMNs, RBCs	moderate in most vascular epithelial cells, ~50% of stromal cells and epithelial cells, some monocytes	modest in ~20% of all cell types but PMNs, RBCs	modest in 10-20% of all cell types but ciliated epithelial cells, RBCs
Muc16	moderate in most epithelial cells, excluding basal cells	modest in most non-basal epithelial cells	moderate in most non-basal epithelial cells	moderate in non-basal epithelial cells, very modest in ~10% of basal epithelial cells	moderate in most non-basal epithelial cells, modest in ~10% of basal epithelial cells
Kmt2d	modest in ~10-20% of all cell types	modest in ~10-20% of all cell types except PMNs, RBCs	moderate in most vascular endothelial and ~50% of epithelial cells; modest in most stromal cells, monocytes, PMNs	modest in 50% of epithelial cells and ~10-20% of all other cell types but RBCs	modest in ~10-20% of all cell types but RBCs

Very modest expression = <0.5 x log2 UMI (transcript)/cell.
Modest expression = 0.5-1 x log2 UMI/cell.
Moderate expression = 1.5-2 x log2 UMI/cell.
Strong expression = 2.5-3 x log2 UMI/cell.
Very strong expression = 3.5-5 x log2 UMI/cell.
PMNs, polymorphonuclear cells; RBCs, red blood cells.

TABLE 6 | Known RNA and protein expression profiles of *FUT2*, *RASIP1* and DE genes in human tissues.

Gene	RNA Expression (GTEx Consortium; Lonsdale et al., 2013)	Protein Expression (Human Protein Atlas; Uhlen et al., 2015)
FUT2	Minor salivary gland, esophagus-mucosa, small intestine-terminal ileum, colon-transverse, stomach, vagina	Medium expression in most organs/tissues including nasopharynx, lung and oral mucosa
RASIP1	Lung, adipose-visceral (omentum), breast-mammary tissue, adipose-subcutaneous, spleen, uterus	Medium expression in gallbladder, kidney, placenta, smooth muscle; low expression in adrenal gland, salivary gland, epididymis, appendix, tonsil, cerebral cortex, colon
FN1	Cultured fibroblasts, artery-aorta, coronary, tibial	High expression in kidney; medium or low expression in many organs/tissues including low expression in nasopharynx, lung and oral mucosa
KMT2D/ MLL2	Expression detected across all tissues/organs; highest in uterus, thyroid, brain-cerebellum	High expression in cerebral cortex, cerebellum, testis, and epididymis; medium or low expression in many organs/tissues including low expression in nasopharynx and lung
MTAP	Highest expression in cells-cultured fibroblasts, nerve-tibial, ovary, uterus	*Unavailable*
MUC16	Minor salivary gland, adipose-visceral (omentum), fallopian tube, testis, lung, cervix-endocervix	High expression in bronchus, fallopian tube, endometrium, uterine cervix; medium expression in salivary gland; low expression in nasopharynx

susceptibility to OM but their functional role in OM pathology has not been fully elucidated. Although *FUT2* has been studied by many groups, to our knowledge this is the first study in which RNA-seq data combined with ME and NP microbiotas have been examined in relation to carriage of the *FUT2* c.461G>A variant. Our results suggest that the *FUT2* variant confers OM susceptibility through its modulation of *MUC16* expression and downstream induction of *FN1* and *MTAP* after microbe binding and pathogen colonization (**Figures 2**, **3** and **Table 2**). These DE findings were supported by similar regulation of expression in NTHi-infected ME of wildtype mice, whether by bulk mRNA-seq or single-cell RNA-seq data (**Figure 4** and **Tables 3**, **4**). Because these genes were differentially regulated in response to OM in the infected wildtype mouse ME, the results of

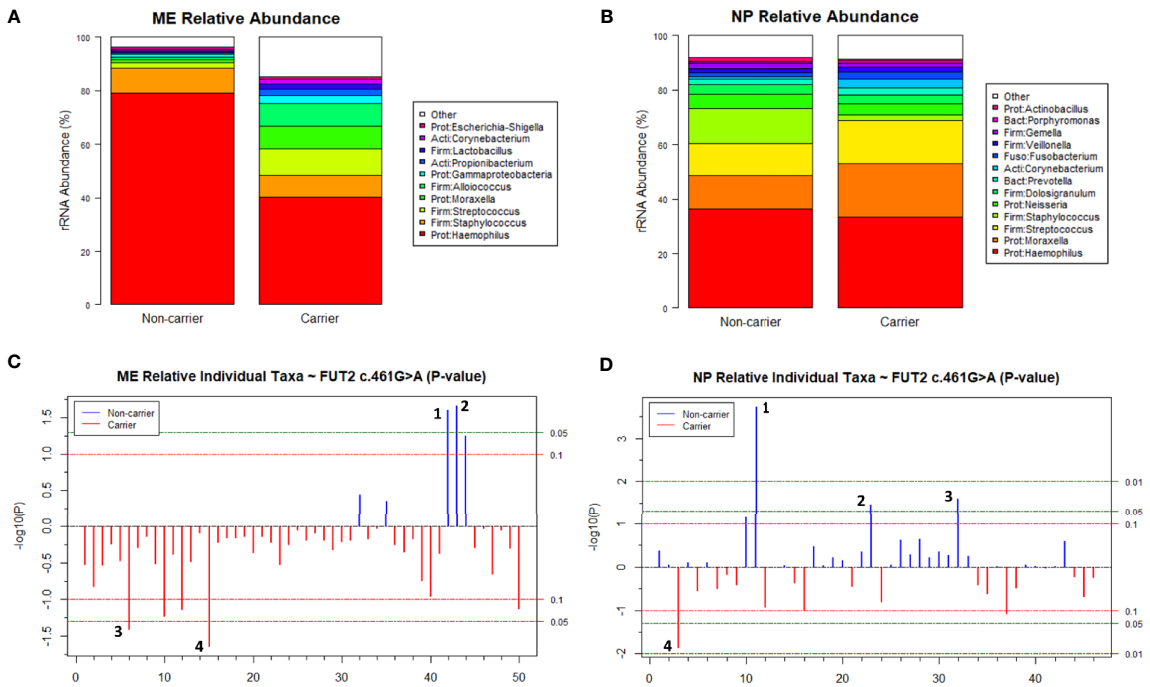

FIGURE 6 | Relative abundance of individual taxa in middle ears (ME) and nasopharynges (NP) of carriers and non-carriers of the *FUT2* c.461G>A variant. **(A)** Cumulative relative abundance profiles in the ME of wildtype (n=8) and carriers (n=26) of *FUT2* c.461G>A. **(B)** Cumulative relative abundance profiles in the NP of wildtype (n=14) and carriers (n=51) of *FUT2* c.461G>A. Plots showing *p*-values for relative abundance of individual bacterial taxa in the **(C)** ME and **(D)** NP of wildtype versus variant carriers after adjusting for batch. *Blue lines* indicate taxa that were increased in wildtype, *red lines* for carriers. *Dashed lines* indicate significance thresholds where the *red line* is unadjusted-*p*=0.1 (non-significant) and *green lines* indicate unadjusted-*p*=0.05 and unadjusted-*p*=0.01. **(C)** In the ME, *Haemophilus* (1) and *Moraxella* (2) were nominally associated with wildtype, whereas *Propionibacterium* (3) and *Anoxybacillus* (4) were nominally associated with variant carriage. **(D)** In the NP, Candidate Division TM7 (1) was significantly associated with wildtype (FDR-adj-*p*=0.009). Additionally, *Selenomonas* (2) and *Actinobacillus* (3) were nominally associated with wildtype whereas *Propionibacterium* (4) was nominally associated with variant carriage.

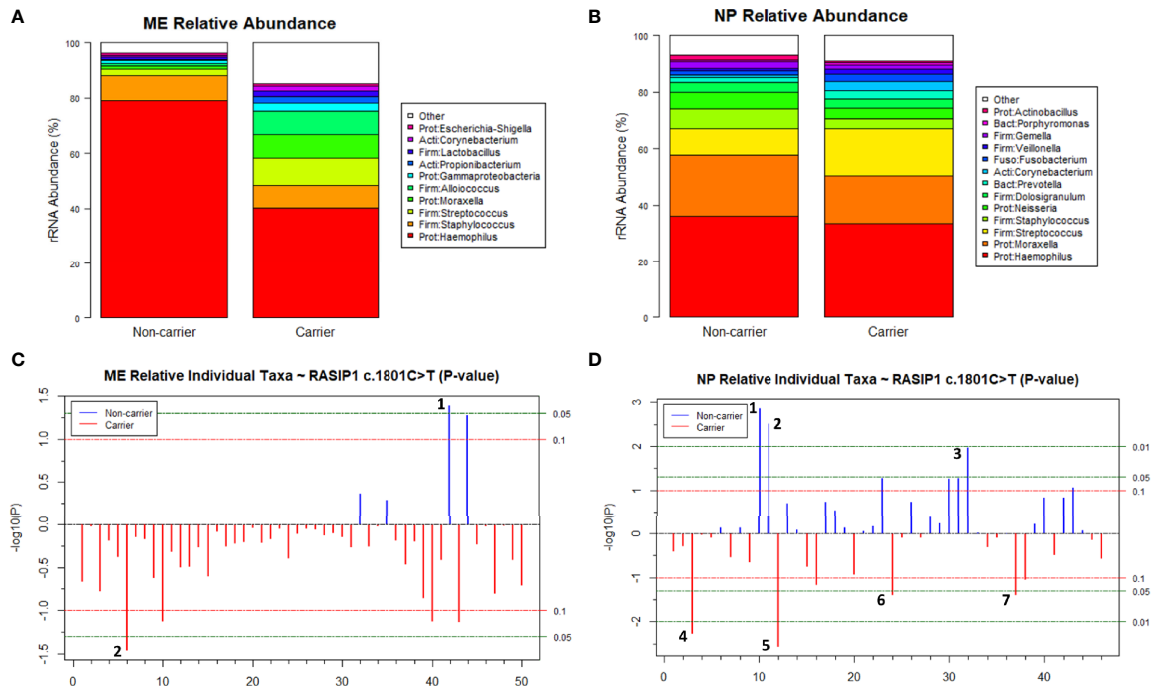

FIGURE 7 | Relative abundance of individual taxa in the middle ears (ME) and nasopharynges (NP) of carriers and non-carriers of the *RASIP1* c.1801C>T variant. **(A)** Cumulative relative abundance profiles in the ME of wildtype (n=8) and carriers (n=26) of *RASIP1* c.1801C>T. **(B)** Cumulative relative abundance profiles in the NP of wildtype (n=15) and carriers (n=50) of *RASIP1* c.1801C>T. Plots showing *p*-values for relative abundance of individual bacterial taxa in the **(C)** ME and **(D)** NP of wildtype versus carriers after adjusting for batch. *Blue lines* indicate taxa that were increased in wildtype, *red lines* for carriers. *Dashed lines* indicate significance thresholds where the *red line* is unadjusted-*p*=0.1 (non-significant) and *green lines* indicate unadjusted-*p*=0.05 and unadjusted-*p*=0.01. **(C)** In the ME, *Gammaproteobacteria* (1) was nominally associated with wildtype, whereas *Propionibacterium* (2) was nominally associated with variant carriage. **(D)** In the NP, Candidate Division SR1 (1) and Candidate Division TM7 (2) were significantly associated with wildtype, and Chloroplast (5) with variant carriage (FDR-adj-*p*=0.05). Additionally, *Actinobacillus* (3) was nominally associated with wildtype, whereas *Propionibacterium* (4), *Staphylococcus* (6) and *Escherichia-Shigella* (7) were nominally associated with variant carriage.

this DE analysis suggest that the *FUT2* c.461G>A variant magnifies the downstream response to infection (for example, downregulated *MUC16*, upregulated *MTAP*), and/or reverses the direction of regulation (e.g. downregulation of *FN1* in carriers of the *FUT2* variant; **Figures 2**, **4** and **Table 4**). Alternatively, DE genes may vary depending on the predominant otopathogen during infection: in other words, whether commensal or otopathogenic bacteria bind to ME mucosal epithelium *via* A antigen, the expression of which is affected by heterozygous or homozygous genotype for the *FUT2* c.461G>A variant (**Figures 6**, **7**; Santos-Cortez et al., 2018).

RASIP1 is expressed in ME endothelial cells and provides another avenue for investigation in relation to *FUT2* c.461G>A variant carriage. *RASIP1* c.1801G>T, previously identified by GWAS to be in LD with *FUT2* c.461G>A (Pickrell et al., 2016), is also in moderate LD with *FUT2* c.461G>A in the sample set. *RASIP1* is part of the PPI immune network including *MTAP*, *KMT2D* and *FN1* (**Figure 3A** and **Table 2**), which led us to question whether the expression and microbiota effects we observed were being driven by the *RASIP1* missense variant rather than the *FUT2* stop variant. When examining the changes in the expression of these genes in wildtype mice after NTHi inoculation, *Rasip1* and *Fn1* expression peaked at 3 hours post-inoculation, whereas *Fut2* peaked at one day post-inoculation, in concordance with *Muc16* and *Mtap* expression (**Figure 4** and **Table 3**). Additionally, in the single-cell RNA-seq data from mouse ME, we observed *Rasip1* and *Fn1* expression in endothelial cells versus epithelial expression of *Fut2* and *Muc16* (**Figure 5** and **Table 5**). When examined together, these expression profiles strongly support *FUT2* as mediating OM susceptibility within the ME mucosal epithelium. In particular, the downregulation of *MUC16* in OM patients with the *FUT2* stop variant might indicate a prolonged recovery phase when *MUC16* is expected to return to normal levels as part of the normal response to acute OM. *MUC16* downregulation is therefore a potential avenue for future research, for example, whether this effect of *FUT2* knockdown is a mechanism for an acute infection to proceed to recurrence or chronicity (Kerschner et al., 2013).

Dysbiosis of the NP and ME mucosal microbiotas is supported by our data here and in our previous studies in which the ME of *FUT2* c.461G>A variant carriers were enriched in potentially otopathogenic taxa such as *Propionibacterium*, and decreased for established otopathogens *Haemophilus* and *Moraxella*, although these associations were nominal (Santos-Cortez et al., 2018). This

TABLE 7 | Summary of relevant knowledge of *FUT2*, *RASIP1* and DE genes.

Gene	Prior findings in literature
FUT2 (alpha-[1,2]-fucosyltransferase), MIM 182100	• c.461G>A variant confers non-secretor status of ABO(H) antigens on mucosal epithelia (Magalhaes et al., 2016) • Non-secretors demonstrate decreased commensal load allowing an increase in bacterial pathogen colonization (Giese et al., 2020) • Non-secretor status affects mucus barrier (Magalhaes et al., 2016)
RASIP1 (Ras interacting protein 1), MIM 609623	• Crucial to formation of vascular structures *via* angiogenesis and vasculogenesis (Xu et al., 2009) • Involved in endothelial barrier function (Xu et al., 2011) • Expressed in middle ear endothelial cells (Ryan et al., 2020)
FN1 (fibronectin-1), MIM 135600	• Glycoprotein found in extracellular matrix and on cell surface (McDonald et al., 1982; Woods et al., 1986) • Involved in cell adhesion, migration, host defense and wound healing (McAuslan et al., 1980; Clark et al., 1982; Hill et al., 1984; Woods et al., 1986) • Expressed in human middle ear epithelial cells & identified as a key modulator of anti-inflammatory response to extracellular stress (Song et al., 2013) • Utilized by *S. aureus* to gain entry to host cells (Fowler et al., 2000)
KMT2D/MLL2 (histone-lysine N-methyltransferase 2B; myeloid/lymphoid or mixed-lineage leukemia protein 2), MIM 602113	• *KMT2D* mutations are the cause of the majority of cases of Kabuki syndrome (KS; MIM 147920) (Ng et al., 2010; Yap et al., 2020) • KS patients have high rate of infections and array of immunological abnormalities (Hoffman et al., 2005) • OM occurs in 55-90% of KS patients (Boniel et al., 2021)
MTAP (S-methyl-5'-thioadenosine phosphorylase)	* $Mtap^{+/-}$ mice had no hearing loss, while $Mtap^{-/-}$ was embryonic lethal (Williamson et al., 2007)
MUC16 (cell-surface associated mucin 16)	• Transmembrane mucin expressed in human and mouse middle ear and airway epithelia (Kerschner, 2007; Kerschner et al., 2010) • Contributes to composition of mucous barrier as part of host defense against infection (Kesimer et al., 2009) • Upregulated in middle ear epithelia of OM patients as compared to normal controls (Stabenau et al., 2021)

could be attributed to the effect of *FUT2* c.461G>A on pathogen binding, wherein those homozygous for the *FUT2* variant are non-secretors of ABO(H) antigens on the epithelia surface (**Table 6**); these antigens can serve as ligands to which some bacteria may bind and thus affect the commensal and pathogen loads of the NP and ME. Interestingly the only bacterial taxon that has a significant association with the *FUT2* variant after correction for multiple testing is Candidate Division TM7, which is also known as *Saccharibacteria* (**Figure 6**). Little is known about *Saccharibacteria* and its reported associations with human mucosal disease have been variable, though there is some evidence that it parasitizes other bacteria and can kill its host bacterium, thereby modulating the overall microbiota (Bor et al., 2019).

The change in relative abundance of chloroplast in the NP corresponding to *RASIP1* variant carriage is an unusual result. This is potentially due to a sequence misclassification of cyanobacteria in the reference database rather than systematic contamination during isolation from the kit or reagents. Though general contamination is a possible explanation, if this were the case its presence would be detected among all samples or the effect would be eliminated by the adjustment for batch during analyses. Furthermore, chloroplast contamination would be negatively correlated with number of reads per sample as contamination would be less prominent in samples with higher bacterial loads. However, we did not observe these in our samples and during analyses. Thus, it is unlikely that the identification of chloroplast as being differentially abundant in carriers of the *RASIP1* variant is due to general contamination, though random, non-systematic contamination cannot be ruled out. Note that the main findings in this work are more likely explained by carriage of the *FUT2* variant and not the *RASIP1* variant.

In addition to the impact on pathogen and commensal binding to epithelia, the DE and network analyses suggest that the *FUT2* c.461G>A variant also has a downstream effect on basic cellular pathways (**Figures 2**, **3** and **Tables 4**, **7**). For example, *FN1* is a modulator of ME anti-inflammatory response (Song et al., 2013) as well as a binding site for otopathogen *Staphylococcus aureus* (Fowler et al., 2000) and group A *Streptococcus* (McNitt et al., 2018). FN1 protein expression was also previously demonstrated to be dysregulated by viral infection (Simon et al., 2015; Qiao et al., 2021); however, viruses are not included in this study due to sample collection methods. Notably we only observed a nominal increase in *Staphylococcus* abundance in the NP (but not ME) of carriers of the *RASIP1* variant (**Figure 7**), but not in carriers of the *FUT2* variant (**Figure 6**). In addition, NTHi inoculation of mouse ME resulted in upregulation of *Fn1* (**Figure 4** and **Table 3**). In contrast, in our OM patients with the *FUT2* stop variant, *FN1* was downregulated (**Figure 2** and **Table 4**), indicating that non-functional *FUT2* might also affect the direction of regulation of the immune network that includes *FN1* and also *RASIP1, MTAP* and *MLL2/KMT2D* (**Figure 3**). It should be noted that *KMT2D* variants are responsible for Kabuki Syndrome which is characterized by increased rates of OM as well as other immunological abnormalities (Hoffman et al., 2005; Ng et al., 2010; Yap et al., 2020; Boniel et al., 2021).

In conclusion, we propose that the mechanistic effects of the *FUT2* c.461G>A variant on OM susceptibility are two-fold: (1) Non-secretor status conferred by this *FUT2* stop variant alters the profiles of bacterial taxa that bind to ME and NP mucosal epithelia and thereby increases susceptibility to bacterial infection in mucosal epithelia; and (2) *FUT2* variants affect expression of genes including downregulation of *MUC16* and those connected to an immune network, which leads to further susceptibility to infection as well as impaired immune responses (**Figure 3**) and basic cellular processes (**Table 2**) within the ME mucosal epithelium. Through increased understanding of the effects of pathogenic variants on dysbiosis and gene regulation in OM, the ability to determine risk for patients due to specific genetic variants may be improved, and thereafter enhance prevention and treatment protocols for OM using more targeted antibiotics for otopathogens associated with these variants.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/gap/, phs001941.v1.p1 https://www.ncbi.nlm.nih.gov/sra/, BioProject ID PRJNA748418.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Colorado Multiple Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by Institutional Animal Care and Use Committee Veterans Affairs Medical Center, San Diego, California.

AUTHOR CONTRIBUTIONS

RS-C conceptualized the study. MS, S-OS, TW, PY, SG, SC, HJ, JP, KC, and NF recruited patients and collected samples. TB performed isolation of human DNA and RNA and microbial DNA samples and submitted them for sequencing. CE and EL performed RNA sequence analyses. CE performed network analyses. JK, CR, and DF performed 16S rRNA sequencing. CE and DF performed analyses of microbiota data. AR performed mouse expression studies. CE, AR, and RS-C wrote the manuscript. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.798246/full#supplementary-material

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Probing Immune-Mediated Clearance of Acute Middle Ear Infection in Mice

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Acute otitis media (AOM) is commonly caused by bacterial pathobionts of the nasopharynx that ascend the Eustachian tube to cause disease in the middle ears. To model and study the various complexities of AOM, common human otopathogens are injected directly into the middle ear bullae of rodents or are delivered with viral co-infections which contribute to the access to the middle ears in complex and partially understood ways. Here, we present the novel observation that *Bordetella bronchiseptica*, a well-characterized respiratory commensal/pathogen of mice, also efficiently ascends their Eustachian tubes to colonize their middle ears, providing a flexible mouse model to study naturally occurring AOM. Mice lacking T and/or B cells failed to resolve infections, highlighting the cooperative role of both in clearing middle ear infection. Adoptively transferred antibodies provided complete protection to the lungs but only partially protected the middle ears, highlighting the differences between respiratory and otoimmunology. We present this as a novel experimental system that can capitalize on the strengths of the mouse model to dissect the molecular mechanisms involved in the generation and function of immunity within the middle ear.

Keywords: otitis media, *Bordetella bronchiseptica*, natural infection, adaptive immunity, protective immunity

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INTRODUCTION

It is estimated that bacterial infections of the middle ears, acute otitis media, affect 80% of the human population, usually at some point in their early lives (Bluestone and Doyle, 1988; Alsarraf et al., 1999; Monasta et al., 2012; Vos et al., 2016; Liese et al., 2014; DeAntonio et al., 2016; Tong et al., 2018). Yet despite its prevalence and a long history of human affliction (Goycoolea et al., 2019; Olivé-Busom et al., 2021), the disease has been difficult to study. A large number of these infections are caused by nasopharyngeal pathobionts that reach and colonize the middle ear *via* the Eustachian tube often during viral co-infections (Bluestone et al., 1992; Soriano, 1997; Marom et al., 2012). Clinical studies indicate that the propensity of infants to suffer repeated episodes of these infections is associated with compromised immunity (Urschel, 2010; Bardou et al., 2020; Pichichero, 2020), reflected in lower detected levels of cytokines, circulating antibody titers (Basha et al., 2014), and vaccine-induced memory B cells (Basha et al., 2014; Basha and Pichichero, 2015). However, efforts to gain a more mechanistic understanding of early interactions between otopathogens and host immunity face experimental challenges, underscored by the limited ability of human-specific otopathogen to efficiently ascend the Eustachian tubes of rodents that are commonly used as animal

models to study otitis media (Piltcher et al., 2002; Melhus and Ryan, 2013; Park and Lee, 2013; Davidoss et al., 2018). To overcome this limitation, pathogens are injected directly into their middle ears to establish infections (Park and Lee, 2013). Despite the biological caveats and technical challenges, direct middle ear inoculations have been productively used to establish and study complex aspects of middle ear infections (Babl et al., 2002; Bouchet et al., 2003; Novotny et al., 2021) and remain the leading approach to gain insight into the innate immune mechanisms of host response and pathogen-specific aspects of middle ear infection (Schilder et al., 2016).

One consequence of direct inoculation of pathogens in the ears is that it bypasses the natural progression of infection that initiates from the nasopharynx, reaching the middle ear *via* the Eustachian tube, leaving gaps in our understanding of the complex interactions and host responses gradually unfolding in this context. In particular, it remains poorly understood how immune mechanisms are engaged and how they function in the prevention/resolution of naturally progressing infection, and how they might be subverted by pathogens to allow persistence. A complimentary experimental system that allows the study of the natural progression of middle ear infections and the corresponding host immune responses could substantially improve our understanding and guide efforts to prevent and cure them (Principi and Esposito, 2020).

We have previously described the ability of *Bordetella pseudohinzii* to naturally establish persistent infections in the middle ears of mice, apparently for life (Dewan et al., 2019). Here, we report that the closely related species, *Bordetella bronchiseptica*, a pathogen and persistent colonizer of the upper respiratory tract of many mammals (Parkhill et al., 2003), also naturally establishes robust infections in the middle ears of laboratory mice starting from low numbers of nasally inoculated bacteria. However, unlike *B. pseudohinzii*, which establishes persistent infections, *B. bronchiseptica* is gradually cleared from the middle ears of immunocompetent hosts, presenting an opportunity to study the immunological mechanisms involved in resolving middle ear infections. Using immunodeficient mouse strains and experimental approaches designed to probe immune mechanisms, we establish important contributions of both B- and T-cell functions to the clearance of middle ear infection. The data from this initial examination highlight the considerable advantages in the use of mouse-specific tools of immunology to experimentally investigate both the basic functioning of effective immunity in the middle ear as well as the complex interactions between bacterium and host immune response in the context of natural middle ear infections.

MATERIALS AND METHODS

Bacterial Cultures and Preparation of the Inocula

Bordetella bronchiseptica RB50 was grown on Bordet–Gengou (BG) agar (Becton Dickson, Ref: 248200) with 10% sheep blood (HemoStat, Ref: 644000-1) and 20 μg/ml streptomycin (Acros, Organics) for 2 days at 37°C. Liquid cultures were obtained by growing the bacterium in Stainer–Scholte broth (Stainer and Scholte, 1970) at 37°C with shaking at 200 rpm (VWR, Model: Advanced 3500 Orbital Shaker) overnight. Serial dilutions were performed in PBS to obtain an estimated 500 colony-forming units (CFUs) in 5 μl PBS for the nasal, low dose–low volume inoculation and confirmed by plating.

Mouse Experiments

Wild-type (C57BL/6/J), B-cell deficient [*Ighm*^{tm1Cgn}/J (alias: muMt^{-})], T-cell deficient [*Tcrb*^{tm1Mom} *Tcrd*^{tm1Mom}/J], and *Rag-1*$^{-/-}$ (*Rag1*^{tm1Mom}/J) female mice were obtained from Jackson Laboratories (Bar Harbor, USA) and were housed in a specific pathogen-free facility at The University of Georgia. Mice were inoculated by gently pipetting an estimated 500 CFUs of bacteria suspended in 5 μl PBS, onto the external nares of mice anesthetized with 5% isoflurane (Pivetal, Ref: P151A).

To test for colonization of organs, mice were sacrificed by CO_2 inhalation (1.5 L/min) for removal of organs. Lungs and trachea were collected via ventral dissection of the the thoracic cavity. For nasal cavities: the dorsal bones, internasal septum and soft tissue of lateral and ventral surfaces enclosing the nasal cavities was collected; middle ears were collected by removal of the ventral jaw to expose the left and right bullae which were then carefully excised. Harvested samples were collected in 1 ml of sterile PBS containing ceramic beads (2.8mm, Omni International) and homogenized using a bead homogenizer (Fischerbrand, Model: Bead Mill 24). Serial dilutions were plated on Bordet–Gengou agar to quantify CFUs. The bacterial colonies were identified by their characteristic pale white color, dome-shaped colony morphology, and typical zone of β-hemolytic clearance on blood agar plates. For the time course of colonization, mice were euthanized at the indicated days post-inoculation (dpi).

Adoptive Transfer Experiments

Immune serum for adoptive transfer was prepared from blood collected by cardiac puncture from convalescent mice (56 dpi) inoculated with a high dose of *B. bronchiseptica* delivered to reach the lungs by inhalation (5×10^5 CFUs delivered in 50 μl of PBS). A total of 200 μl of serum was injected intraperitoneally 2 h before mice were challenged with a low dose (500 CFUs) of *B. bronchiseptica* delivered intranasally in 5 μl PBS by inhalation. Single-cell suspensions of splenocytes for adoptive transfer were prepared from spleens collected in RPMI media 1640 (Gibco) from either naive or convalescent (56 dpi) mice inoculated with 5×10^5 CFUs (delivered in 50 μl PBS) of intranasally delivered *B. bronchiseptica*. Single-cell suspensions of splenocytes were prepared by passing the organ through a 70-μM mesh and pipetting several times. Finally, cells were resuspended in 200 μl of PBS to be intraperitoneally injected into recipient *Rag-1*$^{-/-}$ mice.

Histopathology

Forty-eight, 5-week-old, female C57BL/J6 mice were divided into two groups and inoculated intranasally with either 5 μl of PBS or containing 500 CFUs of *B. bronchiseptica*. Mice were evaluated by histopathology on 3, 7, 14, and 28 dpi. Following fixation in neutral-buffered, 10% formalin solution and subsequent

decalcification in Kristensen's solution, transverse sections were made through the middle and inner ear. Tissues were subsequently processed, embedded in paraffin, sectioned at approximately 5 mm, and stained with hematoxylin and eosin. Histopathological examination consisted of evaluation of the ear for the incidence (presence or absence), severity, and distribution of inflammation.

ELISA Assay

Assays were performed according to established laboratory protocols. Briefly, 96-well Nunc microtiter plates (ThermoScientific, Ref: 80040LE 0910) were coated with heat-killed *B. bronchiseptica* and incubated in a humidified chamber at 37°C for 4 h. Following binding, the plate was then blocked with PBS with 0.1% Tween 20 and 1% BSA overnight at 4°C. Four microliters of serum was used to estimate total IgG using two-fold serial dilutions to titrate out end points using goat–anti-mouse HRP-conjugated antibodies (Invitrogen). Reactions were developed with SureBlue™ (Sera Care, Ref: 5120 0076) and terminated with 1 M HCl. Color intensities were determined at an OD of 450 nm and arbitrary titer values were determined to be the reciprocal of the lowest dilution in which an OD of 0.1 was obtained.

Statistical Analysis

Data generated were statistically evaluated by Student's *t*-test and two-way ANOVA using the statistical analyses package of GraphPad Prism (V2.0).

Study Approval

All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA, United States ("Bordetella-Host interaction": A2016 02-010-Y3-A9).

RESULTS

A Natural Example of Efficient Middle Ear Infection in Mice

We had earlier reported that the mouse respiratory pathogen *B. pseudohinzii* persistently colonizes the respiratory tract and middle ears of mice (Ivanov et al., 2016; Dewan et al., 2019). *Bordetella bronchiseptica*, a broad host-range respiratory pathogen of significant veterinary interest (Parkhill et al., 2003; Mattoo and Cherry, 2005), has also been noted to persist indefinitely in the nasal cavities of laboratory mice, but has not been described as colonizing the middle ear. To examine whether the ability to colonize the middle ear is specific to *B. pseudohinzii*, or is shared with *B. bronchiseptica*, we inoculated groups of C57BL/6J mice ($n = 5$) with an estimated inoculum of 500 CFUs of *B. bronchiseptica*, delivered in a 5-µl droplet of PBS to the external nares. The early colonization profile of the respiratory tract (nasal cavity, trachea, and lungs) and middle ears was then examined over the course of 3 days (**Figure 1A**). *Bordetella bronchiseptica* colonized and grew efficiently to roughly 0.5 million CFUs in the nasal cavities and also reached and grew to lower numbers in the trachea (~2.75×10^2 CFUs) of all mice 3 dpi. Only two mice showed evidence of being colonized in the lungs at 3 dpi. Importantly, we observed that four of the five mice had either one or both their middle ears colonized by hundreds of bacteria by day 3 indicating that the pathogen naturally, efficiently, and rapidly colonizes this organ. To confirm this observation and examine the long-term colonization profile of the pathogen in the middle ears, we inoculated groups of C57BL/6J mice ($n = 4$) and examined bacterial colonization loads for days 1, 2 (for the nasal cavity and middle ears), 3, 7, 14, 28, 42, 56, and 100 (for the nasal cavity, trachea, lungs, and middle ears) (**Figure 1B**). *Bordetella bronchiseptica* colonized and grew efficiently in the nasal cavities of all mice, reaching ~5×10^5 CFUs a week after inoculation, then gradually declining to about 10^3 CFUs, but persisting stably in all mice thereafter. However, the pathogen spread poorly to the trachea and lungs, reaching only a few hundred CFUs at day 3 and was not detected by day 56. As seen earlier, colonization of the middle ears began within 24 h following inoculation and bacterial numbers continued to grow to reach a peak of about 10^5 CFUs at 7 dpi. However, by 14 dpi, these bacterial numbers had substantially decreased and continued to decline thereafter, approaching the limit of detection by 56 dpi and were fully cleared from both middle ears of all animals by 100 dpi.

Histopathological analysis of hematoxylin–eosin-stained sections of the middle ears on days 3 and 7 p.i. (peak bacterial load) revealed the presence of *B. bronchiseptica* in the middle ears with a range of severity of inflammation noted, including the moderately inflamed sections shown (**Figure 1C** and **Supplementary Table 1**). While not all mice showed detectable inflammation, these findings indicate that *B. bronchiseptica* can efficiently reach and grow in numbers within each of the middle ears of the mice and can induce significant inflammation within the middle ears. However, the host is capable of suppressing the continued expansion of the pathogen, and ultimately clears the infection from this organ. Importantly, once infection had been cleared, both middle ears remain free of the pathogen indicating the attainment of bilateral protective immunity, despite the persistent bacterial colonization of the nasal cavity.

Clearance of *Bordetella bronchiseptica* From the Middle Ears Is Dependent on Adaptive Host Immunity

The gradual clearance observed in C57BL/6J mice suggested that adaptive immune mechanisms are involved in protecting the middle ears from *B. bronchiseptica*. To test the roles of T and B cells in this, we examined the persistence of *B. bronchiseptica* in the middle ears of *Rag-1*^{tm1Mom} (*Rag-1*$^{-/-}$) mice, which lack mature T and B cells. Groups of *Rag-1*$^{-/-}$ mice were inoculated with *B. bronchiseptica* as above and the numbers determined in the respiratory tract and middle ears thereafter (**Figure 2**). *Bordetella bronchiseptica* colonized and initially grew in the nasal cavities of *Rag-1*$^{-/-}$ mice similar to the wild type. However, after reaching peak levels of nearly a million CFUs at 7 dpi, the numbers did not decrease but persisted at this peak level.

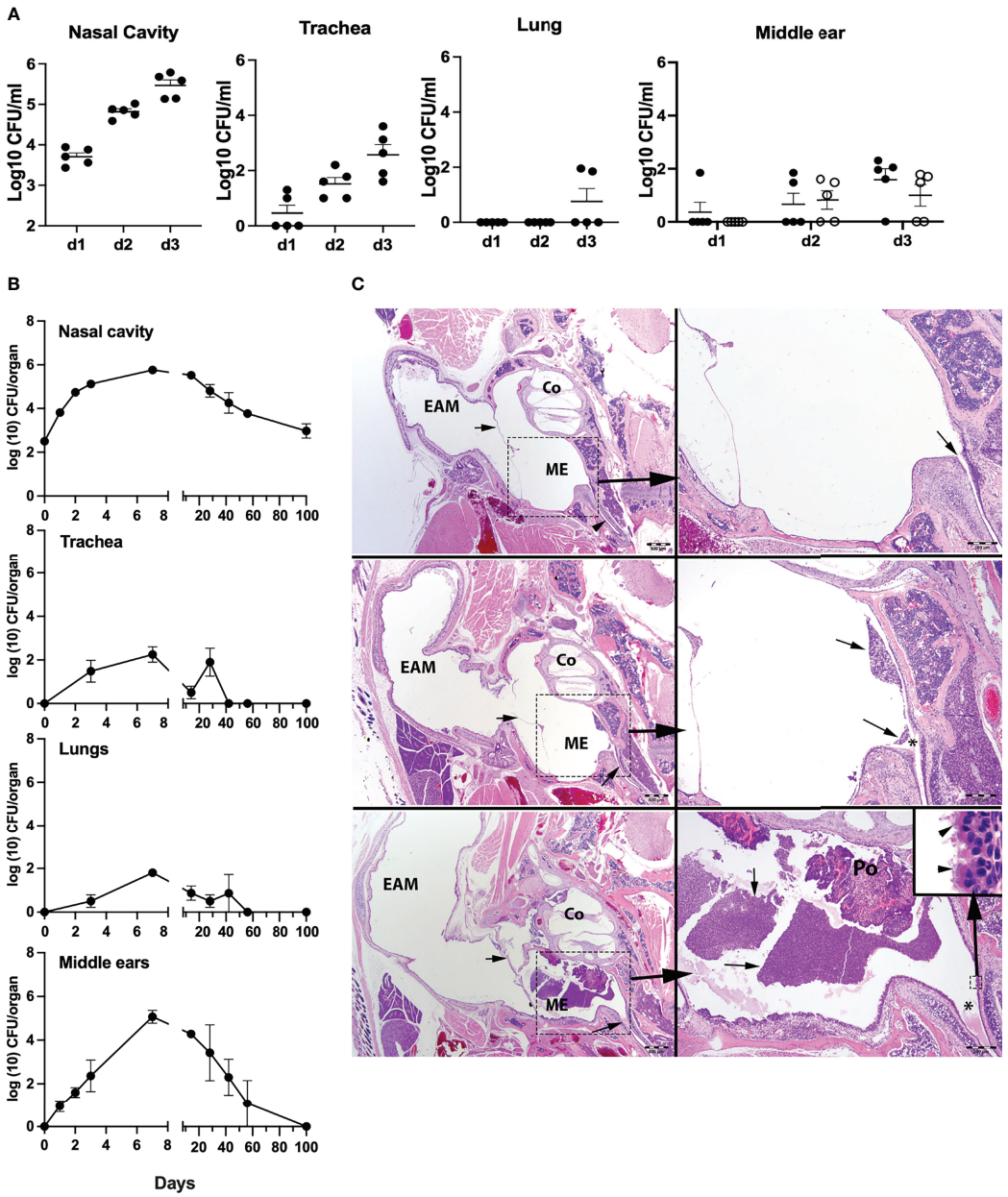

FIGURE 1 | Colonization and growth of *Bordetella bronchiseptica* in the respiratory tract and middle ears of C57BL/6J mice. **(A)** Graphs represent the *B. bronchiseptica* colony-forming units (CFUs) recovered (log_{10}/ml) from the nasal cavity, trachea, lungs, and middle ears (left to right) from individual C57BL6J mice (filled circles, n = 5 per group). For the middle ears, circles represent bacteria recovered from the left (filled circle) and right (open circle) middle ear bulla of an individual mouse. **(B)** Graphs represent the number of CFUs recovered over time from the nasal cavity, trachea, lungs, and middle ears of groups of C57BL/6J mice (n = 4 per group). Filled circles represent the mean value ± SEM. **(C)** Images represent developing inflammation in the middle ears from C57BL/6J mice. HE stains. *Top panel*: uninfected. In the left image, the small arrows point to the tympanic membrane, and in the lower right, to the Eustachian tube as it enters the middle ear. Scale bar = 500 μm. The right-side image represents higher magnification of the dashed boxed region of the middle ear. Scale bar = 200 μm. *Middle panel*: 3 dpi with *B. bronchiseptica*. The left image, the small arrows point to minimal accumulation of inflammatory exudate within the middle ear. Scale bar = 500 μm. The right-side image represents higher magnification of the dashed boxed region of the middle ear. Arrows point to minimal accumulation of neutrophils and macrophages arranged about or near the Eustachian tube (*) as it enters the middle ear. Scale bar = 200 μm. *Lower panel*: 7 dpi with *B. bronchiseptica*. In the left image, the arrowhead points to a moderate accumulation of inflammatory exudate within the middle ear. Scale bar = 500 μm. The right-side image represents higher magnification of the dashed boxed region of the middle ear. Arrows point to a densely cellular collection of neutrophils and macrophages arranged about or near the Eustachian tube (*) as it enters the middle ear, just below an ear polyp (Po; incidental finding). Scale bar = 200 μm. Inset (small dashed box): Bacteria attached to the cilia (arrowheads) on the epithelia lining the middle ear by the entrance of the Eustachian tube. Scale bar = 10 μm. EAM, external auditory meatus; Co, cochlea; ME, middle ear in bony tympanic bulla.

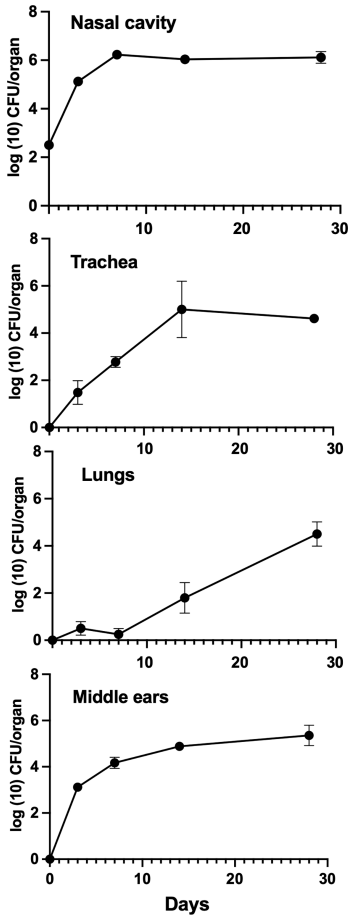

FIGURE 2 | Growth of *B. bronchiseptica* in *Rag-1*$^{-/-}$ mice. Graphs represent the number of *B. bronchiseptica* RB50 CFU recovered from the nasal cavity, trachea, lungs, and middle ears of groups of *Rag-1*$^{-/-}$ mice over time following inoculation. Error bars represent the standard deviation (n = 4).

Bordetella bronchiseptica also colonized the tracheas and lungs of *Rag-1*$^{-/-}$ mice, but unlike in wild-type mice, the numbers of bacteria gradually increased in the lower respiratory tract reaching ~10^4 CFUs by 28 dpi, indicating that T and B cells play a significant role in resisting the spread to and/or growth within the lower respiratory tract. *Bordetella bronchiseptica* also efficiently colonized the middle ears of *Rag-1*$^{-/-}$ mice and grew to similar numbers as the wild type by day 3. Unlike in wild-type mice, where bacterial numbers began to decrease within 2 weeks, the number of CFUs recovered from the middle ears continued to increase, clearly showing that mature B and T cells are critical for controlling and clearing middle ear infections. Bacterial numbers continued to rise beyond day 28 until animals became ill and had to be euthanized (data not shown).

Both T and B Cells Are Required for Controlling Middle Ear Infections

To examine the individual B- and T-cell contributions to the clearance of *B. bronchiseptica*, mice deficient in either B cells or T cells were inoculated with *B. bronchiseptica* as above and bacterial growth and persistence examined over the course of 100 days (**Figure 3**). *Bordetella bronchiseptica* efficiently colonized and grew to similarly high numbers (~10^6 CFUs) in the nasal cavities of both B-and T-cell-deficient mice by 7 dpi and persisted till the end of the experiment (100 dpi). T-cell-deficient mice had higher numbers of *B. bronchiseptica* in the trachea and lungs at most time points, and B-cell-deficient mice appeared to sporadically clear the infection from the lower respiratory tract. However, within the middle ears, *B. bronchiseptica* grew rapidly to over 10^4 CFUs by 3 dpi in both strains of mice. The numbers continued to grow thereafter, plateauing around 10^6 CFU. In summary, T and B cells are of different importance in the lower respiratory tract, but both are required for the control and clearance of *B. bronchiseptica* from the nose and from the middle ear, highlighting differences in immune function in respiratory organs and the middle ear.

Adoptively Transferred Antibodies Partially Protect Middle Ears

Given the important role of B cells in controlling the bacterial load in the middle ear and the contribution of T cells to the efficient generation of an antibody response, we examined the ability of circulating IgG antibodies to protect the middle ears from *B. bronchiseptica*. We had earlier reported (Kirimanjeswara et al., 2003) that adoptively transferred serum antibodies from convalescent mice effectively protected the lower respiratory tract from *B. bronchiseptica* but had little if any effect in the nasal cavity. To examine what protection antibodies could confer to the middle ears, convalescent serum obtained from high-dose *B. bronchiseptica*-infected mice (56 dpi), which generates high titers of anti-*B. bronchiseptica* IgG, was adoptively transferred into groups of *Rag-1*$^{-/-}$ mice (n = 4). Two hours later, the mice were challenged with 5×10^5 CFUs of *B. bronchiseptica* delivered in 50 µl PBS, depositing bacteria in high numbers throughout the respiratory tract. As controls, we included a group of mice that did not receive the antibodies. We then examined the colonization load on 3 dpi.

As shown in **Figure 4**, approximately 10^5 CFUs were recovered from the nasal cavities of both antibody-treated and untreated groups of mice, indicating that serum antibodies had little if any impact in the nose. In contrast, adoptively transferred antibodies nearly and completely cleared *B. bronchiseptica* from the trachea and lungs, respectively. These results are similar to those described by Kirimanjeswara et al. (2003), demonstrating different effects of serum antibodies in the upper and lower respiratory tracts. Interestingly, adoptively transferred antibodies substantially reduced, but did not clear, bacterial numbers in the middle ears. These data indicate that serum antibodies can have a substantial but incomplete impact on *B. bronchiseptica* colonization of the middle ear, and that impact is measurably different from the effects in the upper and the lower respiratory tracts.

Splenocytes Effectively Protect the Lungs and Partially Protects the Nasal Cavity and Middle Ear

As circulating serum antibodies showed limited impact on preventing colonization of the middle ears, we proceeded to

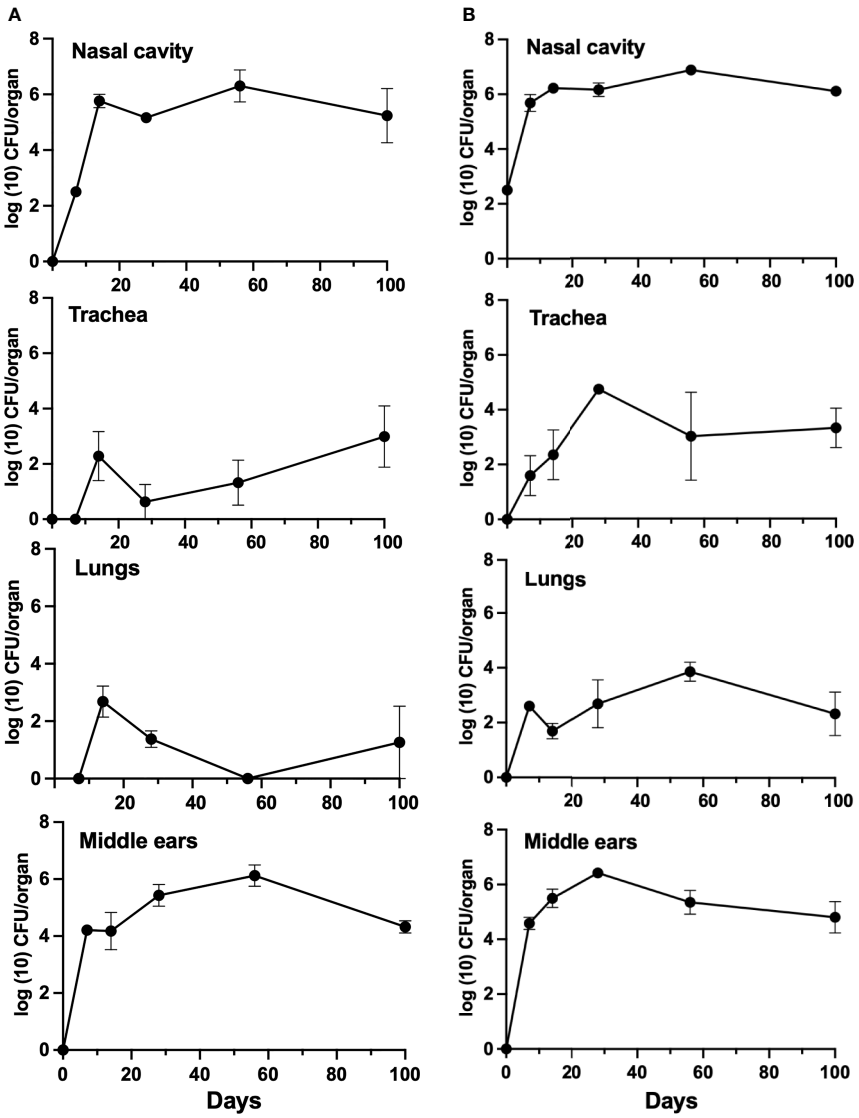

FIGURE 3 | Growth of *B. bronchiseptica* in B- and T-cell-deficient mice. Graphs represent the number of CFU recovered from the nasal cavity, trachea, lungs, and middle ears of groups of B-cell-deficient (left, panel **A**) and T-cell-deficient (right, panel **B**) mice. Error bars represent the standard deviation (n = 4).

examine whether adoptively transferred immune cells of the peripheral lymphoid system were capable of clearing *B. bronchiseptica* from the middle ears. One spleen equivalent of splenocytes from either naive or convalescent (56 dpi) C57BL/6J mice were intraperitoneally transferred into groups of *Rag-1*$^{-/-}$ mice. Two hours after this transfer, the mice were inoculated with 500 CFUs (5 µL PBS) of *B. bronchiseptica* along with an untreated group of mice as a control. After 28 days post-inoculation, the bacterial burden in the respiratory tract and middle ears of the different groups was examined (**Figure 5A**).

While the untreated control group of *Rag-1*$^{-/-}$ mice showed the expected high bacterial burden in the respiratory tract and middle ears, no bacteria could be detected in the tracheas and lungs of immune splenocyte-treated mice and only a few bacteria, close to the limits of detection, were recovered from some mice treated with naive splenocyte (**Figure 5A**). Importantly, for both these groups of treated mice, we observed reduced numbers of bacteria in the nasal cavity and middle ears, indicating that the transferred splenocytes controlled the unrestricted growth of bacteria otherwise observed in *Rag-1*$^{-/-}$ mice and reduced their numbers by ~99% to the levels seen for the wild-type C57BL/6J mice at the corresponding day 28 time point of infection (refer to **Figure 1**). We considered whether this reduction in bacterial load might be antibody mediated, but ELISA assays for total IgG in the sera of these mice showed no significant difference in titers compared with PBS-treated C57BL/6J or untreated *Rag-1*$^{-/-}$ mice (**Figure 5B**).

These observations suggest that middle ear protective immunity is directed *via* T-cell-mediated immune mechanisms,

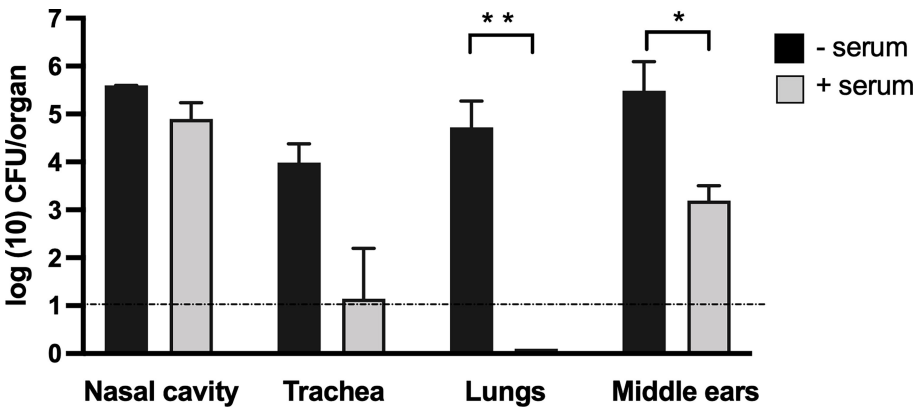

FIGURE 4 | Adoptively transferred convalescent serum protects the lower respiratory tract but is less effective in the nasal cavity and middle ears. Graphs represent the number of CFUs recovered on 3 dpi from the nasal cavity (NC), trachea (TR), lungs (LNG), and middle ears (ME) of groups of C57Bl/6J mice. The adoptively transferred convalescent serum was collected from C57BL/6J mice (light gray columns) or control mice that received no serum (black columns). Dashed line represents the limit of detection. Error bars represent the standard deviation (n = 4). *p < 0.05, ** <0.001.

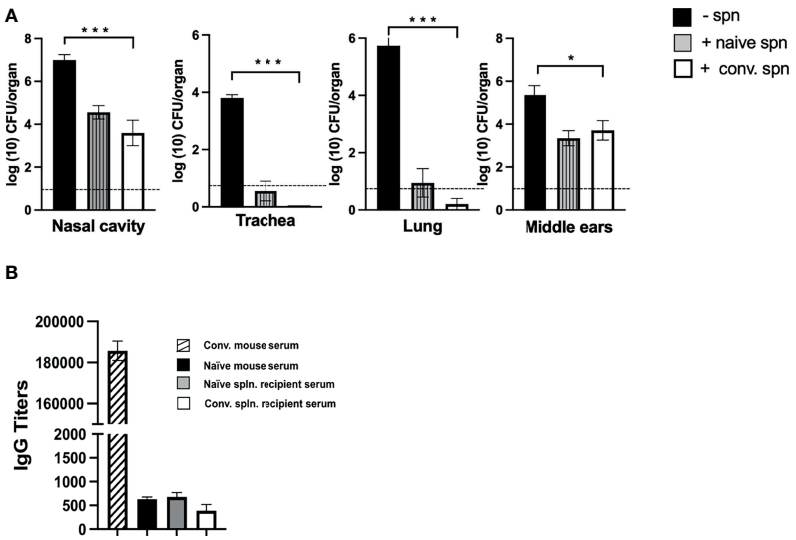

FIGURE 5 | Effect of splenocyte transfer on the growth of *B. bronchiseptica* in *Rag-1*$^{-/-}$ mice. **(A)** Graphs represent the number of CFUs of *B. bronchiseptica* recovered at day 28 post-inoculation from the respiratory tract and middle ears of *Rag-1*$^{-/-}$ mice that either received no splenocytes (black) or mice that received splenocytes from naive mice (dark gray striped column) or splenocytes from convalescent mice (white column) 2 h before being inoculated with 500 CFUs of bacteria (n = 4 mice per group). Error bars represent the standard deviation. Dotted line represents the limit of detection. **(B)** Graphs represent the anti-*B. bronchiseptica* serum IgG titers (arbitrary units) from mice studied in **(A)** [control serum from d56 p.i. convalescent mice (striped column); no splenocytes (black); splenocytes from naive mice (dark gray), convalescent splenocytes (white)]. Error bars: ± SEM; *p < 0.05, *** <0.0001.

likely involving tissue-resident T cells. We did observe a noticeably higher percentage of T-cell populations with resident memory phenotype (CD69^{+}, CD103^{+}) in the middle ears at day 100 compared with uninfected controls (**Supplementary Figure 1**). Although this difference was not statistically significant in this experiment, the possibility arises that these could be contributing to clearance and/or subsequent protection from reinfection of the middle ears which is the focus of ongoing investigations.

DISCUSSION

Clinical studies have associated repeated episodes of middle ear infections with compromised immune status. This is reflected in lower detected levels of cytokines, circulating antibody titers, and memory-specific B cells against vaccine. However, obtaining a more direct mechanistic understanding of the host–pathogen interactions of middle ear infections has been problematic with the lack of models that provide a means to study the progression

of natural disease—i.e., initial colonization, infection, and disease resolution.

Bordetella bronchiseptica, a naturally adapted respiratory tract pathogen in many mammals (Dewan and Harvill, 2019) that is well studied in the respiratory tracts of laboratory mice, also infects their middle ears. When delivered in relatively small numbers in a small volume of PBS that restricts initial colonization to the nasal cavity, infections of the middle ears develop rapidly and consistently, allowing for the study of the natural course of disease progression. We observed that *B. bronchiseptica* rapidly colonizes the middle ears and grows, reaching hundreds of thousands of CFUs in a week: evidence that the bacterium possesses the factors necessary to overcome the physiological and innate immune barriers of the host. What is particularly noteworthy is that despite the bacteria in the nasal cavities persisting at high levels, their numbers in the middle ears begin decreasing after the first week, reaching the limits of detection by 7–8 weeks. These profoundly different outcomes, despite following somewhat similar kinetics of early infection in the nasal cavities and middle ears, suggest qualitative differences in the immune mechanisms between the two associated organs in the upper respiratory tract. Interestingly, the low inoculation dose we employed, while sufficient to launch robust infections in these organs, fails to consistently reach the lower respiratory tract, corroborating the observations that *B. bronchiseptica* is primarily an upper respiratory tract pathogen. In addition, the low inoculation doses used do not generate the high titers of circulating IgG antibodies normally observed when high numbers of the bacteria are inoculated into the lungs, highlighting the potential caveats with using unnaturally large inocula. Associations between low antibody titers in middle ear fluid and acute otitis media were noted decades ago (Sloyer et al., 1976). Our observations that middle ear infections are cleared, despite very low serum IgG antibody titers, suggested that serum IgG antibodies play a modest role in clearing *B. bronchiseptica* from the organ. This fact is further supported by our observation that adoptively transferred convalescent serum, with high titers of *B. bronchiseptica*-specific IgG, cleared >99.9% of the bacteria from the lungs, but had little effect in protecting the nasal cavities and only partially protected the middle ears. However, the observation that muMT^{-} (B-cell-deficient) mice were unable to clear *B. bronchiseptica* from the middle ear suggests that secretory IgA plays an important role. This agrees with the reports of IgA secreting cells being the dominant antibody-producing cells detected by ELISPOT in the middle ear mucosal cells (Suenaga et al., 2001) and with the model of IgA forming B cells being primed in the NALT and migrating to the middle ears to differentiate into plasma cell (Kodama et al., 2000; Kaur et al., 2012).

Interestingly, once *B. bronchiseptica* is cleared from the middle ears, the organ remains protected from subsequent reinfection by bacteria that persist in the nasal cavities, indicative of the acquisition of a middle ear-specific immunity, which, once established, protects the organ from reinfection. T-cell-deficient mice failed to clear middle ear infections indicating a critical role for T cells. Our experiments on the protective effect of adoptively transferred splenocytes in *Rag-1*$^{-/-}$ mice support the hypothesis that subsets of peripheral lymphoid cells are migrating to the upper respiratory tract and middle ears to establish a population of memory cells. We did note a marginal increase in populations with CD4^{+} resident memory cell phenotype (CD69^{+}, CD103^{+}) that arose at later points (100 dpi) in the middle ears of C57BL/6J mice which correlated with the reduction in bacterial loads there (**Supplementary Figure 1**). These may have a role in providing protection from reinfection of the middle ears; however, more detailed investigations on the migration of T cells to the middle ears and of the pathogen specificity of this arising population would need to be investigated. Notwithstanding the increases in resident memory cell populations, the mechanisms involved in the reduction of bacterial loads from the middle ears and those that prevent the organ from being recolonized are unknown and it is the current focus of our investigations.

Although *B. bronchiseptica* is not a primary human pathogen, the efficient colonization of the middle ears of mice and the unparalleled tools available to study the immune response in mice are likely to make this experimental system valuable for the study of the mechanistic detail of the workings of functional immunity in the middle ear. In addition, *B. bronchiseptica* harbors an array of well-studied virulence factors, for which the corresponding deleted mutant strains are available to probe host–pathogen interactions (Cotter et al., 1998; Mattoo et al., 2000; Mattoo et al., 2001; Irie et al., 2004; Dewan et al., 2017; Gestal et al., 2019; Ma et al., 2021). Identifying the factors involved in overcoming physiological barriers to ascend the host Eustachian tube and/or overcome innate host immunity to naturally colonize the middle ears would significantly inform our understanding of how middle ear infections are established. Importantly, as exemplified by the preliminary experiments we conducted here, the mouse model supported with the comprehensive immunological/immunogenetic resources available allows highly incisive questions on features of host immunity that are either induced or suppressed during the control/clearance of middle ear infections.

Finally, it is important to consider the caveats to both the study of human pathogens in non-human hosts they poorly infect and the study of non-human pathogens in their natural hosts (Lairmore and Khanna, 2014). The strengths and weaknesses of each should be acknowledged and carefully weighed against the research question being asked. It may be necessary to embrace the truism attributed to George Box that "All models are wrong. Some models are useful" (Box and Draper, 1987). All models are by default approximations; whether they provide useful information that can inform our understanding and guide our development of improved treatments and preventatives for human disease remains the question.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee, University of Georgia.

AUTHOR CONTRIBUTIONS

KD and EH conceived the study. KD and EH designed the experiments. KD, CS, AC, YS, LM, and UB-M performed the experiments. KD, CS, AC, YS, LM, UB-M, and EH analyzed the data. KD and EH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2021.815627/full#supplementary-material

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Gene Expression Regulation in Airway Pathogens: Importance for Otitis Media

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Otitis media (OM) is an inflammatory disorder in the middle ear. It is mainly caused by viruses or bacteria associated with the airways. *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella catarrhalis* are the three main pathogens in infection-related OM, especially in younger children. In this review, we will focus upon the multifaceted gene regulation mechanisms that are well-orchestrated in *S. pneumoniae, H. influenzae*, and *M. catarrhalis* during the course of infection in the middle ear either in experimental OM or in clinical settings. The sophisticated findings from the past 10 years on how the othopathogens govern their virulence phenotypes for survival and host adaptation *via* phase variation- and quorum sensing-dependent gene regulation, will be systematically discussed. Comprehensive understanding of gene expression regulation mechanisms employed by pathogens during the onset of OM may provide new insights for the design of a new generation of antimicrobial agents in the fight against bacterial pathogens while combating the serious emergence of antimicrobial resistance.

Keywords: gene expression regulation, *Haemophilus influenzae*, *Moraxella catarrhalis*, otitis media, *Streptococcus pneumoniae*

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INTRODUCTION

Otitis media (OM) covers a spectrum of middle ear (ME) inflammatory disorders that are caused by various irritating agents and pathogens. In low to middle-income countries, OM is the main medical condition for antibiotics prescription and surgeries, and deafness in children, respectively. Despite OM is a relatively mild condition that in many cases heals spontaneously, it can cause severe illness and thousands of OM-related deaths have been reported annually (Monasta et al., 2012; Schilder et al., 2016).

Streptococcus pneumoniae (Spn), non-typeable *Haemophilus influenzae* (NTHi), and *Moraxella catarrhalis* (Mcat) are the three main othopathogens isolated from OM patients (Biesbroek et al., 2014; Mills et al., 2015; Lee et al., 2020). The course of othopathogen-dependent OM involves bacterial colonization on the ME epithelium (Hendrixson and St Geme, 1998; Thornton et al., 2011). Bacterial infection in the ME usually occurs as a secondary infection to viral infection in the nasopharynx that subsequently progresses to ME *via* the Eustachian tube (Jossart et al., 1994; Jiang et al., 1999) (**Figure 1**). According to general guidelines in various countries, antimicrobial agents, depending on the age group of children are the treatment of choice of OM. An effective vaccine for

bacterial-related OM is still unavailable (Jalalvand and Riesbeck, 2018). Child immunization programs including conjugated pneumococcal vaccines have, however, decreased the incidence of Spn-related OM in most countries (Littorin et al., 2016; Littorin et al., 2021).

To overcome the hostile environment in the human airway during OM, bacteria are forced to have sophisticated gene regulation expression machineries for optimum survival and adaptation especially in the nasopharynx and ME. In this review, we will mainly focus on the most studied gene regulation mechanisms of Spn, NTHi and Mcat that are associated with OM in the past decade.

Streptococcus pneumoniae

Streptococcus pneumoniae is a Gram-positive diplococcus with capsulated cell wall (Brooks and Mias, 2018). The bacteria asymptomatically colonize (carriage phase) the human upper airway as a part of commensal microbiota in a healthy individual. The carriage phase is essential for pneumococcal pathogenic transition to other sterile sites causing symptomatic infections, such as OM in the ME, in individuals with an immature or weakened immune system (Chao et al., 2014; Coleman et al., 2018; Weiser et al., 2018).

The transition of Spn from nasopharyngeal colonization to ME infection is a multifactorial process. The pathogen needs to adapt to the diverse environmental conditions in the human airway during transition between niches. This includes the varying levels of nutritional status, acidities, oxygen, carbon dioxide, temperature, and a myriad of host antimicrobial factors (Kloosterman and Kuipers, 2011; Manzoor et al., 2015; Paixao et al., 2015a; Paixao et al., 2015b; Man et al., 2017; Aprianto et al., 2018). The establishment of Spn colonization

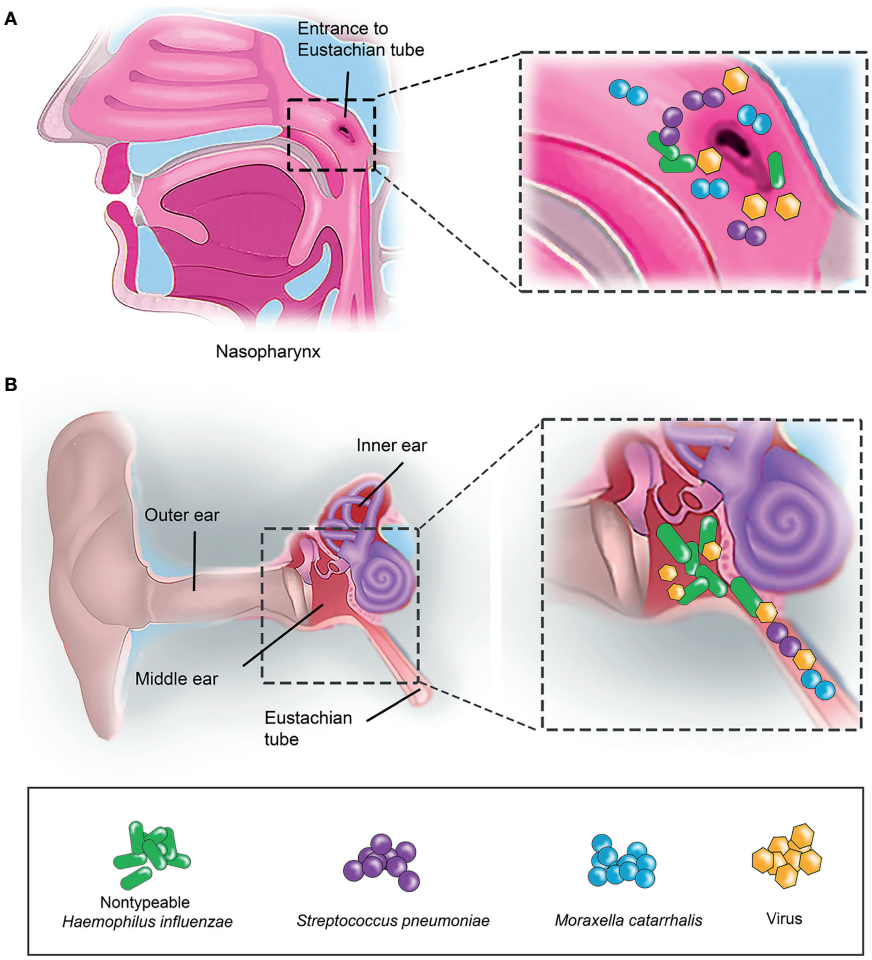

FIGURE 1 | Bacterial colonisation in human nasopharynx and middle ear. **(A)** Nasopharynx is the primary entrance for airway pathogens. Small insert (left panel) indicates the enlarged view of the entrance to Eustachian tube in nasopharynx. Airway pathogens such as nontypeable *H. influenzae* (NTHi) (indicated as green rod), *S. pneumoniae* (Spn) (purple sphere) and *M. catarrhalis* (Mcat) (blue sphere) that initially colonize the nasopharynx as part of the commensal microbiota can, however, migrate into the middle ear (ME) *via* the Eustachian tube. **(B)** Co-infection of airway pathogens and viruses in the ME. Airway pathogens (NTHi, Spn and Mcat) that have successfully entered the Eustachian tube can travel into the ME and colonize as a biofilm. This results in a middle ear inflammation triggered by the host immune response, and subsequently leads to the onset of OM. Virus (yellow hexagon) infection is often preceding the bacterial infection in OM.

in the nasopharynx and ME requires fine-tuned gene expression of a plethora of virulence and metabolism factors in response to the environmental conditions at target niches. Pneumococcal virulence factors, as recently reviewed in detail by Brooks *et al.* and Weiser *et al.*, promote biofilm formation and contribute to pneumococcal adherence to airway epithelial cells, evasion of mucosal clearance by the host immune system, and to outcompete co-colonizing bacteria or resident flora (Brooks and Mias, 2018; Weiser et al., 2018).

Gene Expression During Colonization

Besides the host immune factors, virus infection, the co-colonizing microbiome, and exposure to inhalable particle matters at the nasopharynx and ME can also promote Spn infection in the ME. Host norepinephrine, extracytoplasmic ATP, and nutrients (*i.e.*, N-acetylneuraminic acid and N-acetylglucosamine) released from the damaged nasopharyngeal tissue following a simultaneous viral infection promote pneumococcal dispersal from nasopharyngeal biofilm colonization and migration to the ME (Marks et al., 2013; Chao et al., 2014; Paixao et al., 2015a; Paixao et al., 2015b; Aprianto et al., 2018). Biofilm-dispersing Spn exhibit aggressive growth and virulence phenotypes through their increased production of bacteriocins, virulence factors (capsule, *cbp*A, *psp*A, *ply*, *pcp*A, *nan*A, and *nan*B), proteins of unknown function (SPV_2027 and SPV_2171), and factors related to carbohydrate metabolism, while reducing the expression of genes for competence and adhesion (Gualdi et al., 2012; Allan et al., 2014; Pettigrew et al., 2014; Paixao et al., 2015a; Paixao et al., 2015b; Aprianto et al., 2018). Particle matters (*i.e.*, Asian sand dust, cigarette smoke and black carbon source) induce biofilm formation hence Spn colonization of the human ME epithelium in cell lines and in the ME of mouse OM model (Hussey et al., 2017; Yadav et al., 2020). Genes for biofilm formation (*luxS*), competence (*comA, comB, ciaR*), toxin production (*lytA* and *ply*), detoxification, efflux pumps and osmo-regulator transporters were up-regulated during pneumococcal infection (Cockeran et al., 2014; Manna et al., 2018; Yadav et al., 2020).

Mechanism in Gene Regulation: Pheromone Peptide Signaling

Gene expression in Spn, in response to environmental stimuli, is regulated and synchronized at a population-level through quorum sensing or cell-cell communication systems that are orchestrated by short peptide pheromone-mediated signaling pathways. Three main types of pneumococcal cell-cell communication systems have been discovered so far. They are either mediated by (i) glycine-glycine (GG) peptides, (ii) peptides that signal *via* RRNPP superfamily regulators, or (iii) lanthionine-containing peptides, as depicted in **Figure 2A**. The most well-studied pneumococcal GG peptide signaling is the competence-stimulating peptide (CSP) that is autoinduced hence actively secreted when the surrounding pH, hemoglobin, oxygen levels, antimicrobial stress, and, finally, the bacterial cell density is high (Gagne et al., 2013; Slager et al., 2014; Domenech et al., 2018; Weyder et al., 2018; Paton and Trappetti, 2019; Akhter et al., 2021). A sufficient level of the GG peptide eventually signals the neighboring recipient pneumococci *via* a two-component system (TCS) and alter their gene expression. The CSP signaling is responsible for the induction of (i) competence and transformation in Spn contributing to genetic diversity or as nutrient source; (ii) bacteriocin peptides (CibAB, peptides of the bacteriocin immunity region (BIR), and bacteriocin-inducing peptide (BIP)) for microbial competition; and (iii) biofilm-regulating peptide induced by competence (BriC) that induces the biofilm formation and nasopharyngeal colonization in animal models while altering the fatty acid biosynthesis in the pneumococcal membrane homeostasis for increased adaptation in the host (Valente et al., 2016; Wholey et al., 2016; Aggarwal et al., 2018; Wang et al., 2018; Aggarwal et al., 2021).

Due to a low availability of glucose in the airways, Spn in the nasopharynx and ME relies on the galactose and mannose derived from the airway mucosal glycan lining, as a main carbon source for energy metabolism and virulence (Paixao et al., 2015a; Paixao et al., 2015b). Several recent studies have unveiled the impact of carbohydrate utilization and metabolic processes in Spn virulence, persistence, and infection at nasopharynx, which involves a series of regulators (*gal*K, *gal*R, *hyl, ugl, lacD, nanA, eng*, *raf*K, *est*A, and auto-inducer AI-2) (Afzal et al., 2015; Mclean et al., 2020; Minhas et al., 2021). Sensing of the host carbohydrates for environmental adaptation is mainly carried out by pneumococcal short hydrophobic peptide (SHP) and phosphatase regulator (Phr) that interact with RRNPP superfamily of transcription regulator, regulator gene of glycosyltrasferase (Rgg) and transcription factor regulated by Phr peptide (Tpr), respectively. High abundance of galactose and mannose stimulates SHP144 and SHP939 to autoinduce, whereas induction of PhrA is more related to galactose dependence (Junges et al., 2017; Zhi et al., 2018; Motib et al., 2019). SHP144 and SHP939 imported into the recipient neighboring pneumococci positively regulate their cognate regulator, Rgg144 and Rgg939, respectively. This results in the upregulation of regulons involved in environmental adaptation (*i.e.*, genes for replication and translation, nucleotide metabolism, cell division, ion transport, and capsule production) (Junges et al., 2017; Zhi et al., 2018; Cuevas et al., 2019). The SHP144/Rgg144 signaling also positively regulates the transcription of VP1, a novel virulent GG-peptide of Spn that was highly expressed in chinchilla ME effusions (Cuevas et al., 2017; Cuevas et al., 2019). VP1 activates biofilm development, colonization, hyaluronic acid-dependent attachment of Spn. On the other hand, PhrA imported into the recipient pneumococci inhibits the repressor role of its cognate regulator TprA, activating the transcription of genes for sugar metabolism, neuraminidase activity, and locus of lanthionine-containing peptide for microbial competition during colonization (Hoover et al., 2015; Motib et al., 2017; Motib et al., 2019). Lantibiotic-containing peptides (pneumolancidins, Pld) are another class of pneumococcal bacteriocins that can be autoinduced by histidine kinase receptor signaling in response to the carbon source and cell density (Hoover et al.,

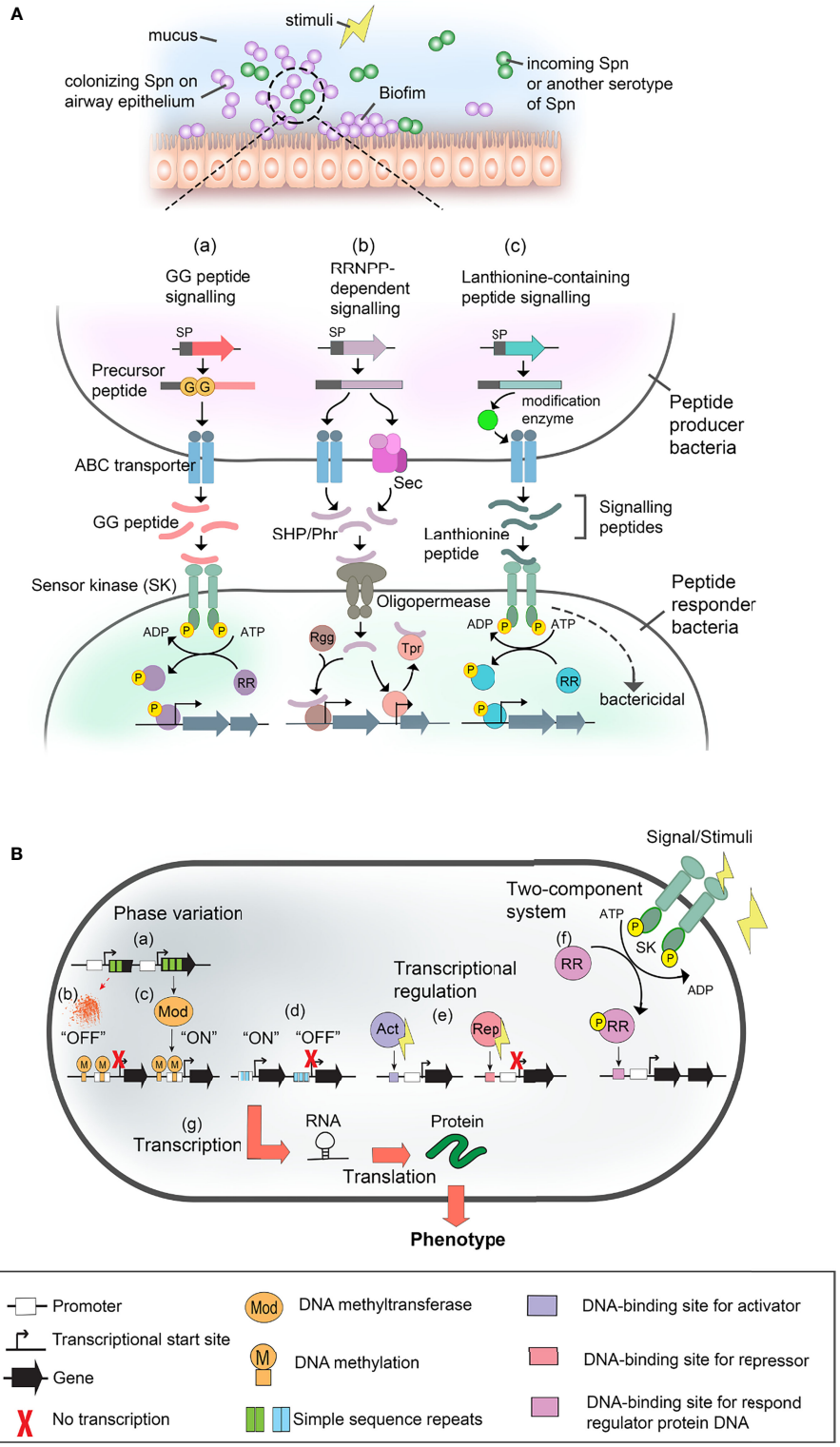

FIGURE 2 | Continued

FIGURE 2 | Gene expression regulatory system in *S. pneumoniae* (Spn), and nontypeable *H. influenzae* (NTHi), and *M. catarrhalis* (Mcat). **(A)** Pneumococcal cell-cell communication systems. Spn colonizing the upper airway mucosa (*i.e.*, at nasopharynx and middle ear) form biofilm and interact with either neighbouring pneumococcal or incoming clone of Spn *via* quorum sensing or cell-cell communication in response to surrounding stimuli (upper panel). Pheromone peptide signalling pathways and their regulatory effect in gene expression during Spn cell-cell communication are shown in the lower panel. Pheromone peptides such as double glycine peptides [GG peptide (*i.e.*, CSP, BriC, VP1)] [as shown in pathway (a)], RRNPP-dependent peptides (*i.e.*, SHR and Phr) [shown in (b)], and lanthionine peptides (*i.e.*, pneumolancidin PldA1-A4) (shown in (c)) are secreted by pneumococci and autoinduced in response to stimuli. Depending on the signal sequence (SP) of the peptides, they are exported *via* ABC transporter or general secretory (SEC) pathway. The precursor peptide is proteolytically processed into active peptide either during transportation by peptidase domain of ABC transporter or membrane-associated proteases, or after secretion *via* unknown extracellular protease. At a sufficient level of the signalling peptide pheromone, they interact with their cognate cell receptors in recipient cells (peptide responder bacteria) such as sensor kinase (SK) of two-component system (TCS) [shown in pathway (a) and (c)]. In canonical TCS, the sensor protein is a histidine kinase (*i.e.*, ComAB, BlpAB and PptAB) that detects exogenous signals, and subsequently, sends a phosphoryl group (yellow sphere) to the cognate response regulator protein (RR). The phosphorylation of the regulator protein results in transcriptional regulation. Alternatively, some peptides such as bacteriocin CibAB and lanthibiotics (pneumolandin) can activate recipient cell directly and induce bactericidal effect. As shown in pathway (b), for RRNPP-dependent peptides such as SHP (*i.e.*, SHP144, SHP939 and RgtS) and Phr (*i.e.*, PhrA), they are transported into responder cell *via* oligopeptide permease system (*i.e.*, AmiACDEF). Once inside the recipient cell, SHPs interacts with their cognate binding partner, Rgg regulators (*i.e.*, Rgg144, Rgg939, abd RgtR), resulting in DNA binding of Rgg and activation of transcription. On the other hand, Phr peptides interact with Tpr regulators that are initially bound to DNA and inhibit the expression of the target gene. Binding of Phr to Tpr results in the releasing of Tpr-mediated inhibition hence activates gene expression. Lastly, lanthionine-containing peptide such as pneumolandin (*i.e.*, PldA1-A4) is processed and translationally modified by intracellular modification enzyme such as LanM before transportation *via* ABC transporter (*i.e.*, LanT) and detected by SK of responder cells (*i.e.*, LanA) [shown in pathway (c)]. This results in either phosphorylation of the response regulator for downstream activation of gene expression, or directly causing bactericidal effect. ABC transporter, ATP-binding cassette transporter; Phr, phosphatase regulator; Rgg, regulator gene of glycosyltransferase; RRNPP, Rap, Rgg, NprC, PlcR and PrgX; SHP, short hydrophobic peptide. **(B)** Schematic representation of gene regulatory mechanism in NTHi and Mcat. Phase variation [shown in (a)] is caused by random mutations in a variable number of simple sequence repeats (SSR) within the open reading frame of DNA-methyl transferases (*i.e.*, ModA and ModM] that alters the gene expression of Mod. Thus, any changes in the variable number of the SSRs as a result of DNA mutation could cause frame shifts in the ORF of *mod*A and potentially lead to premature translation termination and generation of non-functional truncated ModA which is in the "OFF" mode (shown in (b)]. On the other hand, the functional Mod (switched "ON") [shown in (c)] methylates genomic DNA at specific sites that governs transcription in both ways, either by inhibition or activation. The inhibition or activation of transcription depends on the methylated DNA sequence area that potentially bears the recognition sites for regulatory molecules and enzymes. Moreover, the phase-variable number of SSRs [as shown in (d)] can also be found in the transcriptional promoter which, as a result, switches between the "ON" or "OFF" expression status of the target gene (*i.e., hia*). In addition, transcriptional regulators [shown in (e)] can be activators (Act) (*i.e.*, OxyR, Fur) and/or repressors (Rep) (*i.e.*, Fur) of transcription. The regulators are triggered by various stimuli. (f) Another type of transcriptional regulation is a TCS (*i.e.*, FirRS, mesSR, narX/narL) which consists of sensor kinase (SK) and response regulator proteins (RR). The activated regulatory proteins can cause transcription activation and/or repression as described in (e). Finally [indicated in (g)], the genes are transcribed into RNA, followed by translation as protein. Alterations in gene expression are manifested in a bacterial phenotype. Symbols used in panel **(A, B)** are defined at the bottom of this figure.

2015; Maricic et al., 2016). This ultimately benefit pneumococci to outcompete co-colonizing bacteria for space and nutrients during colonization.

LuxS/AI-2-dependent quorum sensing system is also another crucial gene regulatory system in Spn especially for biofilm formation, genetic competence, and fratricide (Trappetti et al., 2011; Yadav et al., 2018). The transcriptomic and mutagenesis studies revealed that LuxS is the central regulator of genes important for Spn virulence and persistence in carriage phase and middle ear infections.

Non-Typeable *Haemophilus influenzae*

Non-typeable *Haemophilus influenzae* (NTHi) is a Gram-negative coccobacillus without capsule that is genetically non-clonal (Erwin et al., 2005; Whittaker et al., 2017). NTHi is part of the nasopharyngeal commensal microbiota. The bacterial species can also, however, cause airway infections such as OM. The currently used vaccine specific to *H. influenzae* type b (Hib) is not effective against NTHi. The pathogen is heme and nicotinamide adenine dinucleotide (NAD) auxotroph, hence requires exogenous supplementation of these elements for growth (Jalalvand and Riesbeck, 2018; Su et al., 2018).

Gene Expression During Colonization

Similarly to Spn, NTHi colonizing the human airway needs to overcome the harsh environmental conditions in the host in order to establish a fulminant infection. Proteomic analysis on NTHi infecting the chinchilla ME revealed the altered expression of 28 bacterial proteins involved in carbohydrate and amino acid metabolism, redox homeostasis, and cell wall-associated metabolic proteins (Harrison et al., 2016). This implies the utilization of glucose by NTHi for aerobic respiration during animal AOM.

Inflammation triggered in the ME upon infection leads to activation of nutritional immunity (Szelestey et al., 2013). This results in the sequestration and restriction of free metal ions available for NTHi iron/heme-dependent metabolic enzymes, regulatory proteins, and aerobic respiration that are essential for bacterial ME colonization (Szelestey et al., 2013; Harrison et al., 2016). Since NTHi is heme-iron auxotroph, the pathogen develops a plethora of iron/heme acquisition mechanisms in response to the host nutritional immunity. This includes the upregulation and expression of a series of core iron- and heme-responsive genes which some are regulated by the NTHi ferric uptake regulator (Fur) (**Figure 2B**) and RNA chaperon Hfq (Harrison et al., 2013; Hempel et al., 2013; Whitby et al., 2013). This in turn promotes the colonization persistence and virulence factor expression in NTHi when infecting the chinchilla ME. Besides the iron uptake genes, Fur also regulates the transcription of small RNA HrrF that is important for molybdate uptake, deoxyribonucleotide synthesis and amino acid biosynthesis (Santana et al., 2014). The high abundance of iron/heme also activates the promoter activity of *pil*A, hence the expression of subunit PilA for the type IV pilus that is essential

for NTHi adherence and biofilm formation (Mokrzan et al., 2019). Moreover, the co-culture with Spn enhances *pilA* expression in NTHi and biofilm formation as well (Cope et al., 2011). Biofilm formation is crucial for NTHi colonization in OM, and the biofilm is also regulated by a TCS transduction system named QseBC/FirRS (ferrous iron responsive regulator/sensor) (**Figure 2B**), which is responsive to low temperature and availability of nutrients (ferrous iron and zinc) (Steele et al., 2012; Unal et al., 2012; Van Hoecke et al., 2016). In addition, high pH in the ME during OM promotes biofilm formation and expression of virulence factors for iron acquisition (Ishak et al., 2014).

Beside the nutritional immunity, NTHi also needs to combat oxidative stress such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated by the airway immune defense. Hence, NTHi upregulated the expression of OxyR regulon (antioxidant enzymes (peroxiredoxin (PgdX) and catalase (HktE)) as shown in the chinchilla ME (Whitby et al., 2012; Parrish et al., 2019).

Mechanism in Gene Regulation: Phase Variation

Phase variation is one of the most studied gene regulation mechanisms in NTHi. It is widely used by NTHi to regulate the expression of virulence factors that are crucial for bacterial colonization at specific niches. Phase variation is a random molecular event that enable a specific gene expression to be reversibly switched "ON" and "OFF". This mechanism is based upon the DNA mutation that results in the formation of variable number of simple sequence repeats (SSR) in the genome (Srikhanta et al., 2005) (**Figure 2B**).

In NTHi, N6-adenine DNA-methyltransferase (ModA) is one of the proteins that have a phase variable expression. The enzyme which is part of the type III restriction-modification (R-M) system methylates bacterial chromosomal DNA at a specific site on the genome, mediating epigenetic regulation (Gawthorne et al., 2012). ModA is encoded by a *mod*A allele. Up to 21 allelic variants of *mod*A (*modA*1-21) exist in NTHi clinical isolates taken from COPD patients and children with ME infection or OM (Atack et al., 2015a; Atack et al., 2019). However, among the identified *mod*A allelic variants, 65% of the isolates carry one of just 5 phase-variable *mod*As, namely allelic variant of *modA*2, 4, 5, 9, or 10 (Fox et al., 2007; Atack et al., 2015a). These alleles contain different numbers of SSRs and hence can alter the expression of ModA. The phase variation of these alleles impacts (i) the gene expression of (a) known NTHi virulence major outer membrane proteins (P2, P5, P6, and HMW) (*modA*2, 4, 5, 9, and 10-dependence) and (b) NTHi proteins involved in antibiotic resistance (mod*A*2, 5 and 10), and (ii) evasion of opsonophagocytic killing (*mod*A4).

In response to the alkaline pH environment occurred in the ME during OM, ModA2 of NTHi mediates the formation of a bacterial biofilm that has greater biomass and stable structure which are critical for NTHi pathogenesis *in vivo* (Atack et al., 2015a; Brockman et al., 2018). NTHi with phase variable *mod*A2 expression (reversibly switch between "ON" and "OFF") has survival and adaptational advantages for colonization at chinchilla ME over an isogenic strain that is unable to phase vary their ModA2 expression. This is explained by the fact that NTHi carrying *mod*A2 that is permanently "OFF" or "ON" is more susceptible to macrophage and neutrophil killing, respectively; whereas strains carrying phase variable *mod*A2 (switchable from "OFF" to "ON") may alter their virulence factor expression for increased antigenic variation hence evasion of the host defense (Brockman et al., 2016; Robledo-Avila et al., 2020). Collectively, the methylation-dependent gene regulation mediated by *mod*A influences the virulence of NTHi especially during infection at ME. In addition, to avoid the host immune system during invasive infection, NTHi also alters the expression of Hia, an immunogenic adhesin, by changing the length of polythymidine tract on the *hia* promoter (Atack et al., 2015b). SSR-dependent phase variation is also used by NTHi for persistence and adaptation in the pathogenesis of other airway infections such as COPD (Poole et al., 2013; Pettigrew et al., 2018; Fernandez-Caldet et al., 2021).

Moraxella catarrhalis

Moraxella catarrhalis is a Gram-negative respiratory opportunistic pathogen. It is categorized into two distinct lineages based on 16S rRNA sequence: (i) ribotype (RB) 1 which comprise of ∼80-90% of isolates associated with adherence and serum resistance; (ii) RB2 and RB3 strains that are detected in ∼10-20% isolates (Bootsma et al., 2000; Verhaegh et al., 2011).

Gene Expression During Colonization

Regulation in gene expression of virulence factors is crucial for Mcat colonization and long-term survival within the host in the human airways. There are a range of extrinsic stimuli that this pathogen must contend with, such as low temperature and iron restricted conditions within the human airway. The environmental factors affect the expression of Mcat major outer membrane proteins that are the key to efficient colonization (Jetter et al., 2010; Spaniol et al., 2011). Notably, genes of resistance-nodulation-division (RND) multidrug efflux systems (*acr*AB and *opr*M) are upregulated at nasopharyngeal temperature (26°C) (Spaniol et al., 2013). Deletion of *acrAB* and *opr*M caused Mcat to lose up to 50% invasion capacity on human pharyngeal epithelial cells (Spaniol et al., 2015).

PilA that mediates Mcat *in vivo* colonization in the chinchilla model was the earliest adhesin to be investigated (Luke et al., 2007). Hoopman *et al*., further elucidated the Mcat colonization *via* DNA microarray analysis which showed the upregulated genes involved in the oxidative stress response, denitrification pathway, in addition to an uncharacterized gene (ORF1550) that is crucial for Mcat persistence (Hoopman et al., 2012). Other well-known adhesin-encoding virulence genes, such as *uspA1*, *uspA2*, *uspA2H*, *mid/hag* and *mcaP*, *mha/mch* and *mcm* also play important roles in protecting Mcat from the serum bactericidal effect by interfering with the host complement pathway factors (Singh et al., 2016; Riesbeck, 2020). A mutation study revealed the impact of an outer membrane lipoprotein (ORF113) on the survival of Mcat in chinchilla (Wang et al., 2014).

Several Mcat virulence factors are growth phase dependent. The strain Mcat RH4 displayed increased expression of *mid/hag* during the lag and stationary phases but fell to lower levels during exponential phase (Riesbeck and Nordstrom, 2006; De Vries et al., 2010). MID/Hag protein is probably essential for initial colonization but not for proliferation. In a separate study, the downregulated expression of *mid/hag* and *uspA2* occurred during infection in chinchilla (Hoopman et al., 2012). MID/Hag expression was also reduced during persistent colonization of Mcat in COPD patients (Murphy et al., 2019). It appears that reducing the profusion of surface proteins could aid Mcat to avoid host recognition.

One of the main modes of Mcat colonizing the ME during infection is *via* biofilms. The synergistic relationship Mcat has with its co-colonizing pathogens increases OM incidence (Blakeway et al., 2018). Mcat secretes beta-lactamase-containing outer membrane vesicles (OMVs) in biofilms which protects co-colonizing pathogens from beta-lactam antibiotics (Armbruster et al., 2010; Schaar et al., 2013). There are interesting facets of biofilm formation which include the quorum-sensing systems found in Spn, NTHi and Mcat. Armbruster *et al.* established the compensation effect of the NTHi *lux*S gene (interspecies quorum signaling factor) in aiding Mcat for persistence in chinchilla since the latter species does not possess a *lux*S homolog (Armbruster et al., 2010; Mokrzan et al., 2018).

Mechanism in Gene Regulation: Phase Variation and Two-Component System

Once Mcat has colonized the host, bacteria regulate the expression of its cell surface components by phase variation. The phase variation mechanism that has been widely studied in Mcat is performed by several DNA methyltransferases including ModM. The DNA methyltransferases are part of R-M system that master mind the gene regulation in Mcat during infection (Blakeway et al., 2018; Phillips et al., 2019) (**Figure 2B**).

Similar to NTHi, Mcat also carries multiple allelic variants of *mod*M (*mod*M1-6). The most common allele of *mod*M present in Mcat isolates is *mod*M2 which regulates genes associated with colonization and host immune evasion (Blakeway et al., 2019). Although the *mod*M3 allele is commonly found within the minor lineage RB2 and RB3 of Mcat strains, the ModM3 methyltransferase is prevalent in Mcat clinical isolates taken from children with OM (Blakeway et al., 2014). Extensive methodologies were used including single molecular real-time (SMRT) methylome analysis to ascertain the methylation activity of ModM3. Also, RNASeq analysis revealed that ModM3 alters the expression of genes involved in biofilm formation, anaerobic tolerance, nitrosative and oxidative stress responses. Specifically, in response to host immune defense-derived nitrosative and oxidative stress, Mcat with "ON" variant of *mod*M3 upregulated the expression of genes *narX/narL*, and a predicted AhpC/TSA family peroxiredoxin RS03200, respectively (Blakeway et al., 2019). Additionally, in a recent study aiming to reveal the correlation between serum resistance and phase variation, Mcat strains that survived in human serum have higher mRNA levels of *uspA2* whereas *mid/hag* and *uspA1* showed reduced expression over a longer time of exposure to the human serum (Tan et al., 2020). Under this selection pressure, variable SSR tract lengths that regulate transcription hence phase variation in *uspA1, uspA2* and *mid/hag* is likely to occur. Antigenic variation of outer membrane proteins is another strategy of Mcat for host immune evasion (Murphy et al., 2019; Tan et al., 2020).

Another gene regulatory mechanism of Mcat is the TCS signaling systems (**Figure 2B**). *mesR* is a gene of this signalling system, and potentially regulates expression of two lysozyme inhibitor genes as a defence against the host immune response (Joslin et al., 2015). Another example of TCS is the *narX/narL* involved in denitrification pathway. This system counteracts reactive nitrogen species produced exogenously and could possibly ensure Mcat survival under anaerobic conditions (Blakeway et al., 2019).

CONCLUSION

The ability to overcome environmental challenges during colonization inside the host is crucial for bacterial survival and persistence, hence establishment of infection. Spn senses the environmental stimuli and regulates its gene expression through short peptide pheromone-mediated signalling pathways and LuxS/AI-2-dependent quorum sensing system. This enables Spn to regulate the switch between biofilm and planktonic phenotype for colonization or invasion, respectively; genetic competence, and fratricide activities, and expression of regulons involved in environmental adaptation and evasion of host immune system. On the other hand, in addition to transcriptional regulators, gene regulation in NTHi and Mcat is mainly mediated *via* phase variation mechanisms that reversibly switches the expression "ON" or "OFF" of the target genes. The system is based on the presence of the variable number of SSRs, as a result of slipped-strand mispairing that occurred either on the promoter or within the ORF, or both, of the target gene. This enables NTHi and Mcat to regulate their biofilm formation, adherence, serum resistance and other virulence phenotype according to growth phase or in response to selective pressure caused by host immune response. The findings discussed in this review could help to improve our understanding regarding the gene expression governing system in these pathogens and can be a potential target of future antimicrobial intervention strategy against Spn, NTHi and Mcat infection.

AUTHOR CONTRIBUTIONS

MJ coordinated the manuscript. MJ, MS, and Y-CS drafted the manuscript. Literature studies of virulence and gene regulation in NTHi, *M. catarrhalis* and *S. pneumoniae* were carried out by MJ, MS, and Y-CS, respectively. Figures were mainly prepared by MJ and MS assisted by Y-CS. All authors edited, critically revised, and approved the final manuscript.

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HB-EGF Plays a Pivotal Role in Mucosal Hyperplasia During Otitis Media Induced by a Viral Analog

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Otitis media (OM), the most common childhood illness, can be caused by bacterial and/or viral infection. Hyperplasia of the middle ear (ME) mucosa is an important component of OM that contributes to its deleterious sequelae. Our previous research revealed that ME mucosal hyperplasia in bacterially induced OM was associated with expression of the heparin-binding epidermal growth factor (HB-EGF) gene, and that HB-EGF induced the proliferation of ME mucosal explants in culture. We used single-cell RNA-Seq to identify ME cells that express *Hbegf* and related genes involved in mediating responses to this factor. To determine the degree to which a viral infection might induce mucosal hyperplasia, and to assess the role of HB-EGF in hyperplasia *in vivo*, we used, Poly(I:C) to simulate a ME viral infection, Western blotting to confirm ME protein expression, and a specific inhibitor to block the effects of HB-EGF during OM. Genes for HB-EGF and its receptor were expressed in the ME primarily by epithelial, stromal and endothelial cells. Poly(I:C) induced prominent ME mucosal hyperplasia, peaking two days after ME injection. Immunostaining revealed that cleavage of proHB-EGF into its soluble form (sHB-EGF) was strongly induced in response to Poly(I:C). Inhibition of the sHB-EGF receptor dramatically reduced the hyperplastic response of the mucosa. The results demonstrate that a synthetic analog of viral double-stranded RNA interaction can induce OM including a strong proliferative response of the ME mucosa, independent of bacteria. They also indicate that HB-EGF is the dominant growth factor responsible for ME mucosal hyperplasia *in vivo*.

Keywords: hyperplasia, EGFR, poly(I:C), mucosal proliferation, heparin-binding epidermal growth factor

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INTRODUCTION

Otitis media (OM), one of the most common childhood infections (Casselbrant et al., 1993), can be a serious disease. In the US, OM causes more pediatrician visits, antibiotic prescriptions, and surgeries than any other condition for children under 5 years of age (Rovers, 2008). It is estimated to cost $5 billion per year in healthcare expenses and lost productivity (Teele et al., 1988; Rosenfeld and

Bluestone, 2003). Chronic and recurrent OM occur in ~15% of children (Pichichero, 2016) and cause persistent hearing loss that has been linked to delays in speech (Friel-Patti and Finitzo, 1993), and language acquisition (Klausen et al., 2000), as well as deficits in learning (Williams and Jacobs, 2009).

OM is frequently polymicrobial, involving multiple bacterial genera/species and/or viruses (Ruohola et al., 2006; Marom et al., 2012). Clinical and experimental studies have shown that infection of the ME induces inflammatory responses, a major feature of which is ME mucosal hyperplasia. The mucosa of the resting ME consists primarily of a simple squamous epithelial monolayer and a minimal stroma, with a thickness of approximately 15-30 μm (Lim and Hussl, 1969; Lim, 1976). During OM, the mucosa rapidly transforms. The epithelium proliferates and remodels into a pseudostratified, respiratory-like epithelium that includes secretory, goblet, and ciliated cells. The stroma also proliferates, adding fibrocytes, connective tissue and neovascularization. The thickness of the mucosa can increase by tens to hundreds of μm in 2-3 days, reducing effective ME volume (Lim, 1979; Hernandez et al., 2008; Leichtle et al., 2010). Increased permeability of the enhanced vascular bed leads to infiltration of the ME lumen by fluid and leukocytes. Mucus and inflammatory mediators are also secreted into the lumen by ME cells (Ryan et al., 2020). Thus, hyperplasia of the ME mucosa is a major driver of inflammatory pathophysiology in OM.

While studies in animals have confirmed that bacterial infection induces mucosal hyperplasia, whether a purely viral infection also does so is less clear. The few experimental studies of virus injection directly into the ME have yielded contradictory results. Respiratory syncytial virus introduction induced very mild inflammation in the guinea pig ME (Berglund et al., 1966; Berglund, 1967), while influenza virus produced a vigorous response in the chinchilla ME, including mucosal hyperplasia similar to that in bacterial OM (Chung et al., 1993).

ME inflammation is generated in acute OM by the interaction of pathogen molecules with innate immune receptors, including the TOLL-like receptors (TLRs) and others (Muñoz-Wolf and Lavelle, 2016). These activate well-known inflammatory pathways leading to the expression of inflammatory mediators such as tumor necrosis factor alpha (TNFα) and interleukin 1 beta (IL-1β) (Kurabi et al., 2016). The mechanisms by which inflammation leads to ME mucosal hyperplasia are unclear. However, by analyzing the ME transcriptome in a mouse model of bacterial OM, we previously found that heparin-binding epidermal growth factor (HB-EGF) is up-regulated with kinetics suggesting a possible role in mucosal growth (Hernandez et al., 2015). Moreover, it was the only up-regulated growth factor capable of strongly stimulating ME mucosal explant expansion in culture (Suzukawa et al., 2014). It remains to be established whether HB-EGF mediates ME mucosal hyperplasia *in vivo*.

HB-EGF is a member of the EGF family of growth factors. It is expressed as a membrane-anchored glycoprotein pro-form (proHB-EGF). Various stimuli, including pro-inflammatory cytokines such as TNFα and IL-1β (Yoshizumi et al., 1992; Dluz et al., 1993), induce proteolytic processing of proHB-EGF to release a mature soluble peptide (sHB-EGF). The soluble form can interact with two EGF receptors, EGFR and ERBB4, in a paracrine and/or autocrine manner to induce responses. sHB-EGF binds to EGF receptors with higher affinity than EGF. These include cellular proliferation and differentiation leading to tissue growth (Dao et al., 2018). Which cells in the ME produce HB-EGF or its receptors is also unknown.

The innate immune response to viruses is distinct from that to bacteria, involving different pathogen receptors. In viral infection, viruses can produce double-stranded RNA (dsRNA) during replication within infected cells (Weber et al., 2006). Host cells recognize double-stranded RNA primarily *via* Toll-like receptor 3 (TLR3) (Matsumoto and Seya, 2008), as well as the nucleic acid receptors retinoic-acid-inducible gene I (RIG-I) and melanoma-differentiation-associated gene 5 (MDA-5) (Yoneyama et al., 2004; Gitlin et al., 2006). Stimulation of TLR3, followed by interaction with the adaptor protein TRIF, or of RIG-1 or MDA5, increases the release of type-I interferons and activates the nuclear factor (NF)-κB pathway leading to inflammatory cytokine production, as a host defense system (Jacobs and Langland, 1996; Matsumoto and Seya, 2008). In the current study Polyinosinic-polycytidylic acid [Poly(I:C)] was employed to mimic viral challenge. Poly(I:C) is a potent agonist of TLR3, MDA5 and RIG-1 (Jensen and Thomsen, 2012) and an established model of viral infection (Miyamoto et al., 2004; Matsumoto and Seya, 2008). In other tissues, exposure to poly(I:C) upregulates the expression of both viral innate immune signaling and effector genes (Li et al., 2012; Parvizi et al., 2012). Whether activation of viral innate immunity in the ME leads to mucosal hyperplasia, and if so whether this involves HB-EGF, remains to be clarified.

Herein, we mined single-cell RNA-Seq (scRNA-Seq) databases of the mouse ME before and after bacterial infection, to identify ME cells that produce HB-EGF and its receptor mRNAs during OM. We then tested the hypothesis that a synthetic analog of viral double-stranded RNA can induce OM that includes mucosal hyperplasia, and that this *in vivo* hyperplasia is mediated by HB-EGF.

MATERIALS AND METHODS

Animals

Studies were conducted using C57BL/6 mice of both sexes, and male Sprague-Dawley rats (60-90 days for each species). Female rats were reserved for breeding, as there are no data suggesting differences in OM pathogenesis due to sex, although incidence is higher in human males (Kvestad et al., 2004). Mice were employed to take advantage of their well-defined genetics. Rats were used for inhibitor studies because their ME volume (50 μl) is much greater than the mouse (5 μl), making these studies more practical. All experiments were performed to National Institutes of Health guidelines and approved by VA San Diego Medical Center IACUC (protocol 13-031 for mouse and 13-032 for rat).

Surgical Procedure

For scRNA-Seq, mouse otic bullae were exposed *via* a ventral approach under deep anesthesia (ketamine 50 mg/kg, xylazine 1

mg/kg, acepromazine 5 mg/kg in.05 ml, IP) as previously described in Melhus and Ryan (2000). A small hole was made using a 23-gauge needle. MEs were injected with $5x10^4$ nontypeable *Haemophilus influenzae* (NTHi) CFUs in 5 µl of saline. During surgical recovery animals were monitored carefully, placed on a warming pad, and given buprenorphine (0.05 mg/kg) through s.c. injection to limit post-operative discomfort. For Poly(I:C) studies, rat MEs were similarly exposed and 500 µg of Poly(I:C) dissolved in 40 µl of sterile saline was injected. Control animals for both species were injected with saline alone.

Single-Cell RNA-Seq

The expression of the *Hbegf*, *Egf*, *Egfr* and *Erbb4* genes in individual cell types of the ME was assessed in an existing murine scRNA-Seq dataset of normal ME (Ryan et al., 2020) and also in data from MEs inoculated 24 hours previously with NTHi. Twenty-four hours was chosen to precede protein and cellular reactions which peaked at 2 days after Poly(I:C) injection. To obtain the data, three groups of six normal or six infected young adults C57BL/6 mice were sacrificed, and the ME tissue and luminal contents harvested from the 12 MEs of each group. Briefly, tissue was enzymatically digested and dissociated into single-cell preparations. Single-cell libraries were prepared using the Chromium Controller (10X Genomics, Pleasanton, CA) according to the manufacturers' instructions and sequenced on an Illumina HiSeq 2500 (Illumina, San Diego). Reads were aligned to a murine reference genome and subjected to principal component analysis (PCA) clustering. Cell types identified based on well-recognized marker genes. Expression of *Hbegf*, Egf, *Egfr* and *Erbb4* mRNA unique molecular identifiers (UMIs) for the various cell type clusters was visualized using the cLoupe program (10X Genomics) cluster and violin plot displays. More detailed methods are available in Ryan et al. (2020).

Measurement of ME Mucosal Thickness in Poly(I:C) OM

Six rat otic bullae were harvested from 3 rats at 1, 2, 3, 4, or 5 days after injection of Poly(I:C). The bullae were immersed in 4% paraformaldehyde (PFA), post-fixed in PFA for 2 days and subsequently decalcified in 10% EDTA with 4% PFA for 1 week. The tissue was paraffin-embedded, sectioned at 8 µm and mounted on silane-coated slides. Deparaffinized and hydrated sections were stained with hematoxylin and eosin (H&E). Mucosal thickness was measured morphometrically (FSX-BSW, Olympus) in three sections through the middle of the ME cavity, at six standardized locations, and the values averaged for each ME.

Immunostaining

Deparaffinized and hydrated sections of control rat MEs, and Poly(I:C)-injected MEs harvested 2 days later, were heated in citrate solution (pH 6.0) for antigen retrieval. After quenching endogenous peroxidases, sections were incubated with anti-HB-EGF antibody (rabbit polyclonal, 1:200, Bioss) that recognizes both forms of the factor for 24 hours. Immunoreaction was detected by ImmPRESS mouse/anti-rabbit secondary antibody (Vector Laboratories) developed with 3, 3'-diaminobenzidine (DAB) chromogen (Vector Laboratories). Slides were counterstained only with hematoxylin.

Western Blot

Rat ME mucosae from 4 MEs were harvested on days 1, 2, 3, 4, or 5 after injection of Poly(I:C) and homogenized in protein extraction buffer: 500 µl of T-PER lysis buffer (Pierce, Rockford, IL) supplemented with protease inhibitors (Roche, Indianapolis, IN) and sonicated briefly on ice. Three independent samples, from 4 MEs each, were generated at each time point. Protein concentrations were measured using a NanoDrop 2000 (Thermo Scientific). Ten µg of protein in 25 µl was loaded in each well of Bis-Tris Mini Gels 4-12% (Life Technologies) and electrophoresed. Proteins were electrotransferred to polyvinylidene difluoride membranes (PVDF) (Bio-Rad). PVDF membranes were probed with anti-β-actin antibody (mouse monoclonal, 1:10000, BD Transduction Laboratories) and the antibody against HB-EGF (rabbit, polyclonal, 1:3000, Bioss) that recognizes both forms of the factor, with which sHB-EGF can be distinguished from proHB-EGF (18.7 kDa) by its lower molecular weight (9.7 kDa). Membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse antibody for β-actin, 1:20000) (anti-rabbit antibody for HB-EGF, 1:10000) and visualized with chemiluminescent detection (GE Healthcare). Light emission was captured with autoradiography film. Labeled band molecular weight was determined by comparison with a standard molecular weight ladder. The intensity of the bands corresponding to sHB-EGF and β-actin were quantified using ImageJ software and sHB-EGF band intensity was β-actin normalized. Values for the three independent samples at each time point were averaged.

HB-EGF Inhibition

Diphtheria toxin uses membrane-bound proHB-EGF as its receptor, mediated by an EGF binding domain in the toxin molecule (Mitamura et al., 1995). CRM197 is a detoxified mutant of diphtheria toxin. It specifically and irreversibly binds the EGF domain of sHB-EGF, preventing its interaction with EGF receptors (Miyamoto et al., 2004). EC50 in reducing the proliferation of cancer cells *in vitro* is ~3 µg/ml (Tang et al., 2012).

After confirmation of the time of maximal mucosal thickness induced by poly(I:C) injection (2 days), the mucosal thickness was evaluated for the following six groups of rats. Group 1 MEs were injected with 40 µl sterile saline. Group 2 MEs were injected with 500 µg of Poly(I:C) in saline. Group 3 was injected with 500 µg of Poly(I:C) plus 4 µg (100 µg/ml) of CRM197 (Reagent Proteins). Groups 4-6 were injected with Poly(I:C) plus 10, 1 or 0.1 µg/ml, respectively, of CRM197.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism 5 software. Data are reported as means ± standard errors of the means (SEM). Two-way ANOVA with Bonferroni correction for multiple comparisons was performed on measures of mucosal thickness. Data normality was evaluated by using the D'Agostino-Pearson omnibus test. Left and right ears in each mouse were considered to be independent of each other, as

previously discussed in detail (Ebmeyer et al., 2005), and therefore were analyzed independently. Western blot relative band intensities were analyzed by Kruskal-Wallis nonparametric ANOVA, also with Bonferroni correction.

RESULTS

Expression of *Hbegf*, *Egfr* and *Erbb4* Genes in Cells of the Murine ME Before and After Bacterial Infection

Single-cell RNA-Seq was employed to determine whether genes related to HB-EGF signaling are expressed in the ME, and to identify the cells involved. In the untreated ME (**Figure 1A**-0h) *Hbegf* was expressed strongly by most cells in five epithelial cell clusters and a substantial minority of vascular endothelial cells, but also by small numbers of other ME cell types. *Egfr* was expressed by a significant minority of epithelial and stromal cells, but not by other cell types. *Egf* mRNA was observed in only a few scattered epithelial, stromal and leukocytic cells and *Erbb4* mRNA in only a few epithelial cells (not shown). At 24 hours after NTHi inoculation (**Figure 1B**), *Hbegf* was observed in all mature epithelial cells, and in a substantial minority of stromal, intermediate epithelial and endothelial cells. Smaller numbers of cells in leukocyte clusters also expressed this gene. *Egfr* mRNA was also observed at high levels in all mature epithelial cells, in the majority of stromal, intermediate epithelial and endothelial cells, and in much small minorities of other cell types. *Egf* mRNA was observed in only a few scattered epithelial, stromal and leukocytic cells and *Erbb4* mRNA in only two epithelial cells (not shown).

Poly(I:C) Induces ME Mucosal Hyperplasia

To determine whether a model of viral infection would induce OM as evidenced by mucosal hyperplasia, 500 μg of the viral analog Poly (I:C) was injected into the rat ME. This resulted in acute hyperplasia of the ME mucosa ($p < 0.01$). Changes in mucosal thickness from normal (16 μm [SEM = 3 μm]) primarily reflected expansion of the mucosal stroma. The mucosal epithelium added somewhat fewer cells, but the epithelial cells assumed a more cuboidal morphology (**Figure 2A**). Mucosal thickness peaked at 2 days after injection at 112 μm (SEM = 27 μm), and then gradually recovered to near normal by day 5 (**Figure 2B**). Compared to bacterial OM (Hernandez et al., 2008; Leichtle et al., 2012), fewer leukocytes were observed in the ME lumen during poly(I:C) OM.

Poly(I:C) Increases ME Processing of HB-EGF Into Its Soluble Form

While our gene expression data confirm ME expression of the *Hbegf* gene during OM, they do not determine whether it is

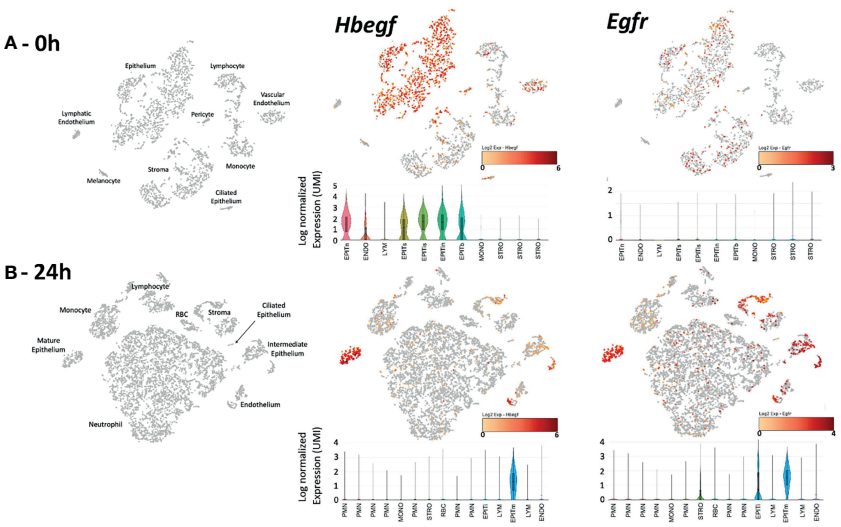

FIGURE 1 | Expression of *Hbegf* and *Egfr* genes in the different cell types of the normal **(A-0h)** and infected **(B-24h)** mouse ME. Clustering of ME cells (in gray) and their identification is displayed in PCA plots, with cells expressing of *Hbegf* and *Egfr* shown in a continuum from pale organ to red indicating relative expression level according to each scale. Violin plots provide quantitative analysis, with height showing relative intensity of expression and width indicating the number of cells (UMIs) at that expression level. **(A-0h)** *Hbegf* is expressed primarily by five clusters of ME epithelial cells representing recognized ME subtypes including basal (EPITb), secretory (EPITs), intermediate secretory (EPITis), intermediate non-secretory (EPITin) and non-secretory including ciliated (EPITn) (Ryan et al., 2020), by vascular endothelial cells (ENDO) and by small subsets of other cells. *Egfr* is expressed by subsets of all epithelial and stromal (STRO) cell clusters. **(B-24h)** Twenty-four hours after NTHi infection, large numbers of infiltrating neutrophils (PMN) in six clusters, monocytes (MONO) and lymphocytes (LYM) dominate the ME, with structural cell types in smaller proportions and epithelial cells in only two clusters: mature (EPITm) and intermediate (EPITi). The different PMN subtypes reflect the increasingly recognized and as yet poorly understood diversity of neutrophils (e.g. Hesselink et al., 2019). As in the normal ME, *Hbegf* is expressed primarily by epithelial and vascular endothelial cells. Normalized gene expression by epithelial cells was higher than in that in the uninfected ME (mean of 2.42 $\log_2$ UMIs versus 1.58 $\log_2$). *Egfr* mRNA is present in most epithelial and stromal cells, but also in vascular endothelial cells. Epithelial cell (0.86 $\log_2$ UMIs) and stromal cell (0.50 $\log_2$) expression was higher than that in the uninfected ME (0.11 $\log_2$ for both) In PCA plot cells, the color orange indicates detectable expression of the gene.

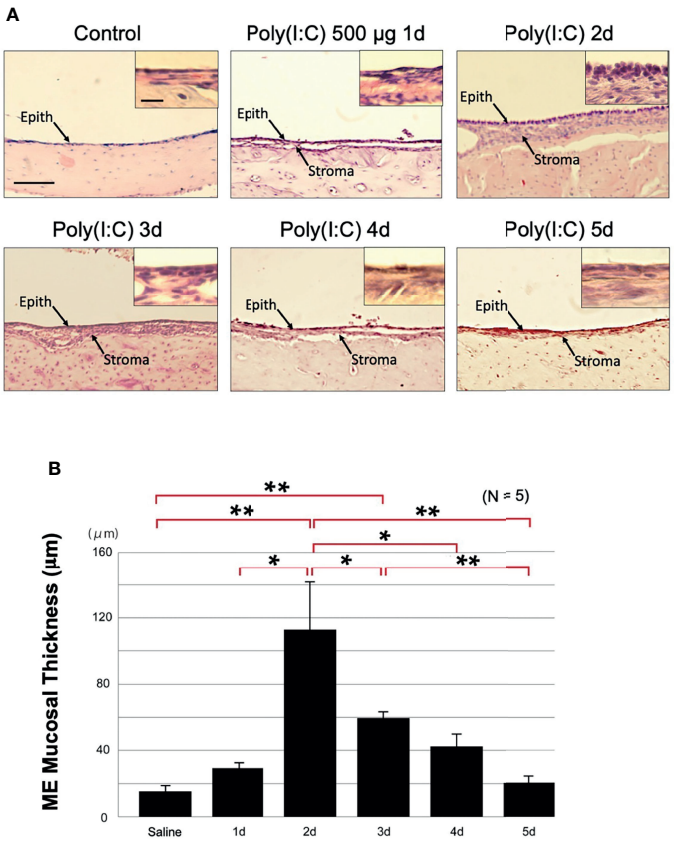

FIGURE 2 | Poly(I:C) enhances rat ME mucosal thickness. **(A)** Representative microphotographs of rat ME mucosa are shown. Control ME is saline-injected at 2 days. Mucosal thickness is maximal 2 days after administration of 500 µg of Poly(I:C) then gradually recovers, becoming similar to the control on day 5. Scale bars: 100 µm for main image, 25 µm for insert **(B)** Morphometrically measured mucosal thickness confirms increase to a maximum at day 2 after administration of Poly(I:C), then a gradual decrease to the control level by day 5. N refers to number of MEs. *$p < 0.05$; **$p < 0.01$.

processed into the soluble form that activates EGF receptors. Western blotting of rat ME tissue with an antibody against HB-EGF (**Figure 3A**) showed essentially none of the soluble form prior to poly(I:C) injection (relative band intensity 2%, SEM = 1%) and a barely detectable band (12%, SEM = 9%) on day 1. This was followed by a strong increase that peaked at 2 days after injection (47%, SEM 19%; $p < .01$), a more than 20-fold increase), and then gradually decreased to almost undetectable at Day 5. Relative sHB-EGF band intensity, shown quantitatively in **Figure 3B**, was highly significant when compared to pre-injection levels. While proHB-EGF was not analyzed by Western, our scRNA-Seq data indicate that expression levels of the *Hbegf* gene by ME epithelial cells increased at 24 hours after bacterial inoculation.

An Inhibitor of sHB-EGF Interaction With EGF Receptors Decreases Mucosal Hyperplasia During Poly(I:C)-Induced OM

To determine the degree to which sHB-EGF might contribute to mucosal hyperplasia, we used a specific EGFR inhibitor. **Figure 4A** shows representative photomicrographs of the mucosa from a control rat ME, and a ME two days after injection of poly(I:C) or poly(I:C) plus 100 µg of CRM197, while **Figure 4B** provides a quantitative analysis of mucosal thickness observed with various CRM197 dosages. The increase in thickness of the mucosa observed after poly(I:C) injection, 112 µm (SEM = 27 µm) was largely absent in the ME injected with poly(I:C) plus 100 µg CRM197, 13 µm, (SEM = 4 µm). This difference was highly significant ($p < 0.01$). Significant reduction was also seen at a CRM197 dosage of 10 µg ($p < 0.01$).

Expression of HB-EGF Is Not Affected by CRM197

CRM197 is a receptor inhibitor, but given the potential for receptor feedback, we used immunohistochemistry to determine whether the inhibitor also influenced the amount of HB-EGF expressed in the ME. **Figure 5** shows representative images of the rat ME mucosa immunostained with an antibody against both forms of HB-EGF. Strong staining was observed in the control ME, the ME injected with poly(I:C) or the ME

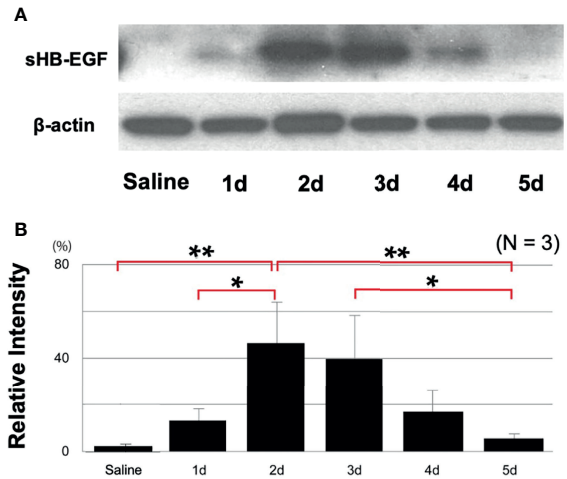

FIGURE 3 | Western blotting demonstrates HB-EGF processing during OM. **(A)** Representative blots for rat MEs are shown. The processed form, sHB-EGF, was distinguished from proHB-EGF by its lower molecular weight, **(B)** Relative band intensity quantification confirms that sHB-EGF increases to a maximum 2 days after administration of 500 μg of Poly(I:C), then progressively decreases. Saline control mucosa at 2 days shows a low sHB-EGF level. N refers to number of samples. *p < 0.05, **p < 0.01.

injected with poly(I:C) plus CRM197, indicating minimal effects of the inhibitor on HB-EGF production.

Processing of HB-EGF Into Its Soluble Form Is Not Affected by CRM197

Receptor feedback might also have influenced the processing of HB-EGF into sHB-EGF. **Figure 4** shows Western blotting with an anti-HB-EGF antibody of bands corresponding to the weight of sHB-EGF, in normal MEs and in MEs injected with poly(I:C) or CRM197 plus poly(I:C). The level of sHB-EGF was low in controls (mean relative intensity 5%, SEM 8%), and increased after Poly(I:C) (43%, SEM 21%; p <.01), a more than 8-fold difference. Moreover, the inhibitor did not affect the level of processed HB-EGF (44%, SEM 16%), indicating no effect on processing.

DISCUSSION

Several novel observations were made in this study. ME administration of Poly(I:C) induced OM that included mucosal hyperplasia and leukocyte infiltration. The ME cells of origin of HB-EGF and its primary receptor were identified, both for the normal ME and during OM. Finally, inhibition of HB-EGF signaling reduced mucosal hyperplasia to a level not significantly different from that in the uninfected ME.

The fact that Poly(I:C) induced the major pathophysiologic elements of OM in the rat ME is perhaps not surprising, since dsRNA interaction with TLR3 and other nucleic acid receptors including MDA5 and RIG-1 induces not only interferons, but also many of the same inflammatory mediators produced by bacterial infection (Jensen and Thomsen, 2012). However, this observation indicates that purely viral OM would likely induce significant ME pathology that was resistant to antibiotic therapy. This conclusion is supported by the results of Chung et al. (1993) that influenza virus inoculation of the ME produced hyperplasia, but not by the results of Berglund et al. (1966; Berglund, 1967) that RSV injection produced minimal ME mucosal growth.

ScRNA-Seq determined that *Hbegf* mRNA is produced primarily by subsets of ME epithelial, endothelial, and stromal cells, as is *Egfr* mRNA. Moreover, as can be seen in **Figure 1**, the

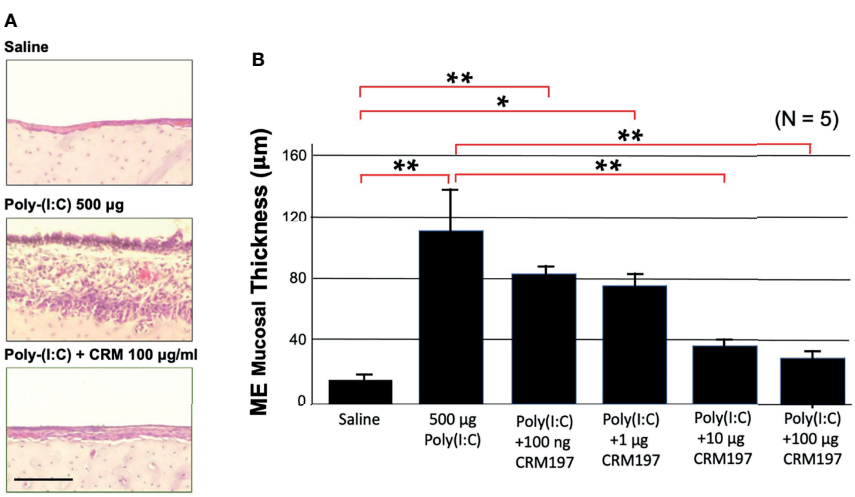

FIGURE 4 | An HB-EGF inhibitor decreases mucosal thickness during OM. Poly(I:C)-induced hyperplasia of rat ME mucosa was inhibited by co-administration of CRM197. **(A)** Representative microphotographs are shown on day 2. Scale bar = 100 μm. **(B)** Mean thickness of saline ME mucosa versus 500 ug of Poly(I:C), without or with various levels of CRM197 on day 2. N refers to number of MEs. *p < 0.05; **p < 0.01.

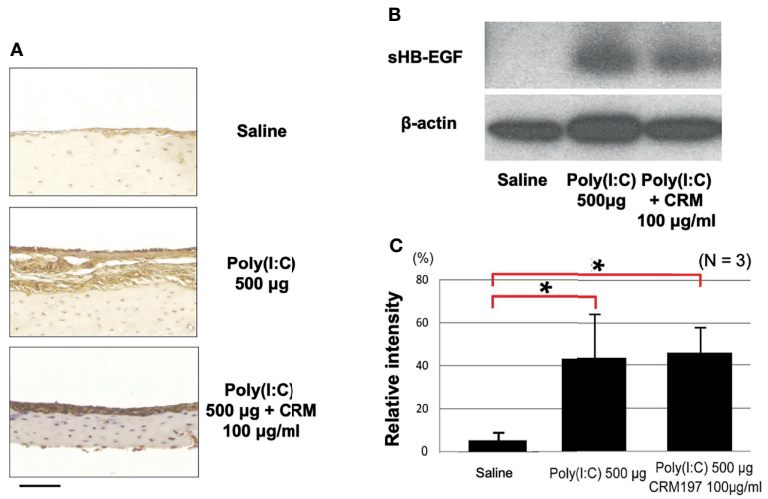

FIGURE 5 | CRM197 does not inhibit HB-EGF expression or processing. **(A)** Representative microphotographs of immunostaining with a pan anti-HB-EGF antibody are shown. HB-EGF was expressed in rat ME mucosa in all 3 groups on day 2. **(B)** Representative Western blot sHB-EGF bands for rat ME, 2 days after saline injection or Poly(I:C) administration with or without CRM197. **(C)** Quantitative analysis showed that sHB-EGF was low in the saline samples but was increased by both Poly(I:C) and Poly(I:C) plus CRM197. N refers to number of samples. *p < 0.05.

cells producing of *Hbegf* and *Egfr* mRNA are very closely matched. This suggests that HB-EGF likely acts in an autocrine manner in the ME. It should be noted that the scRNA-Seq data were generated by bacterial infection of the mouse ME, not Poly (I:C) injection. These data were used since we had an existing database. However, we acknowledge that that Poly(I:C) might induce different cellular responses in the rat ME.

ScRNA-Seq and immunostaining demonstrate that HB-EGF is present in the normal rat ME but increases during Poly(I:C)-induced OM. Increased expression of HB-EGF in the ME by Poly (I:C) is consistent with the induction and processing of this growth factor during the development of poly(I:C)-induced lupus nephritis (Triantafyllopoulou et al., 2010). It also suggests that HB-EGF production is a feature of the response to viral infection. We could find no studies evaluating HB-EGF production during viral infection, although Poly I:C has been reported to up-regulate HG-EGF in kidney cells (Triantafyllopoulou et al., 2010). However, Ras activation can induce expression of the HB-EGF gene (Benn and Schneider, 1994; McCarthy et al., 1995), and many viruses are known to activate Ras signaling (e.g. Schreiber et al., 2020). In the ME, processing into sHB-EGF only appears to occur only after activation of dsRNA receptors by Poly(I:C). It seems highly likely that stimulation of TLR3/TRIF, RIG1 and/or MDA5 in the ME leads to the expression/activation of one or more metalloproteinases, such as MMP9 (Royer et al., 2017), ADAM 9, 10, 12 or 17 (Nanba et al., 2003), that can cleave proHB-EGF and release sHB-EGF to interact with EGF receptors.

The highly similar time courses of ME sHB-EGF level and mucosal thickness change after Poly(I:C) injection implicates a causal relationship between the two. Moreover, the scRNA-Seq data indicate that EGFR, a receptor for sHB-EGF, is expressed by both epithelial and stromal cells, suggesting that both major components of the mucosa can be stimulated. sHB-EGF interaction with EGFR is thus positioned to be a major driver of ME mucosal hyperplasia induction. This was confirmed in the inhibition study, in which the specific HB-EGF receptor inhibitor CRM197 essentially abolished ME mucosal hyperplasia induced by Poly(I:C), reducing growth of both the epithelium and the stroma.

Western blotting revealed that active sHB-EGF was elevated in both Poly(I:C), and Poly(I:C) + CRM197 MEs, compared to control MEs. This is consistent with the inhibitory mechanism of CRM197, which combines with sHB-EGF and prevents binding to EGF receptors (Miyamoto et al., 2004; Yagi et al., 2009) rather than inhibiting HB-EGF processing. It also demonstrates that the procedures used in this study did not reduce HB-EGF expression or its processing due to receptor-mediated feedback.

Taken together, the results of this study provide convincing evidence that sHB-EGF interaction with its EGFR receptor plays a pivotal role in ME mucosal hyperplasia in viral OM *in vivo*. Since many ME infections include viral and bacterial co-infection (Marom et al., 2012), HB-EGF inhibitors could be a useful adjunctive therapy to antibiotics treatment for OM that is unresponsive to antibiotics. They could reduce sequelae such as mucus production and ME volume reduction due to viral co-infectants and/or residual inflammatory bacterial components.

Reduction of pathology in chronic OM could be of value in the developing world, and in vulnerable populations of the developed world, where OM has more serious consequences. It is estimated by the WHO that undertreated OM results in 30,000 annual childhood deaths and one half of the world's burden of handicapping hearing loss (WHO, 2004; WHO,

2020; Leach et al., 2020), due to progression from chronic OM to chronic suppurative OM with tympanic membrane perforation. This allows access to the ME by more pathogenic bacteria that reside in the external auditory canal, including *Pseudomonas aeruginosa* and *Staphylococcus aureus*. These more invasive and destructive pathogens increase damage to the middle and inner ears, leading to permanent hearing loss and risk of progression to meningitis. A treatment that in combination with antibiotics could reduce the pathogenesis of chronic OM might reduce this incidence of this progression and concomitant disease severity.

DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the Science Data Bank repository, accession number: 31253.11.sciencedb.01487 DOI: 10.11922/sciencedb.01487.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the VA San Diego Medical Center.

AUTHOR CONTRIBUTIONS

AK and AR conceived the study. TS, KP, and EC performed experiments. AK wrote the primary manuscript text and prepared the figures. All authors contributed to the article and approved the submitted version.

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Contribution of a Novel Pertussis Toxin-Like Factor in Mediating Persistent Otitis Media

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Chronic otitis media (COM) is the long-term infection and inflammation of the middle ears typically caused by upper respiratory tract pathogens that are able to ascend the Eustachian tube. Our understanding of contributing factors is limited because human otopathogens cannot naturally colonize or persist in the middle ears of mice. We recently described a natural COM in mice caused by *Bordetella pseudohinzii* and proposed this as an experimental system to study bacterial mechanisms of immune evasion that allow persistent infection of the middle ear. Here we describe a novel pertussis toxin (PTx)-like factor unique to *B. pseudohinzii*, apparently acquired horizontally, that is associated with its particularly efficient persistence and pathogenesis. The catalytic subunit of this toxin, PsxA, has conserved catalytic sites and substantial predicted structural homology to pertussis toxin catalytic subunit PtxA. Deletion of the gene predicted to encode the catalytic subunit, *psxA*, resulted in a significant decrease in persistence in the middle ears. The defect was not observed in mice lacking T cells, indicating that PsxA is necessary for persistence only when T cells are present. These results demonstrate the role of a novel putative toxin in the persistence of *B. pseudohinzii* and its generation of COM. This PsxA-mediated immune evasion strategy may similarly be utilized by human otopathogens, *via* other PTx-like toxins or alternative mechanisms to disrupt critical T cell functions necessary to clear bacteria from the middle ear. This work demonstrates that this experimental system can allow for the detailed study of general strategies and specific mechanisms that otopathogens use to evade host immune responses to persist in the middle ear to cause COM.

Keywords: chronic otitis media (COM), *Bordetella pseudohinzii*, PTx-like toxin, T cell function, middle ear

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INTRODUCTION

Otitis media (OM) is the infection and inflammation of the middle ears, and a major reason for doctor visits and antibiotic prescriptions among children (Schilder et al., 2016). Many causative agents of OM begin as colonizers of the nasopharynx and then ascend the Eustachian tube *via* poorly understood mechanisms. While most infections are resolved by host immune mechanisms, some result in persistent infections of the middle ear. A variety of human pathogens have been

identified as responsible for such infections, including *Streptococcus pneumoniae, Haemophilus influenza*, and *Moraxella catarrhalis*, among others (Palmu et al., 2004; Massa et al., 2009). Many of these infections result in pain, inflammation, and fever, sometimes leading to ear drum perforation and hearing loss (Schilder et al., 2016). However, these human pathogens do not naturally establish infections in murine models, a limitation overcome by injecting large numbers of human pathogens directly into the middle ears of mice to study aspects of the severe inflammation that results. But this approach confounds the ability to study bacterial factors and mechanisms that mediate aspects of efficient colonization and long-term persistence in the middle ear.

We have previously described the use of *Bordetella pseudohinzii*, a bacterial species that efficiently colonizes and persists in the middle ears of mice as an experimental model for natural OM progression (Dewan et al., 2019). *B. pseudohinzii* is a recently described species (Ivanov et al., 2016) that, when introduced in very small numbers to the external nares, naturally and efficiently ascends the Eustachian tube, colonizes, and persists in the middle ears of mice for over three months Dewan et al., 2019), apparently for the life of the animal. In addition to its strengths as a model for OM, *B. pseudohinzii* has also been investigated as an upper respiratory tract pathogen that forms biofilms (Clark et al., 2016) and is cytotoxic to ciliated epithelial cells (Perniss et al., 2018). In contrast to *B. pseudohinzii*, a related species, *B. bronchiseptica*, can highly efficiently colonize both nasal cavity and middle ears of mice, and persists for life in the noses but is cleared from the middle ears by processes that involve adaptive immunity (Dewan et al., 2019; Dewan et al., 2022).

Here we investigate the possible mechanisms underlying the immune evasion that allows *B. pseudohinzii* to persist in the middle ear. Genome comparisons revealed numerous putative virulence factor genes unique to *B. pseudohinzii*, of which a set of pertussis toxin (PTx)-like genes were selected for further analysis. Pertussis toxin (PTx) is specifically produced by *B. pertussis* and is a major contributor to the disease process and immunomodulatory effects of *B. pertussis*. PTx is an AB_5 toxin that is made of two major components, the A-promoter (S1 or PtxA subunit) and the B-oligomer (S2-S5 or PtxB-PtxE subunits). PTx-like toxins are characterized by their ADP-ribosyltransferase activities and include toxins produced by several species, such as *E. coli* and *Salmonella spp*, among others (Littler et al., 2017; Tamamura et al., 2017). PTx hydrolyzes NAD to ADP-ribose and nicotinamide as well as transfer the ADP-ribose to the target proteins (Krueger and Barbieri, 1995). Here we report a set of PTx-like toxin genes in the genome of *B. pseudohinzii* that encode a putative novel toxin which is annotated as Pseudohinzii toxin (PSx). To examine the role of PSx in persistence, an in-frame deletion mutant of the gene encoding the putative catalytic component, psxA, was generated. This deletion of *psxA* resulted in a mutant strain that failed to efficiently persist in the middle ears of mice. The predicted structure of this PTx-like toxin subunit and its role in modulating the host immune response in the middle ears of mice suggest PSx is a novel immunomodulatory agent disrupting immune mediated clearance of bacteria from the middle ear.

MATERIALS AND METHODS

Bacterial Strains and Growth

B. pseudohinzii 8-296-03 (Ivanov et al., 2016), and *B. bronchiseptica* RB50 (Cotter et al., 1998) have been previously described. Both bacteria were grown and maintained on Bordet-Gengou (BG) agar (Becton Dickson) supplemented with 10% defibrinated sheep's blood (HemoStat). For mouse inoculations, the bacterial strains were grown at 37°C, with shaking at 200 rpm, to mid-log phase in Stainer Scholte (SS) liquid broth (Stainer and Scholte, 1970). Numbers of bacterial colony forming units (CFU) were estimated by measuring the optical density at 600 nm, validated by dilution in phosphate buffered saline (PBS), plating on BG agar and counting viable colonies after incubation for 2 days at 37°C.

Mutant Generation

The allelic exchange vector pSS4245 was used for the generation of deletion mutants. Briefly, ∼1 kb of DNA flanking each end of gene *psxA* was PCR amplified with primers from IDT (Upstream forward: 5'-AGGGCGGCCGCACTAGGGGTTGAGTTCGCGGGCGAAACCAG-3'; Upstream reverse: 5'-TGGCTGCCAGTTATTGACGCATACCCACGCCATTCCTGCTATG-3'; Downstream forward: 5'-TGGCGTGGGTATGCGTCAATAACTGGCAGCCACGATATGGTG-3'; Downstream reverse: 5'-GATCTGTACACCTAGGGGACGATGAGTACACGCGAATAC-3'), joined and inserted into the allelic exchange vector, pSS4245, by PIPE cloning (Plasmid vector amplification primers: Forward primer: 5'-CTAGTGCGGCCGCCCTAGCATAGG-3'; Reverse primer: 5'-CCTAGGTGTACAGATCCGGACCTGC-3') (Klock and Lesley, 2009). The construct was verified by sequencing, transformed into *E. coli* SM10λ*pir*, and transferred into the parental *B. pseudohinzii* 8-296-03 by mating. Colonies containing the integrated plasmid were selected and incubated on BG agar to stimulate allelic exchange by homologous recombination. Emerging colonies were screened by PCR for replacement of the wildtype by the mutant allele and confirmed by Sanger sequencing.

Genomic and Protein Structure Analysis

Total protein sequences were extracted from the NCBI archive for *B. bronchiseptica* RB50 (RefSeq assembly accession: GCF_000195675.1), *B. pertussis* Tohama I (GCF_000195715.1)) and *B. pseudohinzii* HI4681 (GCF_001698185.1) and 8-296-03 (GCF_000657795.2). Similarities between *B. bronchiseptica* and *B. pseudohinzii* proteins were estimated as H values using mGenomeSubtractor (Shao et al., 2010). H values were determined as the highest BLASTp identity score (i), multiplied by the matching sequence length (lm) divided by the query length (lq) as H = i x (lm/lq). Based on our previous work (Rivera et al., 2019), genes encoding proteins with an H

value < 0.5 were considered absent from the genome. The genomes of *B. pseudohinzii* HI4681 (Spilker et al., 2016) and 8-296-03 (Ivanov et al., 2016) were subjected to pairwise tBLASTx searches against the genomes of *B. pertussis* Tohama I and of *B. bronchiseptica* RB50 (Parkhill et al., 2003), respectively. Pairwise comparisons were visualized using the Artemis Comparison Tool (Carver et al., 2005). The comparisons revealed conserved gene synteny of the entire pertussis toxin operon (*ptx* and *ptl* genes), with the exception of *psxB* that was duplicated in *B. pertussis* and *B. bronchiseptica* genomes. The comparisons also showed that the *B. pseudohinzii psx* operon was inserted at different genomic location compared to the classical *Bordetella* species.

Pertussis toxin protein identity values were determined from pairwise BLASTp comparisons, nucleotide identities of the individual genes were determined using BLASTn. Pairwise nucleotide identity (ANI) of the complete genomes was calculated at https://www.ezbiocloud.net/tools/ani (Yoon et al., 2017). The protein sequences of *B. bronchiseptica, B. pertussis*, and *B. pseudohinzii* toxins were retrieved from NCBI. Protein sequence alignment was conducted by Geneious R10 v 2021.1.1 using global alignment with free end gaps and cost matrix as BLOSUM62. *De novo* and comparative structure predictions were conducted using the Robetta online server (https://robetta.bakerlab.org/). The crystal structure of *B. pertussis* PtxA was retrieved from PDB. The predicted structure of *B. pseudohinzii* PsxA was aligned with *B. pertussis* PtxA and the RMSD value calculated by Pymol v2.5.2.

Mouse Experiments

Four- to six-week-old mixed sex C57BL/6J (000664), B6.129S7-Rag1^(tm1Mom)/J (002216), B6.129P2-Tcrb^(tm1Mom)Tcrd^(tm1Mom)/J (002122) mice were procured from The Jackson Laboratory (Bar Harbour, ME) and bred in the Harvill laboratory mouse colony (University of Georgia, GA). All mice were maintained in specific pathogen-free facilities, and all experiments were conducted following institutional guidelines. Mice were lightly sedated with 5% isoflurane (Pivetal) and inoculated (150 CFU in 5 µL PBS) by pipetting the inoculum as droplets onto their external nares to be inhaled. At the indicated timepoints mice were euthanized *via* CO_2 inhalation. Blood was drawn by cardiac puncture, and the organs were excised. To quantify bacterial numbers colonizing the middle ears, tissues were homogenized in 1 ml PBS, serially diluted, and plated on BG agar. Colonies were counted following incubation for two days at 37°C.

Biofilm Assays

B. pseudohinzii strain 8-296-03 (Ivanov et al., 2016) and the confirmed *psxA* mutant were grown for 48 hours at 37°C on Bordet-Gengou (BG) agar (Becton Dickson) supplemented with 10% defibrinated sheep's blood (HemoStat). The bacterial strains were then grown at 37°C, with shaking at 200 rpm, to mid-log phase in Stainer Scholte (SS) liquid broth (Stainer and Scholte, 1970). Cultures were diluted with sterile 1x PBS to OD_{600} = 0.1. These cultures were serially diluted to a final dilution of 10^{-6} of the original OD_{600} = 0.1 culture. 10 µl of each strain was added to a well of a 96-well plate containing 90 µl of -Mg^{2+} -Ca^{2+} PBS. Three technical repeats were performed. Plates were incubated for 48 hours at 37°C, after which the wells were washed three times with DI water. Following the washes, 125 µl of 0.05% crystal violet was added to each well and the plate was incubated for 15 minutes at room temperature. Excess crystal violet was removed and 200 µl of 200 proof EtOH was added to each well. Absorbance of each well was read immediately at 540 nm (BMG Labtech CLARIOstar multi-mode microplate reader).

Statistics

For experiments determining differences in bacterial loads in the organs of mice, the following statistical analysis were performed using GraphPad PRISM (GraphPad Software, Inc): Two-tailed unpaired Student t-tests, One-way ANOVA and Two-way ANOVA were used to determine statistical differences between two groups. The specific test used is indicated in the Figure Legends.

Ethics Statement

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committees at The University of Georgia at Athens, GA (A2016 02-010-A13 Host-Pathogen Interactions, A2016 07-006-A5 Breeding Protocol). Mice were consistently monitored for signs of distress over the course of the experiments to be removed from the experiment and euthanized using carbon dioxide inhalation to prevent unnecessary suffering.

RESULTS

Timecourse of Colonization of Middle Ears

To evaluate the ability of *B. bronchiseptica* and *B. pseudohinzii* to infect and persist in the middle ears of mice, groups of C57BL/6 mice were inoculated on the external nares with 5 µl of PBS containing a calculated 150 CFU of either wild-type (WT) *B. bronchiseptica* or WT *B. pseudohinzii.* Bacterial load in the middle ears was evaluated at 3, 7, 14, 28, 42, and 56 days post inoculation (dpi) (**Figure 1**). Within 3 dpi, both middle ears of all mice had become colonized with thousands of the respective bacteria, and numbers continued to rise to over 10,000 CFU by 7 dpi, over fifty-times the inoculation dose delivered to the distal nares. The great consistency of rapid colonization and growth indicate that both species are highly efficient in moving from the nasal cavity, up the Eustachian tube to colonize and grow in the middle ears of mice. Mice infected with *B. bronchiseptica* experienced a steep decline (>95% reduction) in bacterial numbers between 7 and 14 dpi. This decline continued to 56 dpi, and *B. bronchiseptica* was cleared from the middle ears by 100 dpi (Dewan et al., 2022) by the generation of a robust adaptive immune response (Dewan et al., 2022). In contrast, *B. pseudohinzii* persisted in the middle ears at high numbers, approximately 10,000 CFU, until at least day 100, the end of this

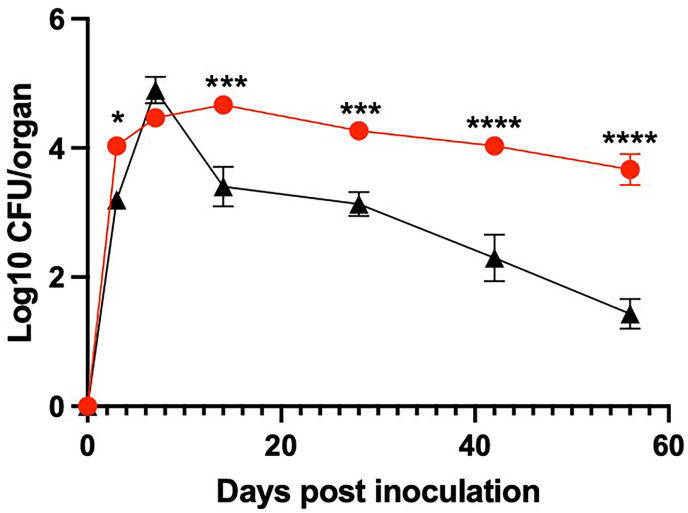

FIGURE 1 | *B. pseudohinzii* efficiently colonizes and persists in the middle ears. Log10 of CFU recovered on days 3, 7, 14, 28, 42 and 56 post inoculation from the middle ears of mice inoculated with either *B. bronchiseptica* (black) or *B. pseudohinzii* (red) bacteria (n=4 per strain per timepoint). Error bar shows the standard error of mean. Statistical significance was calculated by using Two-way ANOVA. *p < 0.0332, ***p < 0.0002, ****p < 0.0001.

timecourse, with no apparent decline, suggesting infection is chronic, potentially life-long (**Figure 1** and data not shown). This contrast in the ability to persist in the face of adaptive immunity suggests *B. pseudohinzii* has specialized mechanisms to avoid immune-mediated clearance that *B. bronchiseptica* lacks.

Differential Genome Content

To assess possible mechanisms that mediate evasion of host immune clearance in the middle ears, the genomes of *B. pseudohinzii* and *B. bronchiseptica* were compared to identify genes particular to the former. The genome of *B. pseudohinzii* contains 4,168 coding genes and 30 pseudogenes, while *B. bronchiseptica* contains 4,768 coding genes and 13 pseudogenes. Utilizing mGenomeSubtractor on annotated genomes provided by NCBI, 1,319 genes were identified that are uniquely present in the genome of *B. pseudohinzii* that are lacking in *B. bronchiseptica* (**Figure 2A**). The annotated genes fall within the categories of metabolism (455), transport (355), regulation systems (159), virulence (114), domains of unknown function (194), and insertion sequences (43). Reasoning that a mechanism that allows for immune evasion and persistence of *B. pseudohinzii* might have homology to a known virulence factor, the 114 genes annotated as related to "virulence" and uniquely present in *B. pseudohinzii* (**Figure 2B**) were selected for further analysis. Of these, one locus of genes stood out as particularly promising based on its weak, but noticeable homology to a known immunomodulatory factor of *B. pertussis*, Pertussis toxin (PTx).

PTx is encoded in a set of genes apparently acquired by an ancestor of the three classical *Bordetella* species (*B. pertussis, B. bronchiseptica*, and *B. parapertussis*), and not shared by any other *Bordetella* species. The PTx-encoding locus of the classical *Bordetella* species and the PSx-encoding locus unique to only *B. pseudohinzii* share low homology, different gene numbers and different locations, contributing to confidence they were not acquired vertically from the last common ancestor. Their operons are composed of five and four genes each, respectively, with both *ptxB* and *ptxC* of the PTx locus sharing homology with *psxB/C* of the PSx locus. Further, the homology between the individual components of the PTx and PSx loci are weak relative to that of their shared core genome (80.34%) with H values between H=0.224 (PsxD) and H=0.500 for PsxA (**Supplementary Table 1**). This evidence suggests that the two toxin operons did not diverge since their vertical inheritance from a last common ancestor. Instead, the *psx* locus of *B. pseudohinzii* appears to have been independently acquired horizontally from a currently unknown source.

B. pseudohinzii PsxA Contains Conserved Sites and Predicted Structural Homology to *B. pertussis* PtxA

To investigate the homology of the *B. pseudohinzii* toxin catalytic unit (PsxA) to the *B. pertussis* toxin catalytic unit (PtxA), sequence alignment and computational modeling were conducted. Despite PsxA only sharing 56% homology to PtxA at the protein sequence level, their ADP-transferase catalytic (H70, E163) (Locht and Antione, 1995) and NAD hydrolase catalytic sites (W60, C75) are conserved (Burns and Manclark, 1989; Cortina and Barbieri, 1989) (**Figure 3**). In addition, the Cys residues involved in disulfate bonding of A-B subunits (C75 and C235) (Burns and Manclark, 1989) are also conserved. Two major differences between PsxA and PtxA reside at the $S_{88}SSR_{91}$ secretion motif and the carboxyl-terminal membrane translocation sequence. Compared to PtxA, the Ser89 of PsxA is replaced by an Arg while the Arg91 is substituted with a Glu. It has been experimentally shown that mutation of PtxA Ser89 to

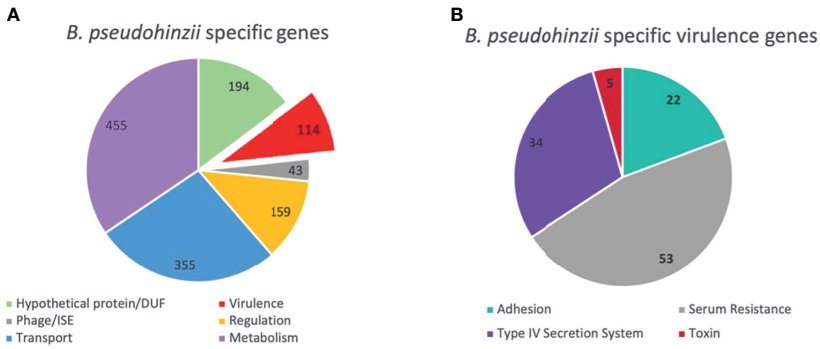

FIGURE 2 | Unique genes present in *B. pseudohinzii* but not in *B. bronchiseptica*. **(A)** 1319 genes expressed in the genome of *B. pseudohinzii* that are lacking in the genome of *B. bronchiseptica* organized by functional category. **(B)** 114 virulence factors genes unique to the genome of *B. pseudohinzii* (from **A**) organized by function.

Gly led to more than 10-fold decrease in secretion of PtxA (Craig-Mylius et al., 2000). In addition, the carboxyl-terminal membrane translocation motif of PsxA is considerably different from that of PtxA. Notably, the carboxyl-terminal sequence of PtxA, especially the terminal Phe269, was previously shown to be critical for membrane translocation of PtxA (Farizo et al., 2002).

To computationally model the 3D structure of *B. pseudohinzii* PsxA, two modelling approaches were used, namely *de novo* modelling and comparative modelling. The *de novo* modelling approach utilized is a machine deep learning based method, RoseTTAFold, which is a recent milestone in *de novo* modelling with high accuracy (Baek et al., 2021). The comparative modelling approach used here, RosettaCM, combined four independent modelling methods (Song et al., 2014) and modelled the PsxA structure based on the published crystal structure of *B. pertussis* PtxA (PDB ID: 1prt) (Stein et al., 1994). As shown in **Figure 4**, the predicted structure of PsxA by comparative modelling (pink) or by *de novo* modelling (purple) is aligned with the known structure of PtxA (tan). To evaluate the structural similarities between modelled PsxA and PtxA, RMSD (root-mean-square deviation) was calculated. Predicted structures of PsxA by both methods display high similarities to PtxA with RMSD values of less than 2 Å (RosettaCM RMSD = 0.602 Å, RoseTTAFold RMSD = 1.655 Å). To evaluate the quality of *de novo* modelling, the lDDT score (local Distance Difference Test on All Atoms) was calculated (Hiranuma et al., 2021). Models with IDDT scores higher or equal to 0.6 is considered correct. The RoseTTAFold prediction has an lDDT score of 0.64, indicating qualified prediction.

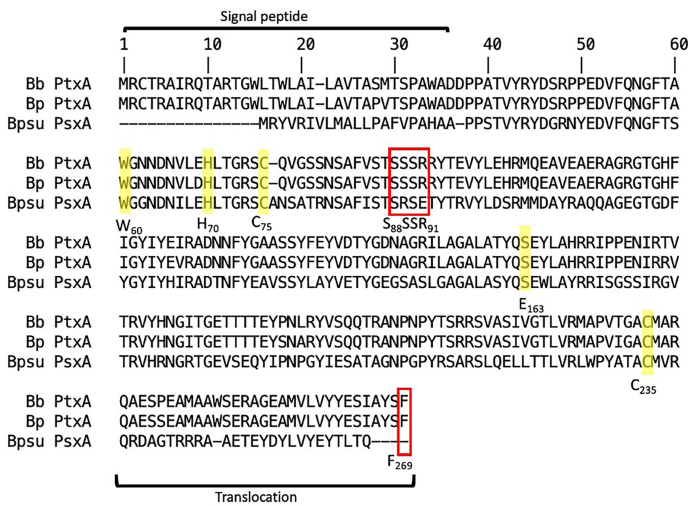

FIGURE 3 | Sequence alignment between Ptx-like toxins of *B. pertussis* (Bp), *B. bronchiseptica* (*Bb*), and *B. pseudohinzii* (*Bpsu*). ADP-ribosyltransferase catalytic sites are notated as H_{70} and E_{163}. NAD-glycohydrolase activity sites are notated as C_{75} and W_{60}. A-B subunit interaction sites are notated as C_{75} and C_{235}. Toxin secretion motif is notated as $S_{88}SSR_{91}$. Outer membrane translocation site is notated as F_{269}. Red box indicates differences and yellow highlight indicates homology.

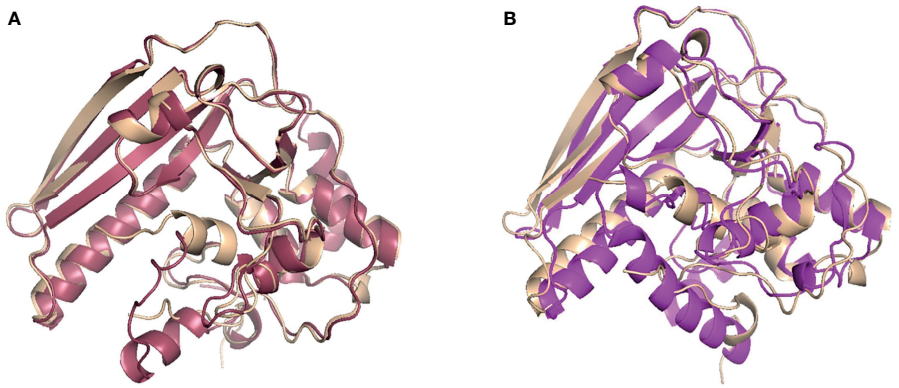

FIGURE 4 | Comparative and *de novo* modeling of PsxA aligned with PtxA. **(A)** Rosetta CM comparative modeling of *B. pseudohinzii* PsxA (pink) based on *B pertussis* PtxA, aligned with *B pertussis* PtxA (tan). RMSD=0.602 Å, confidence= 0.74. **(B)** RoseTTAFold *de novo* modeling of *B. pseudohinzii* PsxA (purple) aligned with *B pertussis* PtxA (tan). RMSD= 1.655 Å, confidence= 0.64.

PsxA Contributes to Persistence in Middle Ears

To determine the role of PSx in the colonization and persistence of *B. pseudohinzii* in the middle ears, an isogenic derivative with an in-frame deletion of the coding region of *psxA* was generated by allelic exchange (**Supplementary Figure 2**). The construct and subsequent integration was verified by Sanger sequencing. Growth curves of the WT and mutant *B. pseudohinzii* were performed simultaneously and no growth defects were observed (**Supplementary Figure 3**). Allelic exchange was performed three times independently to confirm that the effects were not due to random spontaneous mutations (**Supplementary Figure 4**). To assess the impact of PsxA on bacterium-host interactions, C57BL/6 mice were inoculated intranasally with 5 μL PBS containing 150 CFU of either WT *B. pseudohinzii* or *B. pseudohinziiΔpsxA*. The middle ears were harvested from four mice each at days 7, 14, 28, 42, 56, and 100 post inoculation and bacterial numbers were assessed (**Figure 5A**). Overall, the mutant and WT strains had similar numbers of bacteria at early timepoints, but the average numbers of *B. pseudohinziiΔpsxA* were approximately 90% lower than that of the WT strain at 56 and 100 dpi, although inter-mouse variation in multiple comparisons between small numbers (n=4) of animals resulted in differences that did not meet the threshold for statistical significance. A follow-up experiment focusing on 56 dpi with larger numbers of animals confirmed a clear and significant difference, with the *psxA* mutant being present at on average >90% lower numbers than the WT strain (**Figure 5B**). Due to known contributions of biofilm formation in the persistence of bacterial middle ear infections, the biofilm forming abilities of both WT and mutant *B. pseudohinzii* strains were evaluated and no difference was observed between the two strains (**Supplementary Figure 5**). This, along with the prolonged time required to observe a defect reduces the likelihood of PsxA contributing to resistance of innate factors and indicates a role in evading adaptive immune clearance.

The Adaptive Immune Response Contributes to the Control of Middle Ear Infections

The observation that there was no defect in the mutant's ability to colonize, grow, and spread to the middle ears indicate that PSx has no critical roles in these aspects of early infection processes. A defect was only observed after a month of infection, when adaptive immune functions are dominant aspects of the host response, suggesting that PSx may be involved in evading adaptive immune-mediated clearance to allow *B. pseudohinzii* to persist. To determine whether PsxA mediates persistence *via* effects on host adaptive immunity, the persistence of WT *B. pseudohinzii* and *B. pseudohinziiΔpsxA* was assessed in mice deficient in mature B and T cells ($Rag1^{-/-}$ mice). $Rag1^{-/-}$ mice were challenged as above and middle ears were harvested at days 7, 14, 28, 42, 56, and 100 days post inoculation and bacterial load was assessed (**Figure 6A**). WT and mutant *B. pseudohinzii* strains similarly infected $Rag1^{-/-}$ mice at all time points, indicating that PsxA is only required for persistence when B and T cells are present and that PSx is not required to resist the anti-bacterial functions of the innate immune cells present in these mice, including macrophages, neutrophils, and NK cells, among others. The defect of the *psxA* mutant was only observed in the presence of adaptive immunity, indicating that PSx facilitates persistence by mediating evasion of some function(s) of mature B and/or T cells.

PsxA Does Not Affect Production of High Titers of Antibodies

Antibodies are critical aspects of the immune response to *Bordetella spp.* infections and are required for the clearance of both *B. bronchiseptica* and *B. pertussis* from the lower respiratory tract (Kirimanjeswara et al., 2003). The effect of PSx on the antibody response to infection was therefore assessed. Immunocompetent C57BL/6 mice were inoculated with WT or *ΔpsxA* strains, as above, and serum was collected 56 days post inoculation and serum anti-*B. pseudohinzii* IgG titers were analyzed *via* ELISA. These assays

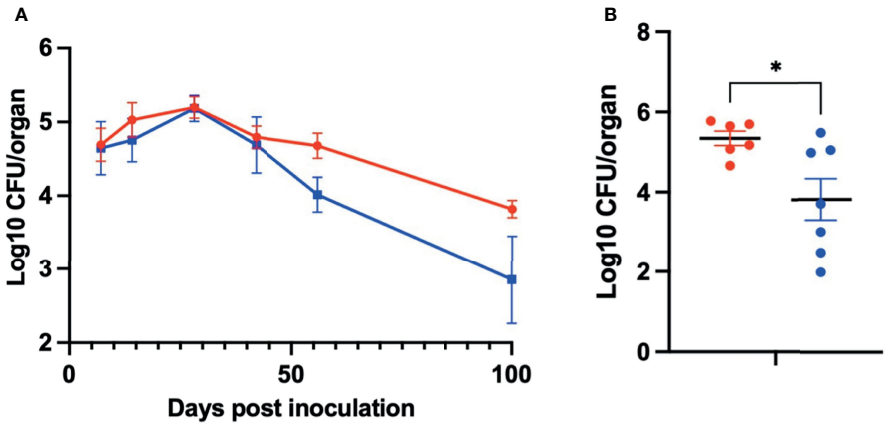

FIGURE 5 | PsxA contributes to persistence of *B. pseudohinzii* in the middle ears. **(A)** Log10 of CFU of WT *B. pseudohinzii* (red) or *B. pseudohinzziΔpsxA* (blue) recovered from the middle ears of C57BL/6 mice on days 7, 14, 28, 42, 56, and 100 post inoculation (n=4 per strain and timepoint). **(B)** Log10 of CFU isolated from the middle ears of C57BL/6 mice on day 56 post inoculation (WT *B. pseudohinzii* n=6 and *B pseudohinziiΔpsxA* n=7). Error bar shows standard error of mean. Statistical significance was calculated *via* Two-way ANOVA. *$p < 0.0332$.

demonstrated that *B. pseudohinzii* and *B. pseudohinziiΔpsxA* induced similarly high IgG titers (**Figure 6B**). These observations indicate that PsxA did not substantially disrupt the production of high titer antibodies, and that high titers of antibodies were not sufficient to clear *B. pseudohinzii* infection from the middle ears.

The psxA Mutant Is More Rapidly Cleared *via* a T Cell Dependent Mechanism

Based on the results in **Figure 6**, we hypothesized that PsxA contributes to persistence *via* evasion of T cell-mediated immune responses. To test this, the persistence of WT and mutant *B. pseudohinzii* in mice lacking both α/β and δ/γ T cells (T cell$^{-/-}$) was assessed. At 56 dpi, the middle ears of these mice were harvested for bacterial enumeration (**Figure 7**). The WT bacterium was recovered in similar numbers from immunocompetent C57BL/6 and T cell$^{-/-}$ mice, indicating that T cells have a limited role in the control of *B. pseudohinzii* infection of the middle ear. In contrast, the *psxA* mutant was recovered from the middle ears of C57BL/6 mice in significantly lower numbers than the wild type *B. pseudohinzii* (**Figure 5**), but this difference disappeared in mice lacking T cells. Together these results indicate that PsxA mediates persistence by disrupting T cell mediated anti-bacterial effects.

DISCUSSION

We have limited understanding of the natural processes involved in chronic OM and the bacterial factors that contribute to

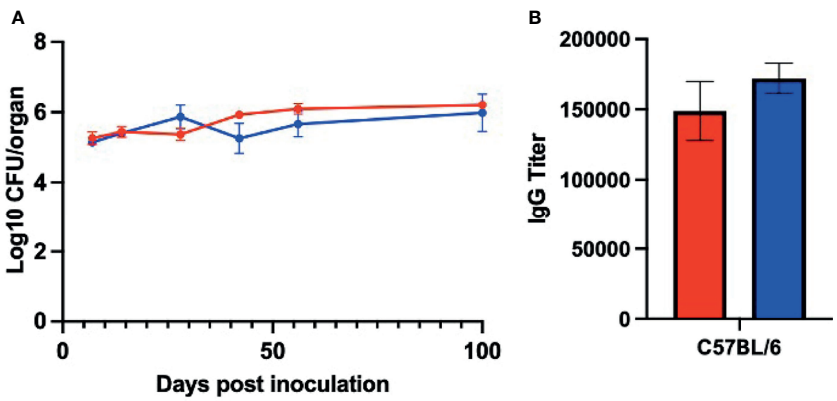

FIGURE 6 | PsxA does not affect persistence in *Rag-/-* mice or serum antibody production. **(A)** Log10 of CFU of WT *B. pseudohinzii* (red) or *B. pseudohinziiΔpsxA* (blue) recovered from the middle ears of Rag1$^{-/-}$ mice on days 7, 14, 28, 42, 56, and 100 post inoculation (n=4 per strain per timepoint). **(B)** Serum from C57BL/6 mice 56 days after inoculation with *B. pseudohinzii* or *B pseudohinziiΔpsxA* was analyzed by ELISA for anti-*B. pseudohinzii* titers (results are the average of two independent assays). Error bars show the standard error of the mean. Statistical significance was calculated *via* Two-way ANOVA.

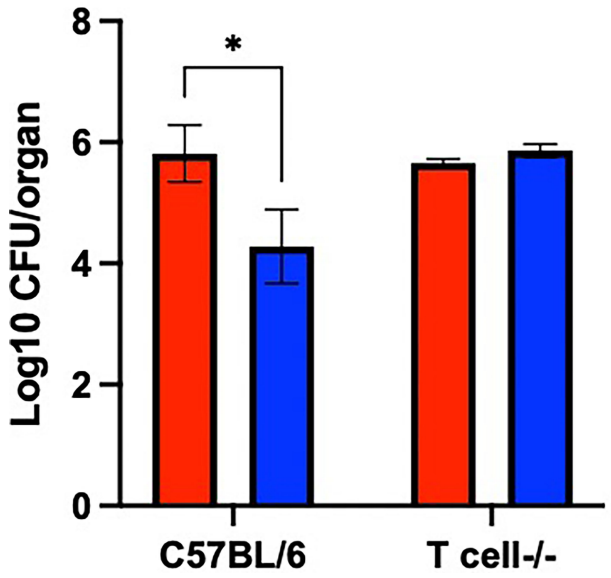

FIGURE 7 | T cell-mediated bacterial clearance is disrupted by PsxA. Log10 of CFU of WT *B. pseudohinzii* (red) or *B. pseudohinzii*Δ*psxA* (blue) recovered from the middle ears of C57BL/6 or T cell-/- mice at 56 days post inoculation. N=3 per mouse type and bacterial strain, assay was performed in duplicate. Error bars show the standard error of the mean. Statistical significance was calculated *via* Two-way ANOVA. *$p < 0.0332$.

modulation of the host immune system to allow persistence. Current experimental systems using human pathogens injected in large numbers into the middle ears of mice or chinchillas can model some aspects of severe acute disease and pathology. However, these systems do not encompass critical aspects of natural bacterium-host interactions occurring during initial establishment or persistence of middle ear infections and cannot reveal the mechanisms involved in those. *B. pseudohinzii* has been previously demonstrated to naturally cause middle ear infection in mice that persist for over 3 months, causing chronic otitis media and progressive hearing loss (Dewan et al., 2019). Here, genes particular to *B. pseudohinzii* and identified as a PTx-like factor, PSx, were investigated and modeling of the catalytic component reveals substantial predicted structural homology to PtxA. A *psxA* deletion mutant of *B. pseudohinzii* demonstrated a significant persistence defect, with a 90% reduction in bacteria recovered from the middle ears of immunocompetent C57BL/6 mice at 56 days post inoculation. This defect was ameliorated in mice lacking both B and T cells and mice lacking only T cells, indicating that T cells are required for the dramatic reduction in numbers of the mutant. These results also indicate that PSx contributes to persistence by mediating the evasion of T cell activities that can otherwise reduce bacterial numbers greatly. This experimental system can allow the mechanisms by which T cells reduce bacterial numbers in the middle ear to be further probed.

The amino acid-level sequence homology between PSx and PTx (56%) demonstrates moderate overall conservation between the two AB_5 toxins. However, the overall homology between the core genomes of *B. pertussis* and *B. pseudohinzii* is much higher, averaging about 80%. The *ptx* loci appears in the very closely related classical bordetellae (*B. pertussis*, *B. bronchiseptica*, and *B. parapertussis*) but not in any of the more distantly related *Bordetella* species, leading to the conclusion that it was acquired horizontally by this lineage as it emerged as successful mammalian respiratory pathogen (**Supplementary Figure 1**) (Parkhill et al., 2003; Linz et al., 2016). The *B. pseudohinzii* genome appears to have acquired, and inserted at a different genome location, a distantly related version of the entire locus, including homologs of all nine genes (*pltA* through *pltI*) that encode the type IV secretion system (T4SS) required for toxin secretion. The corresponding H values ranged from H=0.241 for PtlE to 0.607 for PltA (**Supplementary Table 1**) and support its having been acquired from some distantly related genus. The presence of this system in the genome of *B. pseudohinzii* indicates this organism contains all the machinery known to be needed for the expression and secretion of an intact holotoxin.

Although overall homology is relatively low, the catalytic sites of PTx and PSx are particularly well conserved both in their sequences and predicted 3-dimensional structure, as they are in most AB_5 toxins. This indicates likely similarity in their catalytic activities, suggesting PSx acts like PTx, potentially ADP ribosylating G proteins, although the specificity of their substrates might vary. However, there are significant differences between the protein sequences, notably in the secretion motif and carboxyl terminal sequence. These nonhomologous features could be a result of adaptation of differential secretion and membrane translocation machineries by *B. pseudohinzii*. PTx-like toxins encoded by a diverse group of pathogenic bacteria and their functions have been extensively studied. These include species and strains of *E. coli* and *Salmonella*, among others (Littler et al., 2017; Tamamura et al., 2017), and are characterized as an AB_5 toxin with ADP-ribosyltransferase activity. Importantly, it has been observed that the catalytic A subunit of PTx and PTx-like toxin are conserved though they may not target the same motifs on host G proteins (Littler et al., 2017). Previous observations have identified the multifactorial role of PTx in the activation and subsequent modulation of T cell signaling, leading to T cell death (Schneider et al., 2007). Analysis of the binding motifs of the catalytic A subunit and B subunit could provide insight into the intra- and extracellular host cell motifs that are specifically targeted by PSx.

B. pseudohinzii was described as an otopathogen of laboratory mice in 2016 (Ivanov et al., 2016) and has since been isolated from various rodent sources (Loong et al., 2018). Several investigations into this natural colonizer of mice has revealed key functions and characteristics that allow it to colonize and evade immune clearance as an upper respiratory tract pathogen. This includes the impairment of movement and death of ciliated cells (Perniss et al., 2018). This observation provides an explanation for the progressive hearing loss previously observed in *B. pseudohinzii* infected mice (Dewan et al., 2019) and could also contribute to bacterial persistence. Additionally, T cells have been implicated in the control and clearance of both upper respiratory and middle ear

infections for various *Bordetella* infections (Leef et al., 2000; Harvill et al., 1999a). Here, mice deficient in all T cell subsets were unable to control or clear both WT and mutant *B. pseudohinzii* infections in the middle ears. The bacterial levels of WT *B. pseudohinzii* recovered from both T cell$^{-/-}$ and immunocompetent mice are similar, indicating these infections were unaffected by the presence or lack of T cells. The *psxA* mutant, however, was able to survive to higher levels in T cell$^{-/-}$ mice compared to immunocompetent C57BL/6 mice. This indicates that this mutant strain was uniquely susceptible to T cell-mediated protection and that PsxA likely contributes to the ability of *B. pseudohinzii* to evade T cell mediated clearance. This resistance to T cell action is consistent with the ability of *B. pseudohinzii* to suppress a Th17-mediated allergic response in an asthma model (Jaeger et al., 2020). In addition, these effects are also consistent with published observations on the interactions between PTx from *B. pertussis* and T lymphocytes as well as other published PTx-like toxins, including inhibition of migration and cytokine signaling (Cyster and Goodnow, 1995; Schneider et al., 2009; Brink et al., 2018). These results support the presentation of PSx as a novel PTx-like toxin that contributes to the persistence of *B. pseudohinzii*.

Since *B. pseudohinzii* is well adapted as an efficient middle ear pathogen of mice, we have the unique opportunity to observe the natural process of colonization of the middle ear and the contributions of virulence factors and toxins in these infections. This is an exceedingly powerful tool for the understanding of both host and pathogen factors that contribute to establishment and persistence of chronic middle ear infections. For example, the T cell mediated mechanisms that reduce the numbers of the *psxA* mutant can be probed to better understand how T cells can contribute to immunity in the middle ear. Further, *B. pseudohinzii* lacking PsxA still persists, albeit at lower levels, whereas *B. bronchiseptica* is completely cleared from the middle ear. This is strong evidence that there are other immune evasion elements in the genome of *B. pseudohinzii*, not shared by *B. bronchiseptica*, that remain to be discovered.

Established models of middle ear infections have contributed substantially to our understanding of factors that contribute to pathogenesis, identifying pneumococcal surface proteins that cause inner ear damage (Schachern et al., 2009) and *in vivo* interactions of *P. aeruginosa* with macrophages and middle ear epithelial cells (Mittal et al., 2014; Mittal et al., 2016). As the incidence and burden of chronic otitis media increases, the field may benefit from continued pursuit of alternative models that can provide new windows through which to observe infection. We present this model of a natural otitis media infection as a complementary experimental system to existing models in order to further our knowledge of the complex bacterium-host interactions involved in chronic otitis media.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committees at The University of Georgia at Athens, GA.

AUTHOR CONTRIBUTIONS

LM, KD, BL, and EH conceived the study. LM, CS, YS, KD, BL, and EH designed the experiments. LM, CS, YS, and BL performed the experiments. LM, CS, YS, and BL analyzed the data. LM, CS, YS, BL, and EH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.795230/full#supplementary-material

Supplementary Figure 1 | Structure and chromosomal location of the pertussis toxin-encoding operon in *B. pseudohinzii*. **(A)** Structural comparison of the pertussis toxin operons in *B. pertussis* and *B. pseudohinzii*. The red rectangles and lines show blocks of sequence homology, with a color shading gradient from low (light pink) to high homology (red). The structure of the operon is conserved, with the exception of the duplicated *ptxB/ptxC* genes in the classical *Bordetella* species (*B. pertussis*, *B. parapertussis* and *B. bronchiseptica*). **(B)** Different chromosomal location of the pertussis toxin operon in *B. pseudohinzii* and in the classical bordetellae, here *B. bronchiseptica*.

Supplementary Figure 2 | Genetic context of *B. pseudohinziiΔpsxA* mutant strain.

Supplementary Figure 3 | Similar laboratory growth *in vitro* of *B. pseudohinzii* WT (blue) and *B. pseudohinziiΔpsxA* (orange) bacteria. There were 3 technical replicates in each time point per group. Error bars show the standard error of mean. Statistical significance was calculated using Unpaired t-test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, n.s. > 0.05.

Supplementary Figure 4 | Similar bacterial recovery of *B. pseudohinziiΔpsxA* isogenic mutants from ears. Bacterial recovery of mutant replicates (I-23, I-13, and I-10) from the middle ears 56 dpi from C57BL/6 mice. The mutant here referred to as I-23 was utilized for all experiments with *B. pseudohinziiΔpsxA*. Statistical

significance was calculated using One-way ANOVA. *p<0.05, **p<0.01, ***p<0.001, n.s. >0.05.

Supplementary Figure 5 | Biofilm forming abilities of WT and mutant *B. pseudohinzii*. WT *B. pseudohinzii* (WT Bpsu) (red) and *B. pseudohinziiΔpsxA* (BpsuΔpsxA) (blue) demonstrate similar biofilm forming capabilities after 48 hours in PBS. There are three replicates per group. Statistical significance was calculated using Unpaired t-test. *p < 0.05, **p < 0.01, ***p < 0.001, n.s. >0.05.

Supplementary Table 1 | *B. pseudohinzii* and *B. pertussis* protein homology. Comparison of amino acid sequences of PSx (*B. pseudohinzii*) and PTx (*B. pertussis*) toxin subunits and Type IV secretion system subunits.

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Predominant Bacterial and Viral Otopathogens Identified Within the Respiratory Tract and Middle Ear of Urban Australian Children Experiencing Otitis Media Are Diversely Distributed

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Background: Otitis media (OM) is one of the most common infections in young children, arising from bacterial and/or viral infection of the middle ear. Globally, *Streptococcus pneumoniae* and non-typeable *Haemophilus influenzae* (NTHi) are the predominant bacterial otopathogens. Importantly, common upper respiratory viruses are increasingly recognized contributors to the polymicrobial pathogenesis of OM. This study aimed to identify predominant bacteria and viruses in the nasopharynx, adenoids and middle ears of peri-urban/urban South-East Queensland Australian children, with and without clinical history of chronic otitis media with effusion (COME) and/or recurrent acute otitis media (RAOM).

Methods: Sixty children, 43 diagnosed with OM and 17 controls with no clinical history of OM from peri-urban/urban South-East Queensland community were recruited to the study. Respiratory tract bacterial and viral presence were examined within nasopharyngeal swabs (NPS), middle ear effusions (MEE) and adenoids, using real-time polymerase chain reaction (RT-PCR) and bacterial culture.

Results: At least one otopathogen present was observed in all adenoid samples, 86.1% and 82.4% of NPS for children with and without OM, respectively, and 47.1% of the MEE from the children with OM. NTHi was the most commonly detected bacteria in both the OM and control cohorts within the adenoids (90.0% vs 93.8%), nasopharynx (67.4% vs 58.8%) respectively, and in the MEE (OM cohort 25.9%). Viruses were detected in all adenoid samples, 67.4% vs 47.1% of the NPS from the OM and control cohorts, respectively, and 37% of the MEE. Rhinovirus was the predominant virus identified in

the adenoids (85.0% vs 68.8%) and nasopharynx (37.2% vs 41.2%) from the OM and control cohorts, respectively, and the MEE (19.8%).

Conclusions: NTHi and rhinovirus are predominant otopathogens within the upper respiratory tract of children with and without OM from peri-urban and urban South-East Queensland, Australia. The presence of bacterial otopathogens within the middle ear is more predictive of concurrent URT infection than was observed for viruses, and the high otopathogen carriage within adenoid tissues confirms the complex polymicrobial environment in children, regardless of OM history.

Keywords: otitis media, etiology, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella (Branhamella) catarrhalis*, otitis media with effusion (OME), recurrent acute otitis media (RAOM)

1 INTRODUCTION

Otitis media (OM) is one of the most common infections in young children and is associated with otopathogenic bacteria and/or viruses within the upper respiratory tract (Rovers et al., 2004; Nokso-Koivisto et al., 2015; Phillips et al., 2020; Thornton et al., 2020). Globally, *Streptococcus pneumoniae*, non-typeable *Haemophilus influenzae* (NTHi) and *Moraxella catarrhalis* are the three main bacterial otopathogens of OM. *S. pneumoniae* is identified as the predominant bacterial otopathogen in the middle ear of children experiencing acute otitis media (AOM), while NTHi is more frequently detected in the middle ear of children with recurrent acute otitis media (RAOM) and/or chronic otitis media with effusion (COME) (Ngo et al., 2016). These bacteria are commensal flora in the upper respiratory tract (URT), including nasopharynx and adenoids, which are considered potential reservoirs for both bacterial and viral otopathogens causing middle ear infection (Pettigrew et al., 2012; Stol et al., 2013; Fago-Olsen et al., 2019).

Viral infection of the upper respiratory tract can contribute to OM development, through direct causation of AOM (Chonmaitree and Heikkinen, 1997; Heikkinen and Chonmaitree, 2003; Nokso-Koivisto et al., 2015; Chonmaitree et al., 2016; Schilder et al., 2016; Thornton et al., 2020) and/or initiation of inflammation prolonging middle ear effusion (MEE) (Chonmaitree and Heikkinen, 1997; Heikkinen and Chonmaitree, 2003; Nokso-Koivisto et al., 2015). A range of respiratory viruses, including adenovirus (ADV), rhinovirus (HRV) and respiratory syncytial virus (RSV), have been detected in the middle ear, nasopharynx and adenoids of children with AOM (Heikkinen et al., 1999; Chonmaitree, 2000; Heikkinen and Chonmaitree, 2003; Ishibashi et al., 2003; Monobe et al., 2003; Nokso-Koivisto et al., 2004; Ruohola et al., 2006; Bulut et al., 2007; Drago et al., 2008; Binks et al., 2011; Wiertsema et al., 2011a; Ruohola et al., 2013; Marom et al., 2019; Sawada et al., 2019). Globally, the predominant viruses identified within the middle ear, vary more widely, with RSV reported as the most frequently detected virus in the MEE of children with AOM in the United States (Heikkinen et al., 1999; Chonmaitree, 2000; Marom et al., 2019) and Asian countries (Ishibashi et al., 2003; Monobe et al., 2003; Bulut et al., 2007; Sawada et al., 2019). HRV is identified most often within the MEE of children in Australia (Wiertsema et al., 2011a) and European countries (Heikkinen and Chonmaitree, 2003; Nokso-Koivisto et al., 2004; Ruohola et al., 2006) whilst enterovirus is most frequently identified in Brazil (Buzatto et al., 2017).

Overall, otopathogen detection frequencies appear to relate to geographical location and should be actively considered. For example, in Australia, a majority of studies of OM otopathogen identification have examined rural and/or remote Australian Aboriginal children, who experience significant increased risk of severe OM. These studies include children with severe OM, chronic suppurative otitis media (CSOM) with tympanic perforation and ear discharge (Gibney et al., 2005; Leach and Morris, 2007; Smith-Vaughan et al., 2013). These studies were undertaken in the Northern Territory, a tropical region incorporating many rural and remote communities. Two studies identified bacterial and viral otopathogens within the MEE and nasopharynx of urban children with RAOM (Wiertsema et al., 2011a; Wiertsema et al., 2011b) and were undertaken in the temperate region of Western Australia. The current study aimed to identify predominant bacterial and viral carriage within the nasopharynx, adenoids and middle ears of the upper respiratory tract in peri-urban/urban children undergoing ventilation tube insertion for COME and/or RAOM in South-East Queensland, a subtropical region of Australia. The frequency of bacterial and viral co-infection and their distribution throughout the upper respiratory tracts and middle ear of children with OM were compared to the otopathogen distribution in the upper respiratory tracts of children undergoing adenoidectomy, who had no significant clinical history of OM.

2 METHODS

2.1 Recruitment and Study Cohorts

Children aged 1 to 8 years old who were undergoing ventilation tube insertion (VTI) +/- adenoidectomy for the treatment of COME and/or RAOM were recruited to the OM cohort between December 2008 and December 2010 at Royal Children's Hospital, Brisbane and January and November 2015 at Pindara Private Hospital, Gold Coast, Queensland, Australia. The clinical history of these children included: the presence of MEE for

>3months or recurrent acute OM infection of either 3 episodes within 6 months or 4 or more episodes within 12 months. The cohort without OM history, recruited children of similar age undergoing adenoidectomy as treatment for adenoidal hypertrophy (AH) and/or obstructive sleep apnoea (OSA). These participants had no significant history of OM. All children recruited to this study were fully vaccinated with pneumococcal conjugate vaccines in accordance with the Australian National Immunisation Program schedule [Australian Technical Advisory Group on Immunisation (ATAGI), 2018]. Children with diagnosed immunological abnormality either intrinsic or pharmacological; anatomical or physiological defect; respiratory tract infection; purulent middle ear effusion and any malformations were excluded. All children were examined and clinically well on the day of sample collection and surgery.

This study was approved by the Children's Health Services District Ethics Committee (2008/063 and HREC/14/QRCH/33), Greenslopes Hospital Human Research Ethics Committee (14/18) and the Griffith University Human Research Ethics Committee (MSC/05/08/HREC and MSC/19/13/HREC). Prior, informed consent was provided by each child's parent or guardian.

A series of samples were collected from each child by the surgeon, after anesthesia induction but prior to insertion of VTI or adenoid removal. The samples collected for the OM cohort included NPS, adenoid swab/tissue and MEE, the latter were not collected from participants without OM history. All samples were placed on ice and transferred to the laboratory for processing for RT-PCR and bacterial culture within 4 hours of collection.

2.1.1 Nasopharyngeal Swabs

Nasopharyngeal swabs were collected by trans-nasal insertion of a sterile flexible cotton-wool swab (Copan, Brescia, Italy) reaching the nasopharyngeal space. Swabs were stored in sterile Skim-Milk-Tryptone-Glucose-Glycerol-Broth and placed on ice until processing.

2.1.2 Middle Ear Samples

Prior to MEE collection, outer ear canals were rinsed with sterile physiological saline. An anterior-inferior myringotomy incision was made and MEE from each ear was collected separately using individual sterile Argyle Specimen Traps (Covidien, Dublin, Ireland) connected to a suction system, washed through with 2ml of saline. The MEE was immediately placed on ice until processed. MEE samples were collected from both left and right ears for each OM patient.

2.1.3 Adenoid Tissues

Adenoid tissue was removed using a curette and placed immediately in sterile Hanks buffered saline solution (Invitrogen, Australia) and kept on ice until transferred and processed for bacterial culture. Part of each adenoidal tissue sample was homogenized and placed in sterile Skim-Milk-Tryptone-Glucose-Glycerol-Broth for storage and RT-PCR.

2.2 Bacterial Culture

Following collection, MEE, NPS and adenoid samples were transported on ice to the hospital pathology laboratory and analyzed using standard pathology laboratory culture protocols (Mahon et al., 2014). All predominant bacterial colonies were recorded after 24-72h incubation and visual identification of colonies for *S. pneumoniae*, NTHi and *M. catarrhalis* were confirmed by Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (Shimadzu, Australia). Serotypes for *S. pneumoniae* were initially identified using a Pneumotest kit (Statens Serum Institut – (SSI), Denmark) by Quellung reaction, in accordance with manufacturer instructions. Further identification of serotypes was performed using latex agglutination (Satzke et al., 2015) with latex reagents prepared using antisera from SSI Diagnostica (Ortika et al., 2013) by the Pneumococcal Research Group at the Murdoch Children's Research Institute (Victoria, Australia).

2.3 Bacterial and Viral PCR

Bacterial DNA was extracted from 300µl of MEE, NPS and adenoid samples, as previously described (Smith-Vaughan et al., 2006). RT-PCR detection of *S. pneumoniae*, NTHi and *M. catarrhalis* utilized specific primers for each bacterial target as follows: *S. pneumoniae* (autolysin gene – lytA) (Marsh et al., 2012), NTHi (haemophilus protein D gene – hpd and L-fucose permease gene – FucP) (Binks et al., 2012; Price et al., 2015) and *M. catarrhalis* (outer membrane protein gene – copB) (Marsh et al., 2012), respectively.

Viral otopathogen detection used total nucleic acid extracted using Qiagen x-tractor gene from a 200µl sample spiked with 10^4 copies of Equine Herpes virus, before RT-PCR was performed to detect eight viral pathogens. The viruses included influenza A virus (IAV), influenza B virus (IBV), parainfluenza virus (PIV, including types 1, 2, 3), Human Adenovirus (ADV), Human metapneumovirus (hMPV), Human Respiratory Syncytial Virus (RSV), Human Rhinovirus (HRV) and WU polyomavirus (WU) by Rotorgene instruments, Qiagen Australia, as described previously (Rockett et al., 2013).

2.4 Statistical Analyses

Demographic data including participant age and frequency of OM episodes were compared using independent samples t-tests. Sex, URT presence of bacteria/viruses, patterns of co-colonizing or infecting species were compared between OM and control cohorts using Pearson Chi-square analyses with comparison of detection frequencies between RT-PCR and culture results performed using McNemar's tests. For all statistical analyses, a p-value <0.05 was considered significant and all data analyses were performed using SPSS for Windows, Version 23 (IBM).

3 RESULTS

3.1 Demographics and Sample Collection

A total of 60 children, aged 1-8 years were recruited to the OM group (mean = 3.7 years +/- 1.9 SD, n=43) and the control group without a history of OM (mean = 4.5 years +/- 1.5 SD, n=17).

There were no significant differences in age or sex between the groups, as shown in **Table 1**. The number of episodes of OM differed significantly (p<0.001) between the OM group (Mean = 5.6 +/- 3.2 SD) and control group (Mean = 0.9 +/- 0.9 SD). Overall, the otopathogens identified in all sample types collected from each group are illustrated in **Supplemental Figure 1**. The otopathogens identified in the MEE samples collected and analysed for each group are shown in **Table 2**. Notably within the OM group, a MEE sample was collected from both left and right ears, however one MEE sample was not collected due to pre-existing tympanic perforation. Adenoid samples were only available for 20 children due to OM treatment decisions not including adenoidectomy. Viral RT-PCR results for two children (4 MEE samples) were excluded due to the presence of inhibitors, thus only 81 MEE samples (or 40 paired + 1 single MEE samples) from 43 recruited children were used for viral detection. In addition, one adenoid sample was not successfully collected from a control group participant (n=16) (**Table 1**).

3.2 Bacterial Otopathogens and Viruses Present in the Middle Ear

Within the middle ear effusate (MEE), bacterial otopathogen identification was significantly higher using RT-PCR compared to bacterial culture for each of the 3 predominant bacteria (P<0.001 for each bacterium). Bacterial otopathogens were identified in 47.1% of all MEE samples (n=85) (**Table 2**) compared to 5.9% (n=5) from bacterial culture (**Supplemental Table 1**), thus only RT-PCR data are reported further. NTHi, identified using RT-PCR, was the most common bacterium detected in the 85 MEE samples (25.9%), followed by *M. catarrhalis* (20.0%) and *S. pneumoniae* (17.6%) (**Table 2**). Overall, at least one of the three predominant bacteria were identified within either one or both ears of 26 of the 42 participants with paired MEE samples. Comparison of the left and right ear samples from the same child (n=26) showed that different bacteria were identified in each ear (76.9%, n=20 children) whilst only 6 children had the same bacteria present in each ear **(Supplemental Table 2**).

At least one of the viruses tested was found in 37.0% of MEE samples (n=81), with HRV (19.8%), RSV (7.4%) and WU (6.2%) detected most frequently (**Table 2**). Overall, 22 paired left and right MEE samples had virus present in one or both ears (55.0%, n=40 pairs), with 15 of these pairs having a different virus in each ear (68.2%) compared to 8 pairs showing the same virus in both the left and right ears (31.8%) (**Supplemental Table 3**).

TABLE 1 | Demographic data and samples collected from peri-urban and urban children in South-East Queensland undergoing ventilation tube insertion for otitis media (OM) or adenoidectomy (Control) in the absence of a clinical history of OM.

	OM	Control
Number	43	17
Mean age in year (range)	3.7 (1-8)	4.5 (3–8)
Male (%)	23 (53.5%)	12 (70.6%)
Mean episodes of AOM within last 12 months (range)	5.6 (4-11)	0.9 (0-2)
Middle ear effusion (MEE)	85	–
Nasopharyngeal swabs (NPS)	43	17
Adenoid samples	20	16

TABLE 2 | Bacterial otopathogens and viruses identified by RT-PCR in the middle ears of peri-urban and urban children in South-East Queensland undergoing ventilation tube insertion for otitis media (OM).

	OM(n=85 ears)
Bacterial otopathogens (n=85 ears)	40 (47.1%)
S. pneumoniae	15 (17.6%)
H. influenzae	22 (25.9%)
M. catarrhalis	17 (20.0%)
Viruses (n=81 ears)	30 (37.0%)
Adenovirus	3 (3.7%)
Human metapneumovirus	3 (3.7%)
Influenza B virus	1 (1.2%)
Respiratory syncytial virus	6 (7.4%)
Rhinovirus	16 (19.8%)
WU polyomavirus	5 (6.2%)
No pathogen	30 (37.0%)
Bacterial otopathogens alone	21 (25.9%)
Viruses alone	15 (18.5%)
Bacterial otopathogens and viruses	15 (18.5%)

Number and percentage (between brackets) of samples in which bacteria were detected. Viral detections are from 81 MEE samples due to presence of inhibitors in 4 samples and one sample not collected due to pre-existing tympanic membrane perforation.

Together these data demonstrated that 63.0% (51/81 ears) contained at least one bacteria or virus, with the remaining 30 ears (37.0%) being negative for either the three predominant bacteria causal for OM, or the selected panel of 8 common respiratory tract viruses. Bacterial detection alone (virus negative) or concurrent detection of bacteria and virus was observed in 25.9% of MEE samples, with viral detection alone observed in 18.5% of the MEE samples. With respect to concurrent bacterial and viral presence within the MEE, ADV detection is likely correlated with the presence of NTHi (r=0.321, p=0.001, **Supplemental Table 4**).

3.3 Bacterial Otopathogens and Viruses Present in the Nasopharynx

Bacterial identification within the nasopharynx of children with and without a clinical history of OM by RT-PCR demonstrated that at least one of the 3 predominant otopathogenic bacteria were present in 86.0% and 82.4% of NPS samples respectively. Bacteria were more commonly identified using RT-PCR than using bacterial culture (55.8% and 70.6% for OM and control cohorts respectively) thus only RT-PCR data is reported further. NTHi was identified most frequently in the NPS of children with OM (67.4%, 29/43) and the control cohort (58.8%, 10/17). Neither RT-PCR or bacterial culture data demonstrated any statistically significant difference in otopathogen identification between the OM and control groups (**Table 3** and **Supplemental Table 5**).

Viral identification showed that 67.4% and 47.1% of NPS samples contained one of the 8 viruses investigated in the OM and control groups, respectively. HRV was the most commonly detected virus in NPS samples from both cohorts, with ADV, hMPV, RSV, IAV and WU viruses detected at lower frequencies within the OM cohort only (**Table 3**).

Further examination of the frequency of co-detection of bacteria and viruses was assessed in NPS from both cohorts. Overall, the frequencies where no pathogen, and virus alone were

TABLE 3 | Otopathogens and viruses identified by RT-PCR in the nasopharynx and adenoids of peri-urban and urban children in South-East Queensland undergoing ventilation tube insertion for otitis media (OM) or adenoidectomy in the absence of a clinical history of OM (Control).

Nasopharynx	OM (n=43)	Control (n=17)	*P*
Otopathogens	37 (86.1%)	14 (82.4%)	*0.718*
S. pneumoniae	20 (46.5%)	7 (41.2%)	*0.708*
H. influenzae	29 (67.4%)	10 (58.8%)	*0.528*
M. catarrhalis	23 (53.5%)	8 (47.1%)	*0.653*
Viruses	29 (67.4%)	8 (47.1%)	*0.143*
Adenovirus	5 (11.6%)	0 (0.0%)	*0.142*
Human metapneumovirus	3 (7.0%)	0 (0.0%)	*0.264*
Influenza A virus	1 (2.3%)	0 (0.0%)	*0.526*
Respiratory syncytial virus	2 (4.7%)	0 (0.0%)	*0.366*
Rhinovirus	16 (37.2%)	7 (41.2%)	*0.776*
Parainfluenza virus	5 (11.6%)	1 (5.9%)	*0.666*
WU polyomavirus	8 (18.6%)	0 (0.0%)	*0.056*
No otopathogen	2 (4.7%)	1 (5.9%)	*0.841*
Bacteria alone	11 (25.6%)	8 (47.1%)	*0.114*
Viruses alone	4 (9.3%)	2 (11.8%)	*0.772*
Bacteria and viruses	26 (60.5%)	6 (35.3%)	*0.091*
Adenoids	**OM (n=20)**	**Control (n=16)**	***P***
Otopathogens	20 (100%)	16 (100%)	
S. pneumoniae	13 (65.0%)	12 (75.0%)	*0.517*
H. influenzae	18 (90.0%)	15 (93.8%)	*0.686*
M. catarrhalis	13 (65.0%)	11 (68.8%)	*0.813*
Viruses	20 (100%)	16 (100%)	
Adenovirus	13 (65.0%)	7 (43.8%)	*0.202*
Rhinovirus	17 (85.0%)	11 (68.8%)	*0.244*
Parainfluenza virus	12 (60.0%)	10 (62.5%)	*0.875*
WU polyomavirus	11 (55.%)	5 (31.3%)	*0.154*
No pathogen	0 (0.0%)	0 (0.0%)	–
Bacteria alone	0 (0.0%)	0 (0.0%)	–
Viruses alone	0 (0.0%)	0 (0.0%)	–
Bacteria and viruses	20 (100.0%)	16 (100.0%)	–

Number and percentage (between brackets) of samples in which bacteria were detected. P value was analyzed by Pearson Chi-square analyses.

detected within NPS were low, compared to bacteria alone, however there was no significant difference between children with and without OM (4.7% vs 5.9%, 9.3% vs 11.8% and 25.6% vs 47.1%, respectively) (**Table 3**). The frequency of co-detection of bacteria and viruses in the OM group appeared higher but did not differ significantly from the control group (60.5% vs 35.3%, p=0.091) (**Table 3**).

3.4 Bacterial Otopathogens and Viruses Present in the Adenoids

Adenoid sample collections were not collected from every recruited participant due to clinical treatment decisions however adenoid tissues were obtained from both the OM (n=20) and control cohorts (n=16) (**Table 1**). RT-PCR analyses confirmed concurrent bacterial and viral otopathogens within the adenoids of all children, regardless of OM history. NTHi was most frequently detected in both the OM (90.0%, n=18/20) and control cohorts (93.8%, n=15/16). There were no significant differences in bacterial identifications between OM and control cohorts (**Table 3** and **Supplemental Table 5**).

All adenoid samples, from both OM and control cohorts were positive for at least one of the 8 viruses tested. HRV was the predominant virus identified within OM (85.0%, n=17/20) and control group (68.8%, n=11/17) adenoids. ADV, PIV and WU virus were detected in adenoids from both cohorts but did not differ in frequency between the OM and control groups (**Table 3**).

3.5 Concurrent Bacterial Otopathogens and Viruses Present in the Same Child

Concurrent bacterial and viral detection of the three predominant bacteria, NTHi, *S. pneumoniae* and *M. catarrhalis* and the three most frequently detected viruses, HRV, WU polyoma virus and RSV, were examined within the upper respiratory tract of children with a history of OM, undergoing VTI for OM. For each child, the otopathogens identified within the MEE, NPS and adenoids were mapped and their concurrent presence in multiple upper respiratory tract locations within the same child are presented in **Figure 1**. These data show that when any of the 3 predominant bacteria are present within the middle ear, it is highly indicative of the same bacteria being present within the NPS and adenoids concurrently. Interestingly, only the presence of HRV within the MEE may indicate concurrent presence within the nasopharynx and adenoids, with RSV not identified as frequently in other areas of the upper respiratory tract (**Figure 1**).

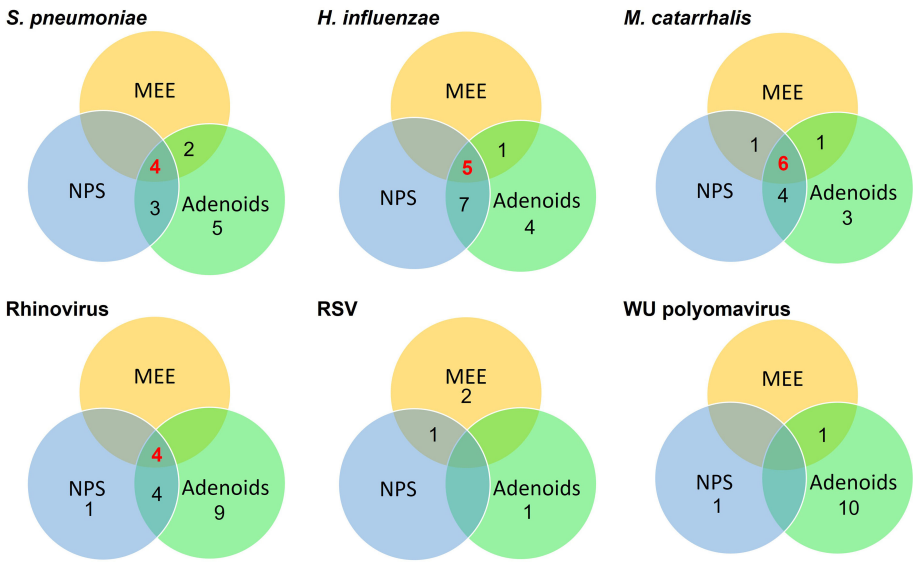

FIGURE 1 | Distribution of concurrent bacterial and viral detection of predominant otopathogenic microbes within the middle ear effusate (MEE), nasopharyngeal swab (NPS) and adenoid samples of the same peri-urban/urban children in South-East Queensland who were undergoing ventilation tube insertion for otitis media (OM). Each circle represents the sample locations within each child and their intersections indicate the number of children with the same microbe identified within two or three of the sample locations.

3.6 Pneumococcal Serotypes

There were 31 pneumococcal isolates serotyped from samples collected in this study. Fourteen (45.2%) were serogroup 23 (serotype 23A was 12.9%, 23B was 32.3%), eight were serotype 11A (25.8%), four were serotype 35B (12.9%), three were serotype 16F (9.7%) and there were single isolates of serotypes 19A and 21 (3.2% each respectively).

4 DISCUSSION

This study identified the predominant bacterial and viral carriage of OM in young peri-urban and urban children of South-East Queensland, Australia undergoing VTI for OM. Otopathogen presence was determined using RT-PCR in MEE, NPS and adenoid samples from the same child, who were clinically well at collection. The frequency of detection of the three predominant bacteria considered causal for OM within the URT, *S. pneumoniae*, NTHi and *M. catarrhalis*, did not differ significantly regardless of the child's clinical history of OM. Rhinovirus was most frequently detected with the MEE of children with OM, with RSV and WU polyomavirus also observed, albeit at slightly, but not significantly lower rates of detection. Overall, regardless of the children's history for OM, otopathogen detection was highest in the adenoids, then comparatively less in the nasopharynx and for children with OM, the middle ear. Despite significantly different geographical location and ethnicity demographics, these findings are consistent with the findings of a study of Australian Aboriginal children from Central Australia. Australian Aboriginal children are reported to experience significantly increased risk of OM and this report identified *Streptococcus* sp., *H. influenzae* and *M. catarrhalis* as common operational taxonomic units (OTUs) found in the adenoids, nasopharynx, and middle ear in these children undergoing treatment for OM (Jervis-Bardy et al., 2015). Similarly, more recent studies in Australia, Finland and Switzerland also found that these OTUs were abundant in the MEE of children with OM (Chan et al., 2016; Sillanpää et al., 2017; Brugger et al., 2019).

Interestingly, a larger study (n=143) conducted in Western Australia reported that children undergoing VTI surgery for RAOM at a younger age than our cohorts, had significantly higher rates of nasopharyngeal colonization with NTHi and *S. pneumoniae* compared to healthy controls (Wiertsema et al., 2011b). These younger children (mean age=1.7 years) were more severely affected by RAOM than observed for the older (mean age=3.7 years) children being treated for RAOM/COME in the current study. These findings perhaps reflect different OM cohorts with the more clinically recognizable, severe and repeated AOM in children undergoing VTI at an earlier age. It is recognized that for most children, OM frequency typically reduces with age, in association with facial growth (Bluestone, 1996; Swarts et al., 2013) but particularly with progressive maturation of their immune system (Wiertsema and Leach, 2009). The smaller cohort sizes in this study and older children recruited may help to explain the lack of significance between the frequencies of otopathogen detection observed between OM affected children and control children, in conjunction with physical and immunological development.

Consistent with previous publications, this study also demonstrated that RT-PCR was more sensitive than traditional culture techniques to the detection of bacterial otopathogens

(Ngo et al., 2016). This is due to the organism's fastidious growth requirements and the presence of viable organisms within a biofilm, which reduce the sensitivity of detection using culture (Thornton et al., 2013; Niedzielski et al., 2021). In the current study, which was undertaken when the children were well, it is not possible to differentiate whether the magnitude of the difference between RT-PCR and culture results was technical or due to a lower bacterial load at the time of sampling. Technically, RT-PCR may detect unviable bacterial fragments, inflating detection frequencies, although previous research confirms that live bacteria present in biofilms, identified using fluorescence in situ hybridisation (FISH) are not always culturable (Thornton et al., 2013).

Co-detection of different bacteria in different locations of the URT in this study has not only confirmed the role of the nasopharynx and adenoids as potential sources of NTHi and *M. catarrhalis* but has also demonstrated that *S. pneumoniae* detection in the middle ear of children with OM is also linked to colonization of the nasopharynx and/or adenoids of the same child. In all cases where *S. pneumoniae* was isolated from the MEE, it was also isolated from either the NPS or adenoids, suggesting that *S. pneumoniae* in the middle ear has originated from the nasopharynx and/or adenoids, although further investigation, including typing studies are needed. Similarly, NTHi and *M. catarrhalis* may also have originated from the nasopharynx and/or adenoids in the current study, but not all NTHi or *M. catarrhalis* identifications within the same child were concurrently identified in the MEE, nasopharynx and/or adenoids. A report by Stol et al. (2013), has shown a genetic match between *S. pneumoniae* found in the middle ear and nasopharynx of children with RAOM/COME. Importantly, these children were undergoing VTI, similar to the current study, and were also not experiencing current active OM infection at the time of sample collection. Furthermore, only 80% of NTHi isolates from the middle ear and nasopharynx were a genetic match (Stol et al., 2013). Together, the current findings and those by Stol, suggest that NTHi or *M. catarrhalis* in the middle ears of children with OM may persist and are not necessarily seeded from those bacteria in the nasopharynx and/or adenoids.

Throughout this study, NTHi was the predominant otopathogen detected in the URT, particularly in the nasopharynx and adenoids of children without a clinical history of OM, in addition to within the middle ear of children with RAOM/COME. A limitation of this study and any other studies using a control group comprised of children undergoing surgery for adenotonsillar hypertrophy/disease, is that this pathogenic process may also impact the high prevalence of NTHi in the control group. Despite this limitation, the current results are consistent with reports from Western Australia and New Zealand (Wiertsema et al., 2011b; Mills et al., 2015; Seppanen et al., 2020) and reflect the regional prevalence of NTHi as the most common otopathogen in the middle ear and nasopharynx of children with RAOM/COME reported in our global systematic review (Ngo et al., 2016).

Importantly in this study, the predominant bacterial otopathogen, NTHi was progressively increasingly resistant to β-lactam antibiotics over the study period, rising from 14.3% (2009-2010) to 67.9% (2015) (**Supplemental Table 6**). In addition, multidrug resistance was not observed for NTHi within the 2009-2010 isolates but was present within the 2015 cohort (12/28 isolates data not shown). These findings highlight the importance of continuous surveillance for antimicrobial resistance to inform antibiotic therapy. Monitoring of serotype variants and their frequency in children of different ages undergoing a range of national immunization programs (NIP) from around the world would assist in evaluation of the potential impact of existing NIP vaccinations on OM prevalence. For example, recently, a 10-valent pneumococcal NTHi protein D conjugate vaccine was reported to reduce the frequency of middle ear infection caused by NTHi in Australian Indigenous communities (Leach et al., 2015). Development of an efficacious vaccine for NTHi has potential benefits for reduction in OM prevalence in both urban children and Indigenous Australian children, the latter children are known to experience significantly increased risk of severe OM development at a young age (Leach et al., 1994; Boswell and Nienhuys, 1996).

Limitations of the current study, in addition to those mentioned previously, include the selection of the comparative control group. Children undergoing adenoidectomy+/-tonsillectomy do not reflect a "healthy" cohort but are a sample of convenience, undergoing ear, nose and throat surgery and anesthesia, permitting collection of comparative clinical samples. Children with a clinically recognizable history of OM were excluded from control group recruitment and this is reflected in fewer OM episodes. The slightly but not significantly higher number of boys in the control group reflects recruitment is consistent with previous studies (Schupper et al., 2018) showing a higher incidence of adenoidectomy for boys. Similarly, the age range distribution between the control and OM groups is reflective of the diagnostic and clinical pathways, which tend to result in surgical resection of adenoids at a later age than for VTI due to RAOM or COME.

In the current study, despite the small sample size, *S. pneumoniae* and *M. catarrhalis* were detected at similar or lower frequencies compared to NTHi in the different regions of the URT in children with and without RAOM/COME. These results are consistent with the results of a systematic review of previous reports from the Pacific region, including Australia and New Zealand (Ngo et al., 2016).

Viral detections within the MEE of the present study occurred within 37.0% of MEE samples. The presence of these viruses, within the middle ears of OM prone children, in the absence of AOM symptomology provides support to the potential role of viruses in COME pathogenesis. Persistence of unresolving infection and presence of otopathogens in the middle ear may contribute to RAOM pathogenesis through dysregulated innate immune responses including inflammation and accumulation of middle ear effusate reflective of COME (Massa et al., 2015). Viral presence within the middle ear of OM-prone children contrasts to the sterile middle ear reported in children and adults without OM (Westerberg et al., 2009). Improved detection of viral otopathogens using RT-PCR may continue to better inform

our understanding of the role of viruses within OM pathogenesis, particularly COME (Massa et al., 2015).

In this study, HRV was the predominant virus detected in all URT locations. The predominance of this virus is consistent with previous reports from Finland, the Netherlands and Western Australia, where viral detection within the middle ear was reported using PCR methods (Pitkäranta et al., 1998; Nokso-Koivisto et al., 2004; Ruohola et al., 2006; Wiertsema et al., 2011a; Stol et al., 2013). In contrast, RSV was the most common virus identified (via PCR) in the MEE of children with OM from Japan and Turkey (Monobe et al., 2003; Bulut et al., 2007). Similar studies from the US also confirmed RSV as the predominant virus within the middle ear, however these studies did not use PCR based detection methods (Heikkinen et al., 1999; Patel et al., 2007). Identification of the predominant virus within the MEE of children experiencing OM clearly varies with the region and recruitment criteria for participants in each study, including age and clinical symptomology. Furthermore, viral incidence may vary at different times within a year or between years as evidenced by a recent report from the US. The study reported significantly increased frequency of RSV detection in the URT of children with OM in the peak season of RSV, compared to the shoulder seasons of RSV (Makari et al., 2015). Therefore, the timing of patient recruitment may impact the study population detection frequencies of the otopathogen, if it occurred during the low-activity RSV seasons rather than the peak period (Chonmaitree, 2006). Each virus may present differently over the year, for example, HRV infections occur year round (Winther et al., 2006), which may increase the opportunity for detection and reporting. Regardless of which virus is predominant, an effective vaccine against that virus would benefit children with OM, who are positive for the virus, unfortunately, for the two most commonly detected viruses, HRV and RSV, vaccine development has been difficult (Campbell et al., 2015; Glanville and Johnston, 2015).

The frequency of viral detection varied by location within the URT, with the frequency of detecting at least one virus present in the NPS of children with OM tended to be higher than observed in control, although not significantly. This trend is consistent with a previous study in which children with RAOM had significantly higher frequency of viral detection in the nasopharynx compared to their control counterparts (Wiertsema et al., 2011a). In contrast, all adenoid tissues from children with or without OM in the current study had at least one virus present and this finding is consistent with previous studies whereby 90-100% adenoid samples from children with adenoidal hypertrophy, RAOM, or with OME had viruses detected (Herberhold et al., 2009; Sato et al., 2009; Szalmás et al., 2013). Interestingly, the current study showed that children with OM had higher frequencies of ADV and WU detection in both the nasopharynx and adenoids compared to children without OM. These findings are consistent with previous studies in Australia conducted on non-Indigenous children with/without OM (Wiertsema et al., 2011a) and Indigenous Australian Aboriginal children with/without the disease (Binks et al., 2011). In addition, those viruses were also detected in the middle ear of children with OM in the current study and further research is needed to investigate the potential role of these viruses in OM pathogenesis.

Finally, bacterial-viral co-infection or colonization in the URT may increase the incidence of OM development in children. Indeed, the current study showed that children with OM tended to show higher frequency of bacterial-viral detection in the nasopharynx compared to children without OM although the difference was not statistically significant (60.5% vs 35.3%). However, the role of co-occurrence of specific microbes in potentially increasing the risk of OM pathogenesis was demonstrated in the present study by the co-detection and correlation of ADV and NTHi. Further exploration of co-infection and its impact on OM pathogenesis has been provided through animal models, with elegant research using the chinchilla often considered the most representative of the human condition. Using the chinchilla model, Suzuki and Bakaletz (1994) demonstrated that intranasal inoculation with both ADV type 1 and NTHi resulted in development of more severe OM than was observed using either agent as a single pathogen (Suzuki and Bakaletz, 1994; Miyamoto and Bakaletz, 1997). In addition, intranasal inoculation of chinchillas using ADV may increase the transduction of NTHi from the nasopharynx to the middle ear and induce NTHi OM (Miyamoto and Bakaletz, 1997). Murine viral pre-infection models that utilize polymicrobial bacterial pathogens including *S. pneumoniae* and *M. catarrhalis* have demonstrated increased infection frequency and severity (Krishnamurthy et al., 2009), whilst bacterial co-infection models using NTHi and *M. catarrhalis* have also shown enhanced bacterial persistence and antimicrobial resistance (Armbruster et al., 2010). Interestingly, a study by Short et al. (2011) demonstrated that infection with influenza virus enabled development and persistence of pneumococcal OM *via* neutrophil extracellular trap (NET) induction in the mouse (Short et al., 2011). Persistent effusion, bacteria and NETs have been reported in children with RAOM (Thornton et al., 2013) and together, viral-bacterial co-infection play significant roles in the development and persistence of OM.

Overall, the current study identified NTHi and HRV as the predominant otopathogens within the URT of peri-urban and urban children, with and without COME/RAOM from South-East Queensland Australia. The presence of multiple otopathogens, both viral and bacterial within the middle ear and the upper respiratory tracts of clinically well children undergoing VTI surgery for COME/RAOM confirm the complexity of vaccine development to reduce the risk and impact of this frequent childhood disease. For the future, improved routine surveillance of otopathogens present in the URT of children experiencing OM, including affected children of differing ages from across the world, will better reflect the impact of existing vaccines and the support the development of new vaccines for OM prevention.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Children's Health Services District Ethics Committee (2008/063 and HREC/14/QRCH/33), Greenslopes Hospital Human Research Ethics Committee (14/18) and the Griffith University Human Research Ethics Committee (MSC/05/08/HREC and MSC/19/13/HREC), in accordance with the National Statement on Ethical Conduct in Human Research 2007. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

HM, RT and AC designed the study. HM, CP and BM developed and planned surgical collection protocols. BM, CP, HM, RT and MN designed recruitment and collection protocols and HM, BM and CP oversaw patient recruitment and sample collections. CN, TS, RT and QPID, MCRI and GCUH Pathology consortia processed samples. CN, HM, RT, TS, AC analysed data. CN and HM drafted the manuscript and all authors contributed to the article and approved the submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.775535/full#supplementary-material

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Immunomodulation as a Protective Strategy in Chronic Otitis Media

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Introduction: Major features of the pathogenesis in otitis media, the most common disease in childhood, include hyperplasia of the middle ear mucosa and infiltration by leukocytes, both of which typically resolve upon bacterial clearance *via* apoptosis. Activation of innate immune receptors during the inflammatory process leads to the activation of intracellular transcription factors (such as NF-κB, AP-1), which regulate both the inflammatory response and tissue growth. We investigated these leading signaling pathways in otitis media using mouse models, human samples, and human middle ear epithelial cell (HMEEC) lines for therapeutic immunomodulation.

Methods: A stable otitis media model in wild-type mice and immunodeficient KO-mice, as well as human tissue samples from chronic otitis media, skin from the external auditory canal and middle ear mucosa removed from patients undergoing ear surgery, were studied. Gene and protein expression of innate immune signaling molecules were evaluated using microarray, qPCR and IHC. *In situ* apoptosis detection determined the apoptotic rate. The influence of bacterial infection on immunomodulating molecules (TNFα, MDP, Tri-DAP, SB203580, Cycloheximide) in HMEEC was evaluated. HMEEC cells were examined after bacterial stimulation/inhibition for gene expression and cellular growth.

Results: Persistent mucosal hyperplasia of the middle ear mucosa in chronic otitis media resulted from gene and protein expression of inflammatory and apoptotic genes, including NODs, TNFα, Casp3 and cleaved Casp3. In clinical chronic middle ear samples, these molecules were modulated after a specific stimulation. They also induced a hyposensitive response after bacterial/NOD-/TLR-pathway double stimulation of HMEEC cells *in vitro*. Hence, they might be suitable targets for immunological therapeutic approaches.

Conclusion: Uncontrolled middle ear mucosal hyperplasia is triggered by TLRs/NLRs immunoreceptor activation of downstream inflammatory and apoptotic molecules.

Keywords: otitis media, immunology, apoptosis, TNFα, NOD-like receptors, TOLL-like receptors

INTRODUCTION

Otitis media (OM) is one of the most common ear diseases and a serious healthcare problem. Furthermore, chronic OM is one of the most important reasons for acquired and preventable hearing loss (World Health Organization, 2004). Yet, the pathogenesis of OM is not fully understood. It is currently assumed that the etiology is multifactorial, with the innate immune system and its reaction during bacterial infections playing an essential role. *Streptococcus pneumoniae* and *Haemophilus influenzae* are the predominant bacterial pathogens of which, in the case of chronic OM, especially non-typable *H. influenzae* (NTHi) is found to be a dominant pathogen (Ngo et al., 2016). Prior viral upper respiratory infection (URI) increases OM incidence, with the middle ear (ME) exhibiting more chronic infection (Chonmaitree et al., 2015). The immune response to pathogens leads to hyperplasia, leukocytic infiltration and apoptosis of the ME mucosa (Leichtle et al., 2011; Wigand et al., 2018). Current therapy for chronic OM consists of topical and systemic antibiotics, improvement of middle ear ventilation with pressure equalization tubes (Leichtle et al., 2017) and/or surgery (Tisch et al., 2020).

The innate immune system is responsible for the rapid recognition of, and defense against, pathogens. Pattern recognition receptors (PRRs) of the ME mucosa recognize bacterial molecules, termed pathogen-associated molecular patterns (PAMPs), leading to the expression of inflammatory cytokines, chemokines, interferons and antimicrobial peptides (Takeuchi and Akira, 2010). Important representatives of the PRRs are the Toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain–like (NOD) receptors (NLRs).

TLRs are membrane-bound proteins that consist of a leucine-rich extracellular domain, a transmembrane fragments and a cytoplasmic domain. They surveil both the cell surface and vesicular cell compartments for the presence of PAMPs. Recognition of PAMPs leads *via* the downstream adaptors myeloid differentiation factor 88 (MyD88) and/or TRIF (TLR domain containing adapter inducing interferon-β) to activation of NF-κB (nuclear factor kappa B) and MAPKs (mitogen-activated protein kinases), which in turn mediate the expression of important proinflammatory cytokines including interleukin-1 beta (IL1β) and tumor necrosis factor alpha (TNFα) (Kawai and Akira, 2009; Kurabi et al., 2016). The importance of the TLRs to OM resolution have been demonstrated in TLR knockout (KO) mice models, in which ME infection always results in chronic disease (Leichtle et al., 2010).

NLRs are cytoplasmic proteins that recognize intracellular PAMPs. They consist of a caspase recruitment domain (CARD), a nucleotide binding and oligomerization domain (NOD) and leucine-rich repeats. The first identified NLRs, NOD1 and NOD2, recognize peptidoglycan derivatives, that contain a diaminophilic acid (TriDAP) and muramyl dipeptide (MDP) (Inohara et al., 2005; Jeon et al., 2012). Recognition of PAMPs *via* NLRs leads *via* the adaptor RIP2 (receptor-interacting-serine/threonine-protein kinase 2) to the activation of NF-κB and MAPK pathways resulting in production of cytokines and chemokines similarly to the TLRs (Kurabi et al., 2016). NLRP3 (NLR family pyrin domain containing 3) acts differently in that it participates in the inflammasome, which processes pro-IL1β and pro-IL18 into their active forms. The importance of NLRs to ME pathogenesis has also been demonstrated using KO mouse model. which show a delayed inflammatory response to infection as well as prolonged OM with mucosal hyperplasia (Lee et al., 2019).

These two independent innate immune systems also interact with each other. Components of the bacterial cell wall such as the muramyl dipeptides (MDP) activate both TLR2 and NOD2, while NOD2 activation *via* RICK (receptor-interacting serine/threonine protein kinase) leads to a down-regulation of the TLR2 signal cascade (Strober et al., 2006). A summary of the different innate immune pathways implicated in OM is illustrated in **Figure 1**.

NF-κB is a central regulator of many cytokine and immunoglobulin genes, and is involved in immune, inflammatory, viral, and acute phase reactions. It consists of the p50 and p65 subunits and preexists in the cytoplasm in an inactive form, complexed to its inhibitor, IκB (Kieran et al., 1990; Baltimore and Beg, 1995). PAMP-induced stimulation and degrading of the inhibitor causes NF-κB to be released and activate the transcription of proteins including ICAM1, TNFα, IL1β, IL6 and IL8 (Fujisawa et al., 1996; Johnson et al., 1997; Nassif et al., 1997; Fakler et al., 2000; Melotti et al., 2001). Several studies have noted a connection between the MyD88/NF-κB pathway and OM (Johnson et al., 1997; Nassif et al., 1997).

TNFα itself can lead to the expression of other proinflammatory cytokines, chemokines and antibacterial peptides (Vitale et al., 2007). TNFα also plays an important role in apoptosis, a critical feature of tissue remodeling during hyperplasia, recovery of normal mucosal structure during OM resolution and clearance of leukocytes from the ME. The binding of TNFα to death receptors of the TNFα receptor family induces the formation of the death-induced signaling complex (DISC) and the activation of caspase-8 and caspase-3, ultimately initiating apoptosis. This extrinsic apoptosis pathway is supplemented by an intrinsic apoptosis pathway, with mitochondria as the central component, that can be induced *via* the extrinsic pathway (Morris et al., 2018). In order to avoid excessive apoptosis, there is also an independent parallel negative feedback loop mechanism in which the activation of NF-κB by TNFα has an anti-apoptotic effect (van Antwerp et al., 1996).

In summary, innate immune responses play an important role in the timely resolution of OM. However, imbalances in the innate immune system network may lead to failure of bacterial clearance, persistent inflammation and chronic OM. A high probability of chronic OM occurs in the absence of central components of the aforementioned pathways, such as TLR, NLR, MyD88 and TNFα (Leichtle et al., 2011). Hence, these pathways are of central importance in the normal recovery from OM and in the pathogenesis of chronic OM. This study assesses the networks of the innate immune system in chronic OM, with a focus on the possibility of new immunological therapy approaches.

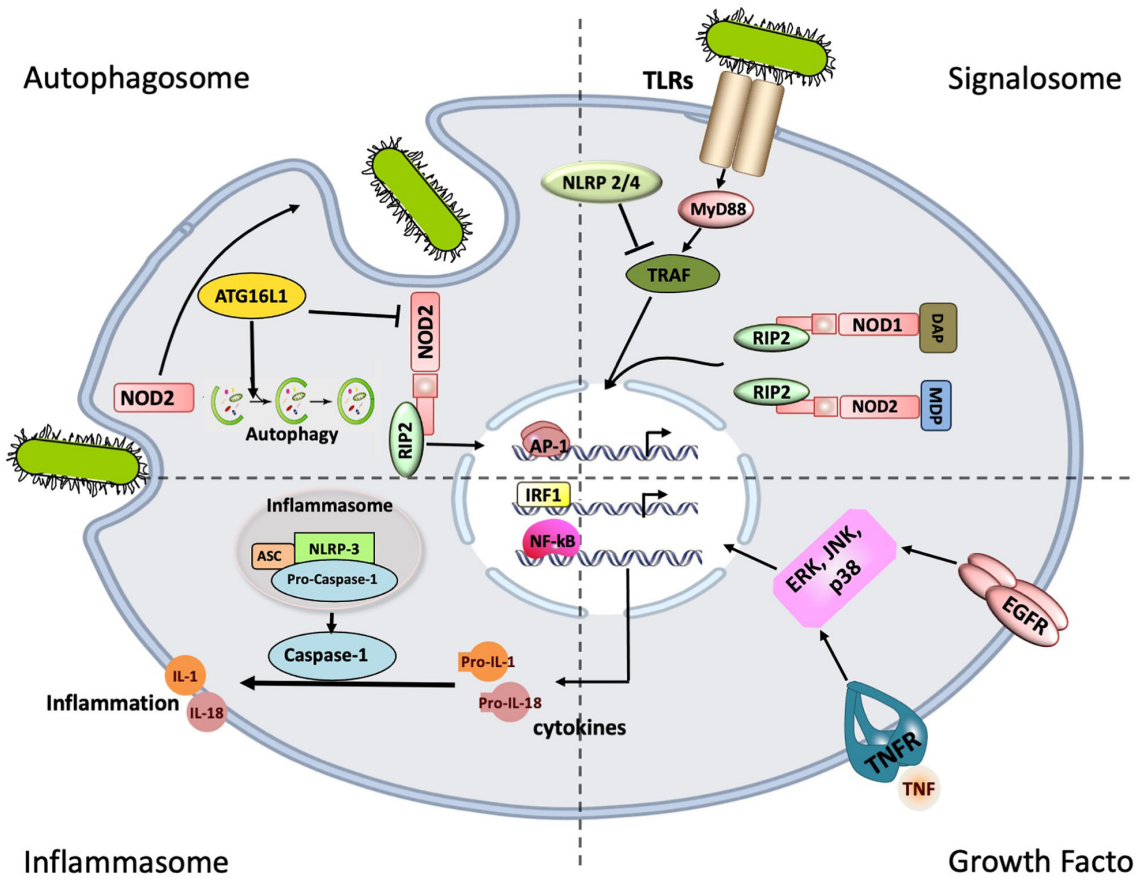

FIGURE 1 | Innate immune signaling pathways implicated in bacterial Otitis Media (OM). The most common receptors involved in pattern recognition are the Toll-like receptors (TLRs), and NOD-like receptors (NRLs) of which signaling leads to the activation of Nuclear factor of kappa-B (NF-κB), the main transcription factor involved in cytokine production. Stimulation of NLRP3 leads the inflammasome to recruit and activate caspase-1, which in turn cleaves the pro-forms of IL1ß, Il18. These pro-inflammatory cytokines are in turn released in their mature/bioactive forms. The intracellular Nucleotide oligomerization domain -1 and -2 (NODs 1 and 2) can also induce inflammation through the RICK/RIP2 pathway. However, NOD2 can also induce autophagy through ATG16L1 shifting the balance of NOD2 downstream signaling. Crosstalk between the different immune elements: TLRs/NRLs, the Inflammasome and Growth factors are believed to modulate the immune responses.

METHODS

Mouse Models

TNF$^{-/-}$, NOD1$^{-/-}$, NOD2$^{-/-}$, RIPK2$^{-/-}$ mice and their age-matched C57BL/6 wild-type (WT) controls were purchased from Jackson Laboratory (Bar Harbor, ME). TL2$^{-/-}$, TLR4$^{-/-}$ and MyD88$^{-/-}$ mice were originally kindly provided to us by Akira and colleagues (Hoshino et al., 1999; Miyao et al., 2006; Leichtle et al., 2009). The animals were kept under specific pathogen-free conditions (BSL2). All experiments were performed according to the National Institutes of Health Guidelines and approved by the Institutional Animal Care and Use Committee of the Veterans Affairs Medical Center in San Diego, CA.

Bacteria

Haemophilus influenzae strain 3655 (nontypeable NTHi, biotype II) was used. In brief, A culture was streaked onto a chocolate agar plate and placed in a 37°C incubator overnight. two colonies were inoculated into 25 mL of brain heart infusion (BHI) media supplemented with 1 mL of Fildes enrichment (BD Diagnostic Systems) and grown for 16 hours at 37°C with shaking. The next day, the bacterial culture was spun down at 7,000 rpm for 10 minutes at 4°C and the pellet resuspended in fresh BHI media. Stock was diluted to 10^5-10^6 CFU/mL and 5 µL was injected per ear to induce infection in the ME of the mice (Hernandez et al., 2008).

Animal Experiments

A total of 60 mice were examined. Groups of 6 mice per strain (TNF$^{-/-}$, NOD1$^{-/-}$, NOD2$^{-/-}$, RIPK2$^{-/-}$, TLR2$^{-/-}$, TLR4$^{-/-}$, MyD88$^{-/-}$ and WT mice) were operated under general anesthesia using ketamine rodent cocktail (Hernandez et al., 2008). A small opening was created in the surgically exposed ME bulla and both sides were inoculated on with NTHi strain described above,

afterwards the intratympanic access was closed with connective tissue and the cervical access closed surgically. Untreated mice group served as a control group at time zero. Data from all mice except RIPK2$^{-/-}$ have been published previously (Leichtle et al., 2009; Hernandez et al., 2015; Lee et al., 2019).

Histology

ME were collected from control and NTHi treated animals on day 1, 2, 3, 5, 7 and 10, as for the KO mice on day 3 and 10. For this purpose, intracardiac perfusion with PBS was carried out under general anesthesia, followed by 4% paraformaldehyde (PFA) at the respective times. The ME of the mice were microsurgically resected, postfixed overnight with 4% PFA overnight, and decalcified in an 8% EDTA and 4% PFA solution over a 14-day period. The bullae were then processed in 30% sucrose followed by OCT media and cut as cryosections. For tissue histology, the ME bullae were embedded in paraffin, sectioned at 7 µm and stained with hematoxylin-eosin (H&E). Sections from the same region of each middle ear were digitally recorded, and the mucosal thickness and the percentage area of the middle ear lumen occupied by inflammatory cells were determined at standard locations. The data were not normally distributed and were analyzed using the Kruskal-Wallace nonparametric ANOVA. Differences between groups were considered significant at $p < 0.05$.

Gene Microarray

Affymetrix gene arrays were used to quantify relative ME gene expression profiles during the course of OM. The MEs of 40 mice per time point were inoculated bilaterally with NTHi. The ME mucosa and exudate were harvested from 20 mice and pooled at each of the following time points: 0 hours (0h, no treatment), 3h, 6h, 24h, 2 day (2d), 3d, 5d and 7d. Total RNA was extracted by homogenizing the tissue in TRIzol (Life Technologies, Carlsbad, CA) and reverse transcribed to generated biotinylated cRNA library that were hybridized onto the MU430 2.0 microarrays per time point sample according to the manufacturer's protocol. This procedure was duplicated for each time point using the other 20 mice. Hence, each data set represents 2 independent biological replicates and 4 Affymetrix arrays. The raw data were median normalized using Variance-modeled posterior Interence Approch (VAMPIRE) and the specific gene transcript fold-level changes were assessed using Genespring GX 7.3 (Agilent Technologies, Santa Clara, CA). Detailed methods are provided (Hernandez et al., 2015).

Cell Culture

The human middle ear epithelial cell line (HMEEC) (Chun et al., 2002) was a generous gift from David J. Lim (Department of Head and Neck Surgery, University of California Los Angeles, CA, USA). According to the protocol (Woo et al., 2014), the cells were maintained in a 1:1 mixture of DMEM and bronchial epithelial basal medium (Lonza, Walkersville, MD) supplemented with bovine pituitary extract (50 µg/ml), hydrocortisone (0.5 µg/ml), hEGF (0.5 ng/ml), epinephrine (0.5 µg/ml), transferrin (10 µg/ml), insulin (5 µg/ml), triiodothyronine (6.5 ng/ml), retinoic acid (0.1 ng/ml), gentamicin (50 µg/ml), and amphotericin-B (50 ng/ml). The cells were cultured at 37°C under 5% CO2 and 95% air atmosphere. For immunomodulation, the HMECCs were stimulated with NTHi, TNFα, MDP, Tri-DAP, SB203580 (p38 MAP Kinase Inhibitor) and CHX (Cycloheximide) alone or in combination for 6, 24, 48 and 72 hours and qPCR or MTT Assay was performed. Cycloheximide was used to inhibit protein synthesis in eukaryotic cells and initiates apoptosis as a positive control, since it works rapidly and strongly *in vitro*.

Human Tissue Samples

After giving consent, samples of chronic OM (COM) (n=20), the external auditory canal skin (EAS) (n=20) and healthy middle ear mucosa (n=20) were taken from patients during middle ear operations at the ENT department of the University of Lübeck (Germany) (see **Table S1** for further clinical details). The median age of the patients with COM was 51.8 years (range, 22 to 82 years). All 20 patients had surgery due to COM with hearing loss. The tissue samples for healthy controls (n= 20 middle ear and n= 20 EAS) were collected from 21 patients with a median age of 56.4 years (range, 30 to 80 years), undergoing surgery for hearing rehabilitation with a cochlear implant, a vibrant soundbridge implant, tympanoscopy or a second-look surgery. All samples, if not processed immediately, were kept in liquid nitrogen and stored at -80° C according to the protocol of our previous publications (Leichtle et al., 2015; Leichtle et al., 2021). All protocols were approved by the ethics committee of the University of Lübeck (10-039, 20-434). All clinical examinations were carried out according to the principles of the Declaration of Helsinki (1964).

Quantitative Real-Time PCR

The procedure was carried out according to the protocol of our previous publications (Lai et al., 2009; Leichtle et al., 2010). In brief, the mRNA was extracted from the human tissue samples (COM: n = 20, ear canal biopsies: n = 20 and healthy middle ear mucosa: n = 20) or from the HMECC using RNeasy Mini Kits (Qiagen, Mississauga, ON, Canada). The amount of RNA was measured with a spectrophotometer. According to the manufacturer's protocol, 0.5 µg of total RNA was converted into cDNA using the first strand cDNA synthesis kit (Fermentas, St.Leon-Rot, Germany). After the reverse transcription reaction (RT), all samples were diluted 1: 4 in ddH2O and subjected to real-time PCR analysis with Maxima SYBR Green QPCR Master Mix (Fermentas, St. Leon-Rot, Germany). 0.3 µM gene-specific primers (TNF, IL1-β, IL8, NOD1, NOD2, RIPK2, TLR2, TLR4, BID, CAS7, CASP3) and GAPDH, for a total reaction volume of 25 µl. For all targets, the cycling conditions were: 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles each consisting of 95°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Integration of the SYBR Green dye into the PCR products was monitored using the ABI PRISM 7000 sequence detection system (Applied Biosystems, Carlsbad, CA, USA). The quantification of gene expression was determined by the comparative $\Delta\Delta C_T$ method. The target gene expression in the test samples was normalized to the endogenous reference GAPDH level and was reported as the fold difference relative

to GAPDH gene expression (Leichtle et al., 2015; Leichtle et al., 2021). All measurements were performed in triplicate and three independent experiments performed for each gene target.

Immunohistochemistry

For LSAB (Labeled (Streptavidin-Biotin) staining, paraffin-embedded, formalin-fixed tissue sections were deparaffinized and re-hydrated in xylene, ethanol and TBS. Endogenous peroxidases (15 min incubation in 3% H_2O_2) and endogenous biotin (Avidin/Biotin Blocking Kit, Vector Laboratories, Burlingame, CA) were blocked. For antigen retrieval, sections were incubated in Proteinase K (DAKO, Carpinteria, CA) for 7 min, blocked with 1% BSA in PBS and incubated with the primary rabbit anti-Nod1 (1:200, Imgenex IMG 5739), anti-Nod2 (1:600, Santa Cruz, sc 56168), anti-RIPK2 (1:200, Abcam, ab 75257), anti-caspase3 antibody (1:100, cell signaling) and anti-cleaved (c) -Caspase3 antibody (1:100, Cell Signaling) in PBS overnight. Washed in 0.1% BSA, washed in PBS and treated with biotin sheep anti-rabbit secondary antibodies (DAKO) and the AEC peroxidase substrate kit (Vector Laboratories) according to the manufacturer's instructions. Positive controls were performed according to the protocol. Negative controls without antibody were used for every experimental epitope target.

In Situ Apoptosis

The apoptosis rate in OM samples with cellular effusion was determined by Image-iTTM LIVE Red Caspase-3 and -7 Detection Kit (Thermo Fisher Scientific Inc., Schwerte) according to the manufacturer's protocol. The removed tissue was directly covered with the 30X FLICA reagent working solution in a dilution of 1:30, incubated for 60 min protected from light at room temperature and rinsed with the 1x wash buffer 1:10. Counter-staining of the cell nucleus was performed with 1mM of Hoechst 33342 (1:1000) and 5mM SYTOX green for 10 min below room temperature. The sample was washed twice with 2 ml of 1X wash buffer. The sample cover-slipped and 2 ml 1X wash buffer and evaluated under the confocal fluorescence microscope (LSM 510, Carl Zeiss and DM IRB, Leica).

MTT Assay

Cellular metabolic activity was determined by a quantitative colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. This assay determines viable cell numbers based on the mitochondrial conversion of MTT. Approximately 5 to 10×10^3 cells were dispersed into each well of a 96-well plate and cultivated with different stimuli (NTHi, TNF, Tri-DAP and MDP). 10 µL of MTT dye (5 mg/mL; Sigma-Aldrich) was added for 1 hour at 37°C and then100 µL of MTT stop solution was added to stop the reaction (Sigma-Aldrich). After gently shaking for 24 hours, absorbance was measured at 570 nm using a Benchmark Plus microplate reader (BioRad, Hercules, CA, USA). All measurements were performed in triplicate and three independent experiments were performed for the assay.

Statistics

Data are reported as arithmetic means ± standard deviation. ANOVA with Bonferroni correction for multiple tests was performed with StatView and GraphPad Prism for normally distributed data. The percentages of the ME area covered by leukocytes were compared by the non-parametric Mann-Whitney U-test. Differences between groups were considered significant at $p < 0.05$. The two ears from each mouse were treated as independent samples since they were found to be independent form each other (Ebmeyer et al., 2005). Sex contingency was compared with a two-sided Fisher's Exact Test.

RESULTS

Gene Microarray

Gene arrays were utilized to provide quantitative evaluation of key innate immune PRR expression levels within the ME. We followed gene expression throughout the course of an episode of OM after NTHi inoculation in the mouse. A variety of receptors that respond to molecules produced by pathogens were evaluated (**Figure 2**). These include the TLR (TLR2, TLR4, TLR6, TLR9) and the NLR (NOD1 and NLRP3) families, as well as several receptors that recognize foreign DNA. Part of these data have been published previously in separate reports (Hernandez et al., 2015; Lee et al., 2019), and are aggregated here with our gene analysis. Most dramatically TLR2, NLRP3 and the IFN regulatory factor (DAI), which functions as a DNA sensor, were rapidly upregulated within 3-6 h post bacterial inoculation. DAI expression remained day 7, when the infection resolved. Detailed data on fold change ranges and statistics are presented in the **Supplementary Table S2**.

Mucosal Hyperplasia

Having observed that NTHi infection regulates the different innate immune receptors and key adaptor components at the gene level using microarrays, we investigated the ME response to NTHi infection in mice lacking TLRs (TLR2, TLR4), NRLs (NOD1, NOD2), the obligatory immune adaptors (MyD88, RIP2), and the major innate immune effector gene TNFα, and compared the ME histopathology to wild-type C57 controls. **Figure 3** shows the histology of OM in WT type mice after NTHi inoculation. The ME mucosal hyperplasia is noted within 24h after NTHi exposure and reaches a maximum at 2-3 days, with leukocytes infiltration peaking about the same time. After which, the hyperplastic response transitions to recovery and healing, with a return to normal thickness observed by day 7. Infection of WT mice by NTHi produces an acute OM lasting only a several days. However, mice with innate immune gene deficiencies exhibit more persistent OM, and for some knockouts lasted several weeks (**Figure 4**). The data, published previously in separate reports (Hernandez et al., 2008; Leichtle et al., 2009; Leichtle et al., 2010; Lee et al., 2019), are aggregated here and compare with mouse models of RIPK2 as well. **Figure 4A** reveals the OM pathogenesis at 3 days and 10 days post NTHi inoculation revealing that ME mucosal healing was altered in

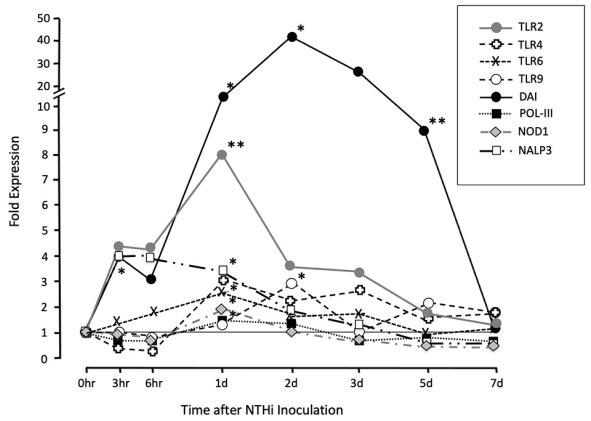

FIGURE 2 | Assessment of genes involved in patter recognition during a complete episode of acute NTHi-induced OM in the mouse, from initiation to recovery, evaluated by gene arrays. The cell surface TLR2, TLR4 and TLR6 were expressed in sentinel mucosa and highly upregulated in early time points (3hrs to 24hrs). The intracellular PRRs TLR9 was upregulated at later time points (2d). Meanwhile, NLRP3 expression peaked between (3hrs to 24hrs). The DNA-dependent activator of IFN-regulatory factors (DAI) and DNA sensor RNA polymerase (Pol-III) expression peaked later at day 2 onwards. *p < .05; **P < .01

the absence of these innate immune molecules. **Figure 4B** illustrates quantitative evaluations of the mucosal thickness in the KO mice plus levels of leukocyte infiltration of the ME cavity. We observed that the mucosal thickness and leukocyte clearance were delayed in the immune deficient mice compared to that seen in WTs, especially in TLR2-deficiency by day 10. Meanwhile, the NLR pathway, in the absence of NOD2 and NOD1, responded hyposensitively at day 3. Compared to the WT phenotype, mucosal growth was lower in the NOD1 and NOD2 KO mice. The percentage of ME lumen occupied by infiltrating leukocytes was also lower on day 3 in theses mice.

Immunoregulation in Human OM. Expression of Innate Immune Receptors in Human COM

Using RT-PCR, the gene expression of the innate immune receptors NOD1, NOD2, RIPK2, TLR2 and TLR4 in human samples of COM were investigated (**Figure 5A**). and compared to healthy middle ear tissue. For normalization, the housekeeping gene GAPDH was used. Significantly increased gene expression of NOD2 and TLR2 was observed in COM. N = 20 each; GraphPad Prism with an unpaired t-test, $p<0.05$. The mRNA expression of NOD2 and TLR2 was significantly increased compared to healthy tissue samples of the ME. However, the gene expression of the patients showed large variability. The gene expression of NOD1 was increased but not significantly expressed when compared to healthy ME tissue. Meanwhile, RIPK2 and TLR4 were constitutively expressed, without up- or down-regulation. Overall, the results show an inhomogeneous gene distribution of the individual patients with high variability, which was related to the different clinical expression of COM and surgical findings intraoperatively (see **Table 1** and **Supplementary Table S1**). For protein expression, immunohistochemical staining using immunoperoxidase (DAB) on cryosections was performed detecting the three NLR-pathway

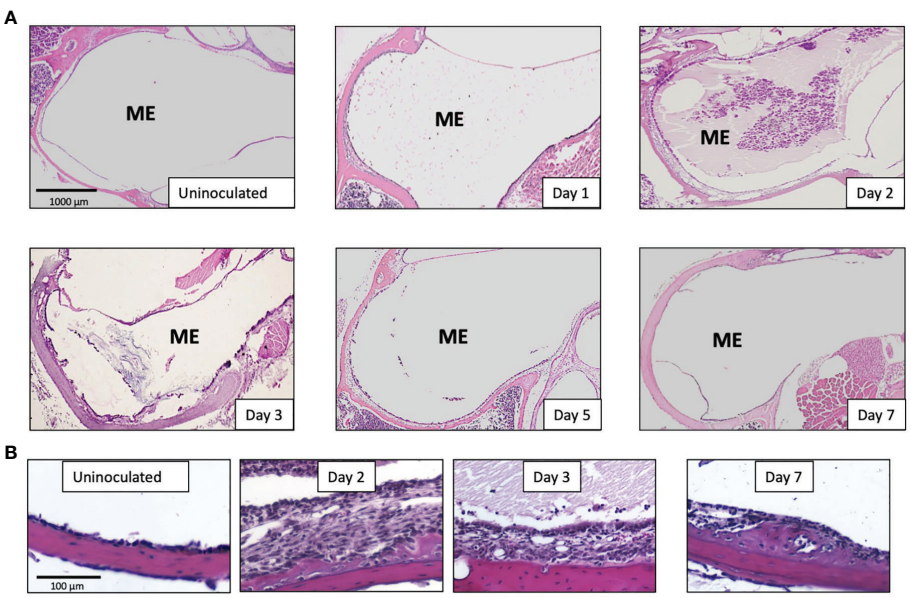

FIGURE 3 | Pathophysiology of the middle ear during a NTHi induced OM in WT mice (C57/BL6). **(A)** OM time course showing cross-sections of the ME bulla and revealing that the ME mucosal hyperplasia and cellular infiltration peaks at 2-3 days after bacterial inoculation and recover by 5-7 days. Cellular infiltrate are evident on day 1, peak by day 2 and return to baseline by day 7 **(B)** Middle ear mucosa thickening is seen at a higher magnification (10x) at the indicated OM time points.

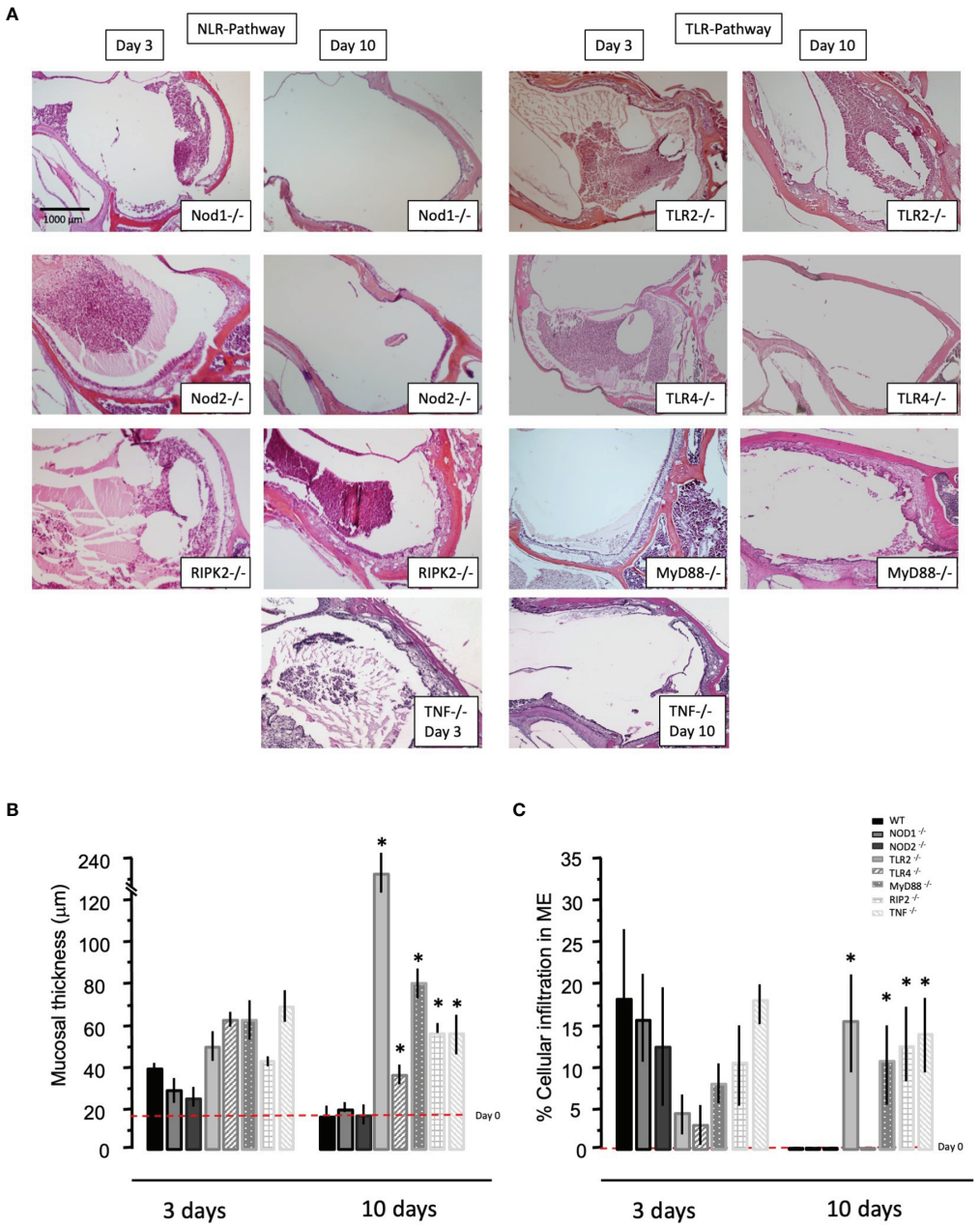

FIGURE 4 | Representative histological changes at 3 and 10 days post NTHi challenge seen in middle ears of different immunodeficient KO mice. **(A)** Considerable inflammation in the ME space (filling the cavity with fluid and inflammatory cells) is observed on day 3 and by day 10 persistent mucosal hyperplasia and cell recruitment is noted in some of the immunodeficient KO animals. **(B)** Quantitative analysis of mucosal thickness and **(C)** the % area of the middle ear cavity covered with inflammatory cells in KO mice. (n=6 ears, $^*p < 0.05$).

receptors in human COM, which is a novel finding. **Figure 5B** shows the expression of NOD1, NOD2 and RIPK2 proteins in human OM and their positive controls. All three receptors could be readily detected intra- and subepithelial in COM. Similar to the gene expression results, NOD1 and NOD2 protein expression in COM was visibly elevated compared to RIPK2 protein expression. Immunohistochemistry also revealed that NOD1 is localized primarily in the epithelial layers of the middle ear tissue, whereas NOD2 and RIPK2 seems to be present in the basal epithelial layer and subepithelium.

Gene Expression of Downstream Genes TNF and IL8 in Human COM

For a more detailed gene evaluation between the different human tissue, we investigated the relative expression of the effector genes TNF and IL8 in healthy ME mucosa; n=20, external auditory

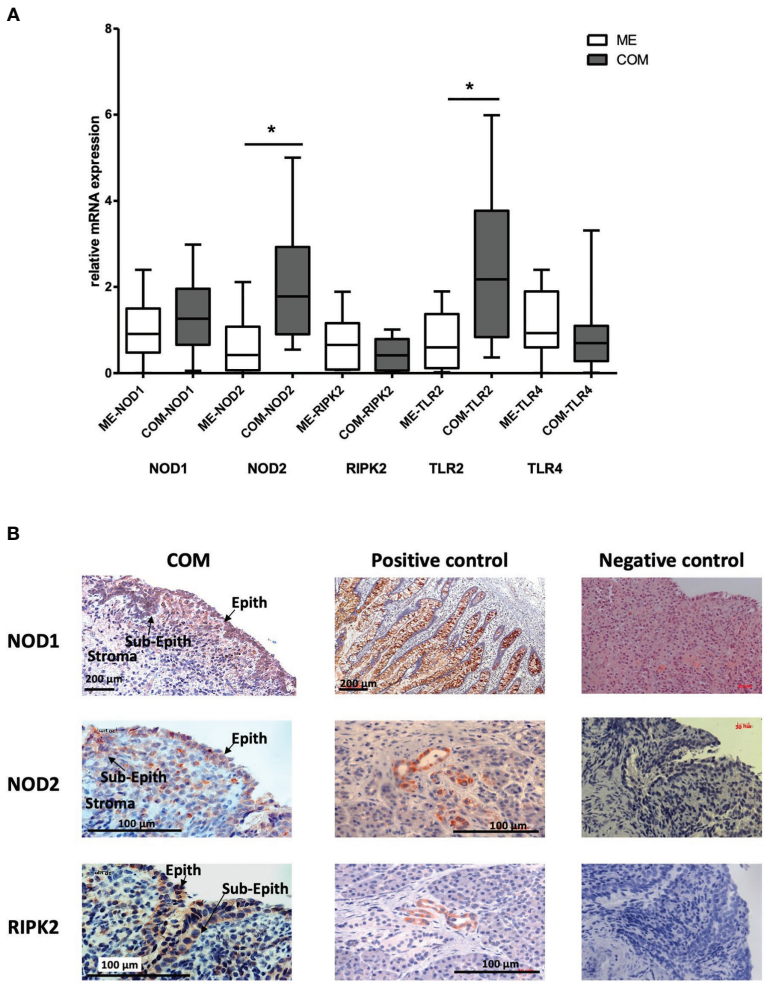

FIGURE 5 | **(A)** Gene expression of Innate Immune Receptors in human COM. mRNA expression of NOD1, NOD2, RIPK2, TLR2 and TLR4 in healthy middle ear samples (ME) and COM relative to GAPDH. Significantly increased gene expression of NOD2 and TLR2 in COM compared to healthy middle ear tissue. For normalization, the housekeeping gene GAPDH. N = 20 each; GraphPad Prism with an unpaired t-test, *p < .05. **(B)** Protein expression of NLR Immune Receptors in human COM. NOD2, NOD1 and RIPK2 could be localized immunohistochemically mainly intra- and subepithelial in COM. The right column displays their positive control in small intestine (NOD1) and in pancreas (NOD2 and RIPK2). Orange/brown represents the target molecule NOD1, NOD2 and RIPK2, blue represents nuclei. Scale is shown by scale bar insert.

skin (EAS); n=20, and COM; n=20, relative to GAPDH (**Figure 6A**). Expression of TNFα mRNA in COM was significant increased compared to the external auditory skin and healthy ME mucosa. The individual gene expression levels in COM show a significantly broader distribution compared to the other tissue samples. The mRNA gene expression was most homogeneous in healthy ME mucosa. In contrast, the IL8 gene expression in the COM show no significance change from external auditory skin or healthy ME mucosa, suggesting that IL8 production was similar in all three samples.

Gene Expression of Apoptotic Genes in Human COM

Given the upregulation of TNF gene expression in COM, the gene profiles of extrinsic and intrinsic apoptotic genes were examined. Relative mRNA expression of Bid, Casp3 and Casp7 were respectively analyzed relative to GAPDH in same as above. The gene expression of Bid (left) and Casp3 (middle) in COM was significant increased compared to the healthy ME mucosa and EAS, displaying a broad distribution (left). A significant upregulation of Casp7 in EAS and COM compared to healthy samples (right) could be detected as well (**Figure 6B**).

Expression of Apoptotic Proteins in Human COM

The localization of the apoptotic protein Casp3 and cleaved-Casp3 (cCasp3) were analyzed using immunohistochemical staining in human COM (**Figure 6C**). Both proteins were easily detected in the epithelium and subepithelial in COM. Casp3 showed distinct staining intra- and subepithelially. Active

TABLE 1 | Clinical and demographic information from patients utilized in this study.

	COM n =20	Healthy n=21	*p value* significant at $p < .05$
Mean age in years (range)	51.8 (22-82)	56.4 (30-80)	0.19
Female Gender	12	8	0.22
n (%)	60%	38%	
Hearing loss	20	15	0.51
n (%)	(100%)	(71%)	
Vertigo	6	2	0.48
n (%)	(30%)	(10%)	
Diagnoses/Procedure			
COM	16	–	
COM with facial palsy	2	–	
COM with mastoiditis	2	–	
Acute suditas for tympanoscopy	–	4	
Cochlear implantation	–	8	
Vibrant Soundbridge implantation	–	4	
Second-Look	–	4	
Meatoplasty	–	1	

cCasp3 protein expression was mainly located in the subepithelial layer. Negative controls, without antibody, and positive controls (using tonsil tissue), were performed to validate the epitope staining. To detect active caspases, the Image-iTLIVE Detection Kit Red for Caspase-3 and -7 was used on fresh samples from COM with cell effusion directly excised from surgery (**Figure 6D**), based on a fluorescent inhibitor of caspases (FLACA) method. The remaining red fluorescent signal showed a direct measure of the amount of active caspase present at the time the inhibitor was added. Caspase3 and 7 activity was detected in COM middle ear effusions, as disseminated red staining of single cells.

Immunomodulation in the Human Middle Ear Epithelial Cell Line (HMEEC)

Following our observations regarding the involvement of TLR and NODs in COM in murine models (*in vivo*), and the human tissue data implicating TLRs and NODs in inflammation and apoptosis, we set out to explore the functional stimulation of these receptors using HMEEC cells *in vitro*. **Figures 7A, B** shows the gene expression of TNFα and IL1β in HMEEC at 6h and 24h post stimulation with NTHi, TNF, Tri-DAP, MDP, SB203580, CHX or double-stimulation. (**Figure 7A**) At 6h, the mRNA expression of TNFα in HMECC stimulated with NTHi, TNFα and CHX or their double stimulation with NTHi was significantly increased compared to medium alone (negative control). As well, TNFα expression at 24h showed a significant induction after treatment with NTHi, TNFα, Tri-DAP, MDP, SB203580, CHX or their double stimulation with NTHi in HMEEC compared to medium control. The strongest increase displayed was using MDP and CHX, representing the TLR-NOD2 and apoptosis pathways. Interestingly double-stimulation with NTHi and TNFα, or Tri-DAP, or MDP or SB203580 responded in contrast to their single molecule stimulation with a decrease of TNFα gene expression at 24h (depicted with red arrows), in **Figure 7B**. Compared to TNF, the mRNA expression of IL1β in HMECC at 6h was only induced after stimulation with CHX, or double stimulation with NTHi and TNFα, TriDAP, MDP and CHX compared to the media alone control. However 24 h after stimulation, all stimuli, except SB203580 (a p38 MAP Kinase Inhibitor), caused a significant increase in mRNA expression of IL1β compared to the negative control (media only). The strongest increase in single stimulus displayed were again NTHi plus MDP or CHX with apoptosis induction. In contrast to TNFα, double-stimulation with NTHi and TNFα, and NTHi + Tri-DAP or + MDP resulted in no remarkable change in IL1β expression. Moreover HMEEC single or double stimulation with SB203580 resulted in lower IL1β expression at 24h compared to the NTHi stimulus alone.

Cell Viability in HMEEC After Immunostimulation

MTT assays were used in order to determine whether specific immunomodulation led to an altered cellular viability of HMEEC (**Figure 7C**). Samples treated with NTHi, TNFα, TriDAP and MDP, and their combinations were analyzed to quantify the cellular metabolic activity as an indicator of cell viability at 24, 48 and 72 hrs. The addition of NTHi to cells stimulated cellular proliferation with an increase in % cells. Single stimulation with TNFα, NOD ligands MDP and TriDAP did not have an effect on cell viability (negative control remained at 100%). Double Stimulation of the HMEECs with TNFα and NTHi decreased the cellular proliferation significantly. In response to the double stimulation with MDP or TriDAP with NTHi, no effect was observed on cell viability after 72h monitored.

DISCUSSION

Animal studies have shown that innate immunity plays an important role in the pathogenesis of OM. Pathogenic OM features including inflammation, mucosal hyperplasia and leukocyte infiltration are initiated within hours after ME infection, consistent with innate immune response to

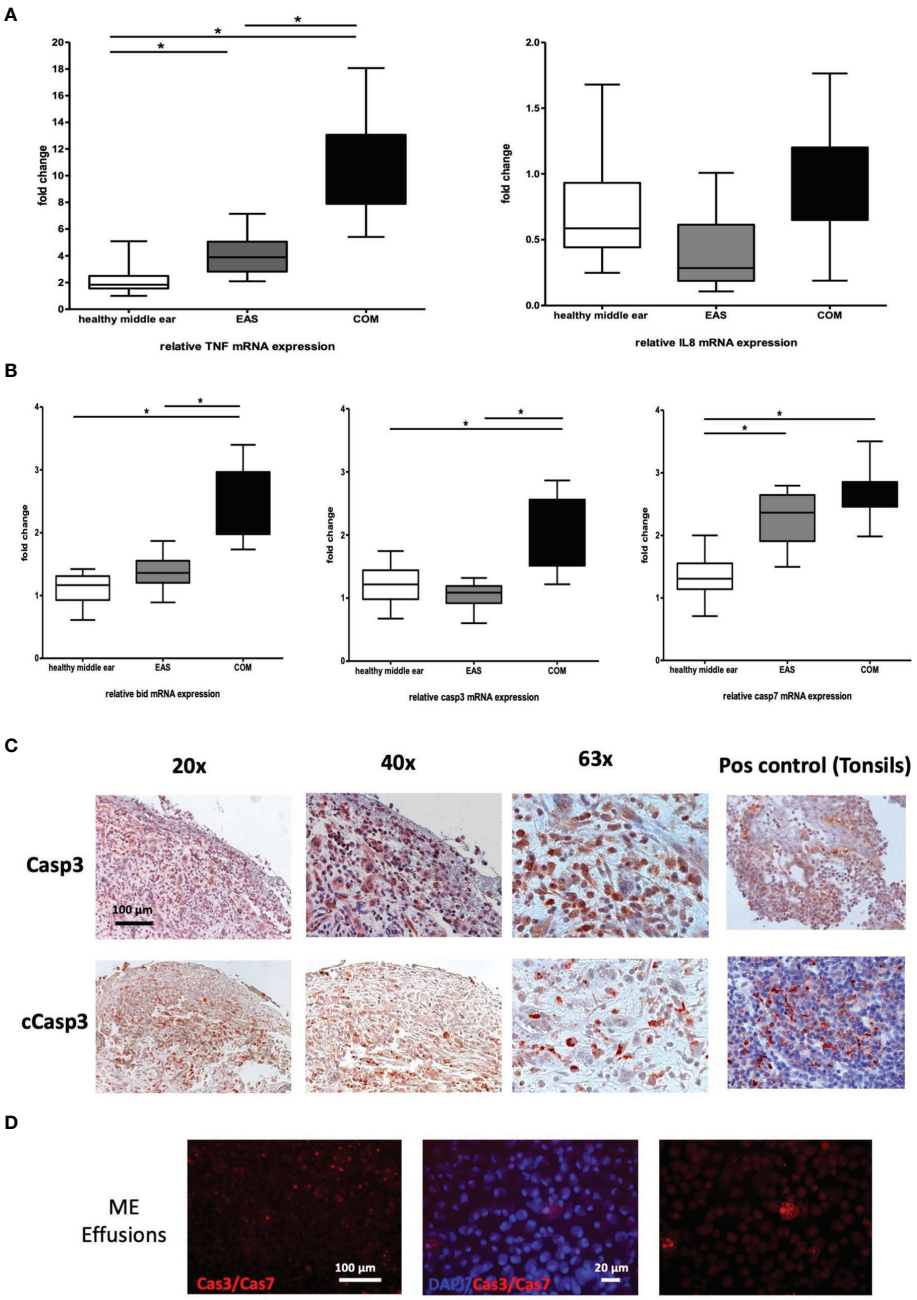

FIGURE 6 | **(A)** Gene expression of TNF and IL8 in human COM. TNFα (left) and IL8 (right) mRNA expression in healthy middle ear samples, external auditory skin (EAS) and COM relative to GAPDH. Significantly increased gene expression of TNFα in COM compared to EAS and healthy middle ear samples. No significant mRNA regulation of IL8. **(B)** Gene expression of apoptotic genes in human COM. Bid, Casp3, and Casp7 mRNA expression in healthy middle ear samples, external auditory skin (EAS) and COM relative to GAPDH. Bid and Casp3 mRNA expression in COM is significantly increased to EAS and healthy middle ear samples (left and middle). Significant upregulation of Casp7 in EAS and COM to healthy samples (right). Normalization with GAPDH; N = 20 each; GraphPad Prism with an unpaired t-test, *p < .05. **(C)** Expression of apoptotic proteins. Immunohistochemistry by immunoperoxidase of Casp3 and cCasp3 as brownish-orange staining in human COM. Casp3 is broadly stained in the epithelium and subepithelial, whereas active cCasp3 protein expression shows staining mainly in the subepithelial layers. 20x, 40x and 63x magnification. **(D)** Caspase3 and 7 livestain in COM with cell effusion shows a disseminated red staining of single cells mainly subepithelial. 20x and 63x magnification.

pathogens (Hernandez et al., 2008). The essentially immediate innate immune response to pathogens suggests that many innate immune genes are constitutively expressed by ME cells. This has been confirmed by single-cell RNA-Seq of the normal mouse ME (Ryan et al., 2020). Genes encoding the TLR and NLR families, as well as several receptors that recognize foreign DNA, are also

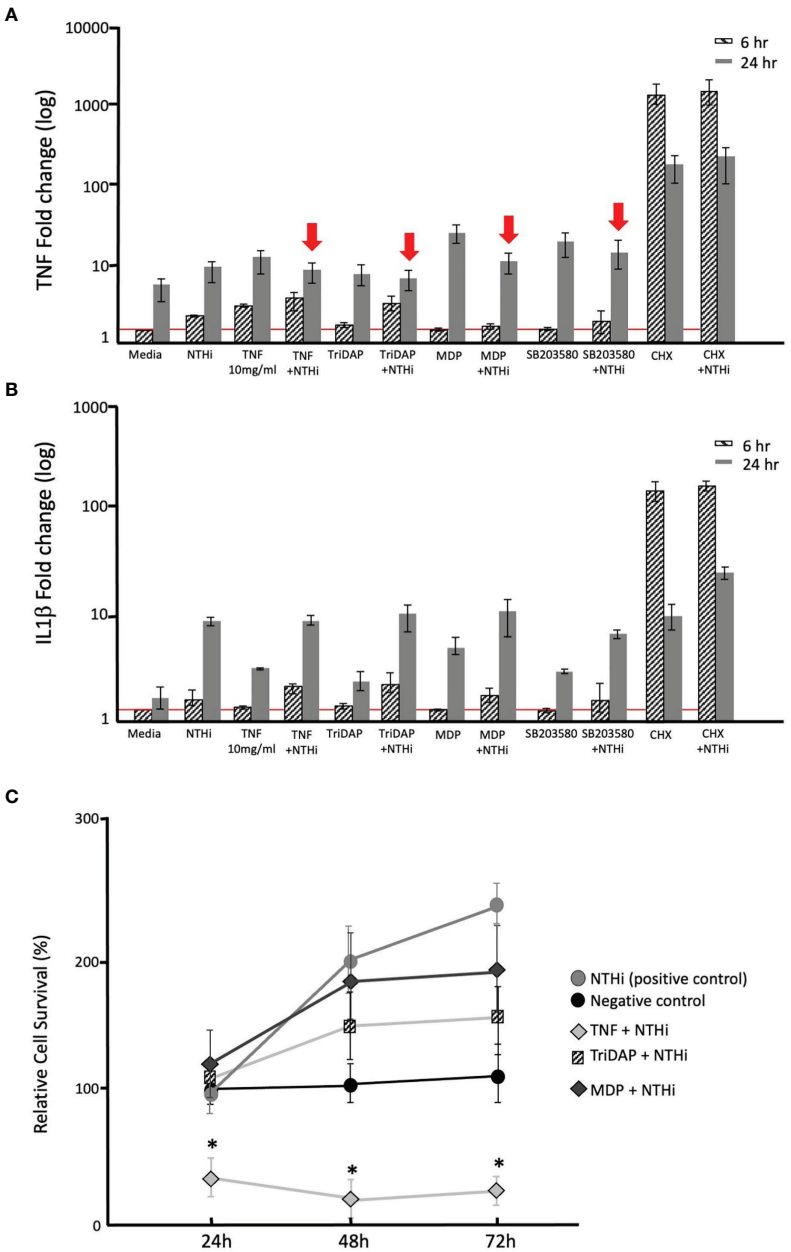

FIGURE 7 | Immunmodulation in the Human Middle Ear Epithelial Cell Line (HMEEC). Gene expression of TNF **(A)** and IL1β **(B)** in HMECC at 6h and 24 h after stimulation with NTHi, TNF, Tri-DAP, MDP, SB203580, CHX or double-stimulation, was evaluated using qPCR. For normalization, the housekeeping gene GAPDH was used. GraphPad Prism with an unpaired t-test, *p<0.05. **(A)** Increased gene expression of TNFα after stimulation with NTHi, TNFα and CHX or their double stimulation with NTHi at 6h in HMEEC compared to medium. At 24h induction of all stimuli or their double stimulation with NTHi in HMEEC compared to medium. However, double-stimulation with NTHi and TNFα, or Tri-DAP, or MDP, or SB203580 compared to the direct single stimuli molecule showed hyporesponse at 24h, with a reduction in TNFα expression (red arrows). **(B)** Significantly increased gene expression of IL1β only after stimulation with single stimulation with NTHi, or CHX; or double stimulation with NTHi and TNFα, TriDAP, MDP and CHX at 6h compared to controls. At 24h significant IL1β induction was observed under all conditions compared to controls. However, double-stimulation with NTHi and TNFα, Tri-DAP, MDP or SB203580 responded with no change in IL1β gene expression when compared to stimulation with NTHi alone at 24h. **(C)** Effects of NTHi, TNF, TriDAP, MDP and their combinations on the cellular viability of cultured HMEECs assessed by MTT assay. TNFα had a significant effect on cellular viability after incubation with NTHi Significant difference ($p < 0.05$) is marked with *.

significantly up-regulated in the ME during animal OM (**Figure 2**).

In addition to pathogenesis, innate immunity plays a significant role in OM resolution (Massa et al., 2021). In the normal child, OM typically recovers within a few days, too soon for adaptive immunity to be engaged (Leichtle et al., 2011). Animal studies have shown that imbalances in innate immune activity can lead to failure of bacterial clearance and hence COM (Hernandez et al., 2008; Leichtle et al., 2009; Leichtle et al., 2010; Underwood and Bakaletz, 2011; Kurabi et al., 2016; Lee et al., 2019). In humans, several studies have associated polymorphisms in several innate immune genes with proneness to COM (Toivonen et al., 2017; Emonts et al., 2007; Ilia et al., 2014; Nokso-Koivisto et al., 2014). Moreover, ME responses to bacteria are disabled in mice deficient in innate immune receptors or effectors (**Figure 4**). Infected with the otopathogen NTHi, uncontrolled mucosal growth and leukocyte infiltration, combined with significantly pronounced inflammatory components, are observed in the ME, similar to the histopathological features found clinically during ME surgeries for OM. Especially, mice deficient in the innate immune receptors TLR2 and NOD2, their adaptor molecules Myd88 and RIPK2, and downstream TNFα demonstrate highly persistent OM after NTHi infection (Hoshino et al., 1999; Leichtle et al., 2009; Leichtle et al., 2010; Lee et al., 2019). Apoptosis also contributes to OM recovery. For example, OM pathogenesis is enhanced in mice deficient in Fas-mediated apoptosis (Rivkin et al., 2005).

Recent studies in other systems have demonstrated that a functional immune system is important in keeping inflammatory responses and apoptosis under control (Man and Kanneganti, 2016; Birkle and Brown, 2021). In the infected mouse ME, negative regulators of inflammation are expressed rapidly and simultaneously with pro-inflammatory innate immune effectors (Hernandez et al., 2015). This finding underscores the importance of host control over the pathogenic effects of inflammation mediated by innate immune response to bacteria.

Given prior ME data from the mouse models, association of human innate immune gene polymorphisms with OM proneness, and the documented expression of genes in the human ME during OM (**Figures 2**, **4**), the network of TLRs, NLRs, and their upstream and downstream signaling pathways during OM take on translational significance. In ME tissue from OM patients we found that TLR2 and NOD2 mRNA expression was significantly induced during COM compared to healthy ME tissue, although variability between patients was high (**Figure 5**). TLR2 upregulation in human COM tissue has been described previously by other research groups at the gene and protein levels (Hirai et al., 2013; Jesic et al., 2014; Kaur et al., 2015; Jung et al., 2021). For example, TLR2 expression was higher in the inflamed mucosa of COM MEs compared with the normal control MEs (Jesic et al., 2014), with significantly more TLR2 mRNA in bacteria-positive compared to bacteria-negative MEs (Kaur et al., 2015). Similar differences in ME bacterial load might explain the inter-individual variability in innate immune receptor expression in our COM patient data. In contrast to COM, no difference in TLR2 mRNA or protein levels was observed in prior studies of patients suffering from chronic suppurative OM (CSOM) (Si et al., 2014; Tuoheti et al., 2021). It is possible that the latter result reflects differences in response to the distinct pathogens of this disease, which typically originate from the external auditory canal after tympanic membrane rupture. It is perhaps more likely that a lack of TLR2 response disposes toward this more serious OM type. Certainly the association of distinct innate immune gene alleles with COM susceptibility, as discussed above, argues for a genetic basis of a portion of OM phenotypic variation.

Compared to cell-surface expressed TLR receptors and its downstream signaling molecules role in OM (Jung et al., 2021), less is known regarding the importance of the cytoplasmic PRRs in sensing and responding to bacterial infections in the ME (Kurabi et al., 2016). In this study, we identified NOD2 as significantly upregulated at the gene and protein level, where it was localized intra- and subepithelially in COM (**Figure 5**). NOD2 upregulation is consistent with activation of this receptor, which would require internalization of bacterial PAMPs or bacteria themselves. Internalization of bacteria into phagocytes clearly occurs in OM. However, both *Streptococcus pneumoniae* (Novick et al., 2017) and nontypeable *Haemophilus influenzae* (Hardison et al., 2018) are known to invade epithelial cells under circumstances that occur in OM, and NOD2 activation could also occur epithelially.

We observed that TNFα was significantly upregulated in COM compared to healthy ME mucosa and EAS, compared to the absence of upregulation for IL8 (**Figure 6A**). TNF is well known as a key regulator of not only inflammation but also apoptosis (Chen and Goeddel, 2002; Aggarwal, 2003; Ebmeyer et al., 2011). Indeed, evidence of apoptosis was apparent in our OM patient samples. Expression of the extrinsic and intrinsic apoptotic genes Bid, Casp3 and Casp7 was significantly increased compared to the healthy ME mucosa. A distinct differential localization of Casp3, cCasp3, and Casp7 was observed in live-stained mucosal epithelium and subepithelial in COM (**Figures 6C, D**). In comparison, studies of cholesteatoma (COM epitympanalis) found no regulation of caspase signaling by various research groups (Miyao et al., 2006; Klenke et al., 2012; Leichtle et al., 2021). Cellular substrates of apoptosis in the ME include remodeling or recovering mucosal cells and immunocytes at the end of their life cycle.

We evaluated innate immune stimulation and cell viability using HMEEC, to assess specific immunomodulation and protection strategies (**Figure 7**). Gene expression of TNFα and IL1β, induced by TLR2- and NOD2- activating stimuli, indicated rapid TLR signaling and apoptosis activation (**Figure 7A**), followed by later IL1β and continued TNFα induction. These results confirm that stimulation through TLR2 and NOD2 can regulate the ME inflammatory process in human middle ear cells. Our results further suggest that NOD2 is more involved in the recognition of NTHi PAMPs than NOD1 in HMEECs, mice and patients. Others (Woo et al., 2014) have also demonstrated that NOD2 recognition of NTHi produces human β-defensin 2 upregulation in HMEEC.

The most remarkable finding in our immunostimulation cell studies was that the double-stimulation with NTHi and either TNFα, Tri-DAP or MDP resulted in a decrease of TNFα gene expression. This hyporesponse is consistent with a negative feedback protective mechanism to strong immune reaction/stimulation. Such negative innate immune regulation has been noted by others. For example, peptidoglycan-activated NLRC4 interferes with the activation of NOD2, as a negative feedback regulator (Hruz and Eckmann, 2008).

Based on our results, inhibition of the inflammatory effects of TLR2, NOD2 and/or TNFα could be beneficial in reducing OM pathophysiology. Inhibitors of TLR2 (e.g (Grabowski et al., 2018)), NOD2 (Rickard et al., 2013) and TNF (Monaco et al., 2015) maybe useful therapeutics to explore. Obviously, given strong evidence that lack of the innate immune genes encoding these innate immune molecules leads to COM (Leichtle et al., 2009; Leichtle et al., 2010; Lee et al., 2019), any of these inhibitors would need to be used as adjuncts to antibiotic therapy, where they could be advantageous. They could ameliorate the inflammatory effects of PAMPs during active middle ear infection. Moreover, given that Eustachian tube dysfunction is often present in OM, they could also reduce inflammation due to dead bacteria and their components that persist in the middle ear due to poor drainage of the tympanic cavity.

In conclusion, this study elucidates interactions of key innate immune signaling pathways present in ME mucosal tissue and infiltrating cells, in response to individual and combined infections. Using a translational approach including cellular, animal model and clinical studies, we demonstrated that during COM, activation of TLR2-NOD2-TNF signaling axes (highlighted in **Figure 1**) controls the ME inflammatory response *via* a complex network of positive and negative regulators. This complexity provides opportunities for individual differences to modulate the mucosal response in OM, leading either to OM resistance or proneness. It also provides multiple opportunities for pharmacological intervention to reduce pro-inflammatory signaling or enhance negative regulation.

DATA AVAILABILITY STATEMENT

The data presented in the current study can be found at the Science Data Bank Online repository. https://www.scidb.cn/en/s/YR3M7n, DOI: 10.11922/sciencedb.01553.

ETHICS STATEMENT

All protocols were approved by the ethics committee of the University of Lübeck. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by The Institutional Animal Care and Use Committee of the Veterans Affairs Medical Center in San Diego, CA.

AUTHOR CONTRIBUTIONS

AL and AK designed and supervised the project, designed experiments, conducted experiments and contributed to the writing of the manuscript. DL and MD performed qPCR, IHC and protein interaction. CD conducted animal experiments. AR and K-LB supported the execution of the experiments, editing the manuscript, and conducting the data analysis and interpretation. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.826192/full#supplementary-material

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GLOSSARY

Bid	BH3 interacting-domain death agonist
Casp	Caspase
cCasp	cleaved caspase 3
COM	Chronic Otitis Media
CHX	Cycloheximid
EAS	External auditory skin
HMEEC	Human Middle Ear Epithelial Cell
IL8	Interleukin 8
KO mice	Knockout mice
MDP	Muramyl dipeptide
NLR	NOD-like receptor
NOD1/2	Nucleotide-binding oligomerization domain receptor1/2
ME	Middle ear
MyD88	Myeloid differentiation primary response 88
NTHi	Not typable Haemophilus influenza
OM	Otitis Media
PAMPS	Pathogen associated molecular patterns
PRR	Pattern Recognition Receptors
RIPK2	Receptor Interacting Serine/Threonine Kinase 2
SB203580	Pyridinyl imidazole and p38 MAP Kinase Inhibitor
TLR	Toll-like receptor
TNF and TNFα	Tumor necrosis factor (α)
Tri-DAP	L-Ala-γ-D-Glu-mDAP
URI	Upper respiratory infection
WT mice	Wild-type mice

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Australian Aboriginal Otitis-Prone Children Produce High-Quality Serum IgG to Putative Nontypeable *Haemophilus influenzae* Vaccine Antigens at Lower Titres Compared to Non-Aboriginal Children

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Background: Nontypeable *Haemophilus influenzae* (NTHi) is the most common bacterial otopathogen associated with otitis media (OM). NTHi persists in biofilms within the middle ears of children with chronic and recurrent OM. Australian Aboriginal children suffer exceptionally high rates of chronic and recurrent OM compared to non-Aboriginal children. NTHi protein vaccines comprised of antigens associated with both adhesion and persistence in a biofilm are under development and could be beneficial for children with chronic and recurrent OM. Understanding the ontogeny of natural antibody development to these antigens provides insight into the value of vaccinating with particular antigens.

Methods: An in-house multiplex fluorescent bead immunoassay was used to measure serum IgG titres and avidity for three putative vaccine antigens: recombinant soluble PilA (rsPilA), ChimV4, and outer membrane protein 26 (OMP26) in sera from Australian Aboriginal otitis-prone children (n=77), non-Aboriginal otitis-prone children (n=70) and non-otitis-prone children (n=36). Serum IgG titres were adjusted for age, and geometric mean concentrations (GMCs) were compared between groups using a univariate analysis model. Antibody avidity was calculated as a relative avidity index and compared between groups using ANOVA.

Results: Australian Aboriginal otitis-prone children had lower serum IgG titres to rsPilA and ChimV4 than non-Aboriginal otitis-prone children ($p<0.001$), and non-otitis-prone children ($p<0.020$). No differences were observed between serum IgG titres from non-Aboriginal otitis-prone children and non-otitis-prone children. There were also no

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differences in the proportion of high avidity IgG specific for these antigens between these groups. Serum IgG titres to OMP26 were similar between all groups (p>0.670) although otitis-prone children had a higher proportion of high avidity antibodies to this antigen.

Conclusions: Australian Aboriginal otitis-prone children had lower serum IgG titres to 2/3 major NTHi vaccine candidate antigens, suggesting these children are unable to develop persistent IgG responses due to repeated NTHi exposure. These reduced IgG titres may relate to earlier and more frequent exposure to diverse NTHi strains in Aboriginal children through carriage or infection. These data suggest that Aboriginal children may benefit from immunisation with vaccines containing these antigens to increase titres of protective antibodies.

Keywords: otitis media, Australian Aboriginal children, IgG, nontypeable *Haemophilus influenzae*, avidity, immunology

INTRODUCTION

Otitis Media (OM) affects approximately 80% of children by the age of 3 years, making it one of the most common reasons for a child to visit their general practitioner, receive antibiotics and have surgery in industrialised countries (Monasta et al., 2012). One third of children with acute OM develop chronic OM with effusion (lasting ≥3 months) and/or recurrent acute OM (Rovers, 2008); in our paper these children will be referred to as otitis-prone. First nations children are generally more severely affected by OM, including Australian Aboriginal and Torres Strait Islander children, respectfully referred to as Aboriginal from herein, who have one of the highest rates of chronic OM worldwide (Morris et al., 2005). In Aboriginal children, OM starts earlier, is more prolonged, and more severe than in non-Aboriginal Australian children (Williams and Jacobs, 2009; Williams et al., 2009). Most data on OM prevalence in Aboriginal children has been reported from those living in remote and rural areas (Leach et al., 1994; Morris et al., 2005; Leach and Morris, 2007; Wenxing et al., 2012; Leach et al., 2016) however, a recent Western Australian study has shown that urban Aboriginal children also experience disproportionality high rates of OM, with over 50% of 6 month old children suffering from OM (Swift et al., 2020). Nasopharyngeal carriage of otopathogens is a prerequisite for development of bacterial OM (Wenxing et al., 2012). Australian Aboriginal children experience extremely early nasopharyngeal carriage of otopathogens, including nontypeable *Haemophilus influenzae* (NTHi), which is the leading pathogen associated with chronic and recurrent OM (Faden et al., 1991; Leach et al., 1994; Ngo et al., 2016). Recently, we have demonstrated that the presence of NTHi in the middle ear of children at the time of ventilation tube insertion is linked to an increased risk for repeat surgery (Seppanen et al., 2020).

NTHi persists both intracellularly and within polymicrobial biofilms on the middle ear mucosa and in effusion from otitis-prone children (Thornton et al., 2011; Thornton et al., 2013). These biofilms act as infectious reservoirs and enable bacteria to resist host immune responses and traditional antimicrobial therapies (Kania, 2016). NTHi has particular proteins that inititate formation and maintenance of biofilms, including type IV pilus proteins and the DNABII protein family (Jurcisek et al., 2017). Development of vaccines targeting proteins that are important for persistence are likely to be required to both prevent disease, and reduce chronic and recurrent infections in established OM. Other antigens being considered for vaccine development are adhesins that are important in establishing infections such as, Protein D (PD), outer membrane protein P5 (OMP P5), and Protein E (PE), as well as other conserved surface proteins such as, outer membrane protein 26 (OMP26) (Murphy, 2015).

While no NTHi-specific vaccines are licensed, two promising NTHi vaccines containing the PilA protein (the major subunit from the type IV pilus) are in development (Jurcisek et al., 2007; Novotny et al., 2009; Ronander et al., 2009). The first putative vaccine is a multicomponent adjuvanted protein vaccine, NTHi-10-AS01E, which is in phase 3 clinical trials in adults and contains 3 NTHi proteins: recombinant soluble PilA (rsPilA) that is fused with PE, as well as PD and the *Moraxella catarrhalis* antigen Ubiquitous surface protein A2 (UspA2) (Wilkinson et al., 2019). Sera from mice vaccinated with the rsPilA-PE fusion protein prevented NTHi attachment and biofilm formation *in vitro*, as well as initiation of biofilm dispersal resulting in clearance of established biofilm (Ysebaert et al., 2019). Furthermore, passive transfer of sera from chinchillas vaccinated with rsPilA-PE to unvaccinated animals prevented NTHi OM development (Ysebaert et al., 2019). In clinical trials, the NTHi-10-AS01E vaccine increased antibody titres and T-cell responses to all 4 component antigens (Wilkinson et al., 2019). The second NTHi vaccine in development is ChimV4, a chimera of protective epitopes from both rsPilA and OMP P5, which protects against development of NTHi-induced OM and resolves established NTHi biofilm in the chinchilla OM model (Novotny et al., 2013). The potential vaccine antigen, OMP26, has been demonstrated to enhance pulmonary clearance of NTHi in a rat immunisation model (Kyd and Cripps, 1998), but has not advanced to clinical trials.

Vaccines containing NTHi antigens involved in adhesion, biofilm formation and maintenance could be of particular benefit

to Australian Aboriginal children who acquire NTHi carriage in early infancy and suffer from high rates of NTHi OM (Leach et al., 1994; Wenxing et al., 2012). Both population and antigen specific differences in natural antibody titres have been demonstrated to exist in otitis-prone children (Pichichero et al., 2010; Kaur et al., 2011a; Kaur et al., 2011b; Wiertsema et al., 2012; Kaur et al., 2016; Thornton et al., 2017a; Thornton et al., 2017b). This was specifically observed for anti-Protein D IgG, where Aboriginal otitis-prone children had significantly lower antibody titres than non-Aboriginal otitis-prone children and non-Aboriginal non-otitis-prone children (Thornton et al., 2017b). In this study, we measured natural antibody titres to putative vaccine antigens rsPilA, ChimV4 and OMP26 in sera from Aboriginal and non-Aboriginal otitis-prone children, as well as non-Aboriginal non-otitis-prone children. We hypothesised that 1) otitis-prone children have reduced antibody titres to NTHi antigens involved in biofilm formation and persistence compared to non-otitis-prone children, and 2) this deficiency will be more pronounced in Aboriginal otitis-prone children, particularly those from rural and remote locations.

METHODS

Recruitment of Otitis-Prone Children

Children between 1 and 15 years of age were recruited at the time of admission for ventilation tube insertion or tympanoplasty from public hospitals in Perth, Broome, and Derby in Western Australia between 2003 and 2008. Children who met clinical criteria to undergo ventilation tube insertion for either chronic OM with effusion (cOME: middle ear effusion for 3 or more months) or recurrent acute OM (rAOM: 3 or more acute OM episodes in 6 months, or 4 or more episodes in 12 months) or tympanoplasty for chronic perforation of the eardrum following chronic suppurative OM (CSOM: perforation of the tympanic membrane with persistent discharge from the middle ear for 6 or more weeks) were included (Kong and Coates, 2009). Diagnoses were recorded at the time of surgery. Children were identified as Aboriginal or non-Aboriginal based on parental report.

Approval for this study was obtained from the Princess Margaret Hospital for Children (ethics approval number 831EP), Armadale-Kelmscott Memorial Hospital Ethics Committee, Osborne Park Hospital Ethics Committee, the Western Australian Aboriginal Health Ethics Committee, and Aboriginal community-controlled health services in the Kimberley and Eastern Goldfields regions.

Recruitment of Non-Otitis-Prone Children.

Non-Aboriginal children between 2 and 15 years of age were recruited through the Vaccine Trials Group at the Princess Margaret Hospital for Children (Perth, Western Australia) between 2007 and 2009. Children who were healthy and had no history of recurrent or chronic OM, sinusitis, rhinitis, obstructive sleep disorder, pneumonia or chronic lung disease and no previous ear, nose, or throat surgery were eligible. Approval for this study was obtained from the Princess Margaret Hospital for Children Ethics Committee (ethics approval number 1385EP).

Exclusion criteria for both cohorts included any chromosomal or craniofacial disorders, known immunodeficiency, or receiving immunosuppressive therapy. Written informed consent was obtained from the parents or guardians. Clinical data were collected using parental questionnaires and from medical records.

Sample Collection

Blood was collected into clot tubes (Vacuette® Greiner Bio-one, Frickenhausen, Germany) from otitis-prone children during surgery, and from non-otitis-prone children during clinic visit. Sera were separated by centrifugation for 10 min at 3200g, aliquoted, and stored at −80°C until analysis.

Measurement of IgG Titres Specific for NTHi Proteins rsPilA, ChimV4 and OMP26 Using Multiplex Bead-Based Immunoassays

As described previously for PD (Thornton et al., 2017b), the multiplex bead based-immunoassays were modified to include the antigens ChimV4, rsPilA and OMP26. Two final assays were run, the first including OMP26, rsPilA and PD, and the second being a single-plex assay for ChimV4. Modification included testing for cross-reactivity and specificity to determine assay composition. Specificity was determined to be >80% for each antigen. As expected, >80% cross-reactivity was demonstrated between rsPilA and ChimV4, with little cross-reactivity observed with/between other antigens (<10%). All antigens were conjugated to fluorescent carboxylated microspheres for measurement of serum IgG-specific titres (Martinovich et al., 2021). Briefly, samples were diluted 1:200 in serum diluent (phosphate buffer saline, 2% bovine serum and 0.05% tween20). The serum samples were then incubated with 4000 beads per antigen for 30 min at room temperature in the dark, before adding R-phycoerythrin conjugated anti-human IgG (Jackson ImmunoResearch Laboratories, Pennsylvania, USA) and incubated for a further 30 min. The fluorescence of 100 beads within each specific bead region were measured on the BioPlex® 200 System (Biorad). Inter-assay variability was assessed by calculating the percentage coefficient of variation (CV) of the mean concentration of quality control samples (<22% CV). Mean fluorescence intensity (MFI) data was acquired electronically in real-time and analysed using Bio-plex Manager 6.1 software. Data in arbitrary units (AU/mL) were generated from a standard curve of the in-house reference human serum pool and plotted as the geometric mean concentration (GMC) with 95% confidence intervals (CI). Out of range values were repeated using appropriate higher or lower serum dilutions.

Measuring Avidity of IgG Antibodies Specific for NTHi Proteins PD, rsPilA, ChimV4 and OMP26

Antibody avidity was measured using a modified multiplex bead-based immunoassay. Following the incubation of sera and beads

as in the original IgG assay, duplicates of samples were then incubated for 10 min with 25µL of 1M sodium thiocyanate (treatment) or phosphate buffered saline (untreated). R-phycoerythrin conjugated anti-human IgG was added (Jackson ImmunoResearch Laboratories, Pennsylvania, USA) and incubated for a further 30 min and assessed on the BioPlex® 200 System (Biorad). Relative avidity index (RAI) was calculated using the following equation: (MFI of treated sample)/(MFI of untreated sample) x 100. Out of range values were repeated using appropriate higher or lower serum dilutions.

Statistical Analyses

Host and environmental risk factors were compared between Aboriginal and non-Aboriginal otitis-prone children and non-otitis-prone children using Student's t-tests for continuous variables (age) and Pearson's chi-square analyses (asymptotic significant 2-sided P values) for categorical variables (gender, day care attendance, frequencies of diagnoses and surgery). Serum IgG titres were expressed as arbitrary units (AU)/mL against a reference human serum pool and reported as age-adjusted antigen-specific GMCs, with 95% CI. Adjusted GMCs were calculated by using logarithmically transformed, adjusted antibody concentrations. Univariate analyses using a general linear regression model and adjusting for age, were used to assess differences in anti-NTHi antibody titres between Aboriginal and non-Aboriginal otitis-prone children and non-otitis-prone children; and between Aboriginal otitis-prone children from urban and remote areas of Western Australia. RAI were compared between Aboriginal and non-Aboriginal otitis-prone children and non-otitis-prone children using ANOVA; and between Aboriginal children from urban and remote Western Australia using a Mann-Whitney U Test. The IBM SPSS Statistics 26 for Windows software package (IBM, Armonk, NY, USA) was used for all statistical analyses. Data were plotted using GraphPad Prism 9.0 (GraphPad Software Inc., La Jolla, CA, USA).

RESULTS

Demographics

A total of 173 children were recruited, including 69 Aboriginal and 72 non-Aboriginal otitis-prone children undergoing surgery for OM, and 32 non-Aboriginal non-otitis-prone children. None of the non-otitis-prone children had any significant history of respiratory infection or OM. For otitis-prone children, 74% of the non-Aboriginal children and 62% of the Aboriginal children were undergoing surgery for ventilation tube insertion, and 19% of the non-Aboriginal children and 33% of the Aboriginal children were undergoing tympanoplasty (**Table 1**). Most non-Aboriginal otitis-prone children were undergoing surgery because of a diagnosis of cOME (76%) and/or rAOM (44%), whereas 62% of the Aboriginal otitis-prone children had a diagnosis of cOME and/or rAOM (25%), and 32% had CSOM (**Table 1**). Significantly more non-Aboriginal otitis-prone children were on antibiotics at the time of surgery and received concurrent adenoidectomies (p=0.015). One Aboriginal otitis-prone child and one non-Aboriginal otitis-prone child were diagnosed with cholesteatoma at the time of surgery. Age was significantly different between Aboriginal, non-Aboriginal otitis-prone children and non-otitis-prone children and was therefore considered a potential confounder and adjusted for in all statistics conducted on normalised IgG titre data.

Natural Serum IgG titres for NTHi PilA Derived Proteins Were Significantly Lower in Aboriginal Otitis-Prone Children Compared to Non-Aboriginal Otitis-Prone and Non-Otitis-Prone Children

Australian Aboriginal otitis-prone children had lower serum IgG titres to rsPilA and ChimV4 (GMC: 88.94 and 429.66 AU/mL respectively) compared to non-Aboriginal otitis-prone children (GMC: 240.57 and 1210.76 AU/mL respectively), and non-otitis prone children (GMC: 227.69 and 872.18 AU/mL respectively), $p<0.05$ (**Figure 1A**). There were no significant differences between non-Aboriginal otitis-prone children and non-otitis-prone children. Serum IgG titres specific for OMP26 were similar between groups (GMC: Aboriginal otitis-prone children 1060 AU/mL, non-Aboriginal otitis-prone children 1054 AU/mL and non-otitis-prone children 820.2 AU/mL). Antigen-specific IgG titres were correlated with age to assess IgG development during childhood in each group. Weak correlations were observed between age and NTHi specific IgG titres in each group (R = -0.1-0.33; **Table 2**).

Serum IgG titres for each antigen were plotted against each other to compare relationships between different antigen-specific IgG titres. There was a strong correlation for IgG titres specific for rsPilA and ChimV4 (R= 0.8; $p<0.001$) (**Supplementary Figure 1A**). There was also a strong relationship between rsPilA and ChimV4 and previously reported PD titres (R= 0.9 and 0.77 respectively $p<0.001$) (**Supplementary Figures 1B, C**). Aboriginal otitis-prone children had the lowest IgG titres of all groups for rsPilA, ChimV4 and PD, which were distinct from the other 2 groups (**Supplementary Figures 1A–C**). The non-Aboriginal otitis-prone children and non-otitis-prone children had higher IgG titres, which overlapped in distribution. IgG titres specific for rsPilA, ChimV4 and PD had a weak relationship with titres against OMP26 ($R <0.4$ $p<0.001$; **Supplementary Figures 1D–F**).

Otitis-Prone Children Produce IgG Against Important Biofilm Proteins With Equivalent Avidity Indices to Non-Otitis-Prone Children

Australian Aboriginal otitis-prone children, non-Aboriginal otitis-prone children, and non-otitis-prone children had similar IgG RAI for rsPilA and PD (median RAI: 59%-62% and 89%-92% respectively). In contrast, the median RAI of non-otitis-prone children for IgG specific for OMP26 (58%) was significantly reduced compared to Aboriginal (74%) and non-Aboriginal (70%) otitis-prone children (**Figure 1B**). Australian Aboriginal and non-Aboriginal otitis-prone children showed a

TABLE 1 | Study population.

Characteristic	Non-Aboriginal non-otitis-prone children	Non-Aboriginal otitis-prone children	Aboriginal otitis-prone children	Aboriginal otitis-prone children	
			Total	Urban	Remote
Number [N]	32	72	69	38	31
Age in years [mean (range)] [+]	8.3 (1.6:14.4)	5.2 (1.1:13.6)	6.7 (1.7:12.7)	5.4 (1.7:9.7)	8.2 (2.1:12.7)
Female [N (%)]	20 (62)	36 (50)	31 (45)	15 (39)	16 (52)
Had attended day care [N (%)]	18 (56)	40 (56)	27 (39)	16 (42)	11 (35)
Located in remote WA[a] [N (%)]	0 (0)	2 (3)	31 (45)	0 (0)	31 (100)
History of CSOM[+] [N (%)]	0 (0)	6 (8)	26 (38)	12 (32)	14 (45)
Current surgery [N (%)]					
Ventilation Tube Insertion	NA	53 (74)	43 (62)	24 (63)	19 (61)
Myringotomy	NA	2 (3)	4 (6)	3 (8)	1 (3)
Tympanoplasty[b]	NA	14 (19)	23 (33)	11 (29)	12 (39)
Adenoidectomy[+]	NA	11 (15)	1 (1)	0 (0)	1 (3)
Principal diagnosis [N (%)]					
OME	NA	55 (76)	43 (62)	25 (66)	18 (58)
rAOM*	NA	32 (44)	17 (25)	12 (32)	5 (16)
CSOM*	NA	4 (6)	22 (32)	7 (18)	15 (48)
Immunisations up to date[c] [N (%)]	32 (100)	64 (93)	51 (98)	30 (97)	21 (100)
Receiving antibiotics at time of surgery[d+] [N (%)]	0 (0)	6 (9)	2 (3)	0 (0)	2 (7)

[+]*P-value <0.05 between non-otitis-prone, non-Aboriginal and Aboriginal otitis-prone children.*

**P-value<0.05 between non-otitis-prone, non-Aboriginal and Aboriginal otitis-prone children, and between Aboriginal otitis-prone children from urban and remote areas.*

[a]*Non-otitis-prone children, n = 32; non-Aboriginal otitis-prone children, n = 71; Aboriginal otitis-prone children, n = 69.*

[b]*Seven tympanoplasties were performed due to failure of tympanic membrane healing after ventilation tube insertion.*

[c]*Non-otitis-prone, n = 32; non-Aboriginal otitis-prone children, n = 69; Aboriginal otitis-prone children, n = 52, Aboriginal otitis-prone children from Urban n=31 and Remote n=21.*

[d]*Non-otitis-prone, n = 31; non-Aboriginal otitis-prone children, n = 70; Aboriginal otitis-prone children, n = 65, Aboriginal otitis-prone children from Urban n=37 and Remote n = 28.*

NA, not applicable.

trend towards reduced RAI to ChimV4 compared to non-otitis-prone children (median RAIs 45%, 54% and 63% respectively), however, this did not reach significance (p=0.195). RAIs for ChimV4 and rsPilA showed a moderate correlation (R= 0.5-0.7; p<0.001) for all 3 groups, however, not for other antigens (R=-0.2-0.4). Overall, the RAI to PD was highest compared to other antigens, followed by OMP26, with the RAIs for rsPilA and ChimV4 being the lowest.

Very weak correlations between RAIs and age were observed for all groups and antigens (R=-0.3-0.4) (**Table 3**). Furthermore, only weak relationships between antibody titres and RAI were observed for each antigen (R=-0.2-0.4) (**Table 4**).

Australian Aboriginal Otitis-Prone Children From Remote Areas Undergoing Surgery for OM, Have Similar Serum IgG Titres and RAIs to Those From Urban Areas

This cohort contains Aboriginal otitis-prone children from both urban and remote areas (**Table 1**). IgG titres were compared between these groups. Antibody titres specific for rsPilA, ChimV4 and PD were similar between Aboriginal otitis-prone children who lived in remote areas and those who lived in urban areas (GMCs: rsPilA = 91.1 vs 89.0; ChimV4 = 471.6 vs 415.5 and PD = 828.3 vs 984.6; p> 0.05) (**Figure 2A**). However, anti-OMP26 IgG titres were two-fold higher in children from remote areas when compared to those from urban areas (GMCs: 1188.2 vs 648.5; p=0.012) (**Figure 2A**). When antibody avidity was assessed, the RAIs of IgG specific for rsPilA, ChimV4 and OMP26 were similar in all children regardless of whether they lived remotely or in an urban area (median RAI remote versus urban: 58% vs 59%, 50% vs 44%, and 73% vs 75% respectively) (**Figure 2B**). However, children from urban areas had higher RAIs to PD compared to those from remote areas (93% versus 87% respectively, p<0.05).

DISCUSSION

In this cross-sectional study of 173 Australian Aboriginal and non-Aboriginal children, we examined naturally acquired serum IgG titres and avidity to potential NTHi vaccine antigens: rsPilA, ChimV4 and OMP26. Avidity indices were also measured for PD-specific IgG, for which PD IgG titres have been previously reported for this cohort (Thornton et al., 2017b). We found that naturally induced IgG titres to all NTHi antigens were similar between non-Aboriginal otitis-prone and non-otitis-prone children. In contrast, serum IgG titres against rsPilA and ChimV4 were significantly lower in Australian Aboriginal otitis-prone children compared to non-Aboriginal children regardless of diagnoses. IgG titres against OMP26 were similar for all groups. Notably, there were no differences in the avidity of antigen-specific IgG for rsPilA, ChimV4 or PD, however non-otitis-prone children had lower avidity IgG to OMP26 when compared to otitis-prone children. Weak relationships were observed between IgG titres, avidity indices and age for any antigen.

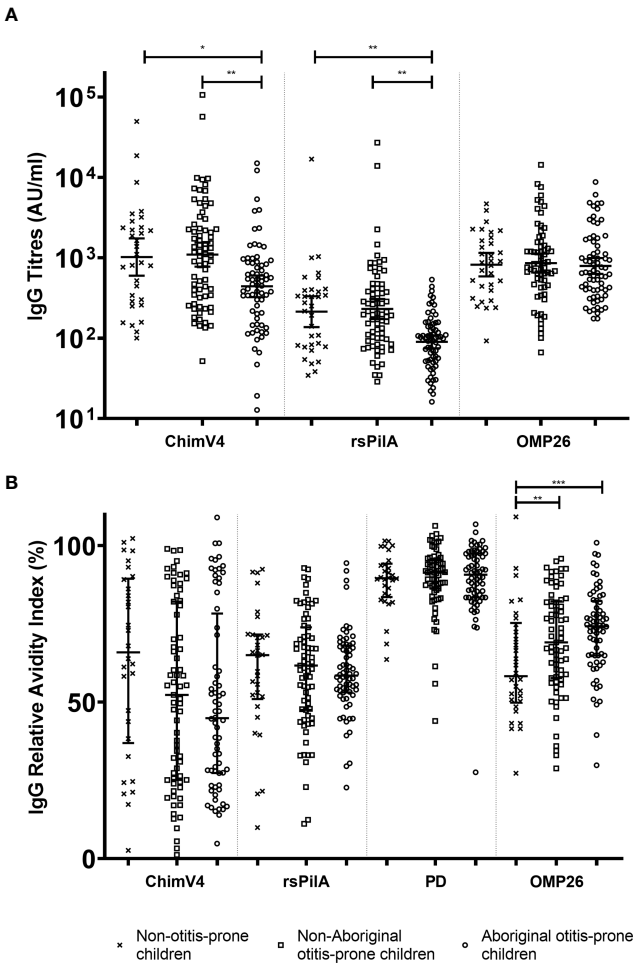

FIGURE 1 | Comparison of serum IgG titres and relative avidity indices between Aboriginal otitis-prone, non-Aboriginal otitis-prone and non-otitis-prone children. **(A)** Comparison of serum IgG titres between study groups. Levels of serum IgG against NTHi proteins are presented for each individual child, with the horizontal bars depicting the GMC ± 95% CI (not age adjusted). Statistical analyses were conducted on the log transformed GMC data, adjusted for age. **(B)** Comparison of serum IgG RAI between study groups. RAI serum IgG against NTHi proteins are presented for each individual child, with the horizontal bars depicting the median ± Interquartile Range. ChimV4, chimeric protein V4 (rsPilA+protein 5), rsPilA, recombinant soluble PilA; PD, Protein D; OMP26, outer membrane protein 26. *P ≤ 0.05; **P ≤ 0.01, ***P ≤ 0.001.

Australian Aboriginal children have been shown to develop chronic OM in infancy and this is associated with early, frequent and diverse NTHi carriage (Leach et al., 1994; Wenxing et al., 2012; Pickering et al., 2014). Despite repeated exposure to NTHi, serum IgG titres specific for rsPilA and ChimV4 were 50% lower in Aboriginal children. Interestingly, no differences were observed for IgG titres against OMP26. These antigen-specific differences are consistent with previous data from this cohort, which showed lower IgG titres to PD but similar levels for outer membrane proteins P4 and P6 in Aboriginal children when compared to both non-Aboriginal otitis-prone and non-otitis-prone children (Thornton et al., 2017b). Previous studies have shown that *in vitro* and *in vivo* OM models antibodies specific for rsPilA can prevent biofilm formation and disrupt established biofilms (Novotny et al., 2015). This specific reduction in IgG titres to rsPilA epitopes (which are also included in ChimV4) may leave children more susceptible to chronic and recurrent infections due to their essential role in the formation and maintenance of biofilms (Jurcisek et al., 2007). Data from murine models have also shown that a certain threshold of PilA antibodies are required to prevent NTHi nasopharyngeal colonisation (Ysebaert et al., 2019). Currently, it is unknown what antibody titres are needed to disrupt biofilms at the mucosal surface *in vivo*. This raises the possibility that a vaccine containing PilA such as, NTHi-10-AS01E, which is immunogenic in adults (Wilkinson et al., 2019; Van Damme et al., 2019), may have a role in preventing NTHi OM in Aboriginal children.

The reduced IgG titres to rsPilA and ChimV4 observed in Aboriginal otitis-prone children compared to non-Aboriginal otitis-prone children may be due to earlier and more dense NTHi colonisation (Leach et al., 1994; Wenxing et al., 2012) and colonisation with multiple strains in this population (Pickering et al., 2014). This early colonisation during development of tolerance to self-antigens may mistakenly also result in tolerance to NTHi antigens (Renz et al., 2018). An alternative explanation may be that these children are able to produce antibody, but these are depleted due to high pathogen exposure; this would require a longitudinal study with concurrent microbiological specimens to assess. Interestingly, unlike in our previous work for PD, no differences were observed in the rsPilA and ChimV4 antibody titres between non-Aboriginal children with and without OM. Previous data by Novotny et al. demonstrated that proportionately, otitis-prone children in the USA had reduced antibody titres to the protective epitopes of PilA compared to non-otitis-prone children (Novotny et al., 2009). This may suggest that in our cohort, while IgG titres between non-Aboriginal otitis-prone and non-otitis-prone children are similar, the proportion of effective antibody may be different. Together these data suggest that vaccination strategies to induce higher IgG titres against protective epitopes of PilA could be considered in these populations.

When we assessed relationships for IgG titres between different antigens to understand antibody development, IgG against PD (previously measured) (Thornton et al., 2017b) positively correlated with anti-rsPilA and anti-ChimV4, but not anti-OMP26 IgG titres. This may indicate that the biological mechanisms leading to reduced antigen-specific IgG titres for rsPilA, ChimV4 and PD are similar. Understanding these immune mechanisms will be crucial to the development of vaccines that can boost protective antibody levels in Aboriginal children.

Although Aboriginal children had reduced serum IgG titres, the relative avidity of PD, rsPilA and ChimV4 IgG were similar between the groups, suggesting that otitis-prone children can produce high-quality antibody. Despite this, the lower antibody titres produced by Aboriginal otitis-prone children may result in

TABLE 2 | Correlation of antigen specific IgG titres with age.

Antigen	Group	Correlation Coefficient	P-value
ChimV4	Non-otitis-prone children	0.279	0.123
	Non-Aboriginal otitis-prone children	0.251	0.033
	Aboriginal otitis-prone children	0.079	0.518
rsPilA	Non-otitis-prone children	0.123	0.501
	Non-Aboriginal otitis-prone children	0.257	0.029
	Aboriginal otitis-prone children	-0.093	0.448
OMP26	Non-otitis-prone children	0.078	0.671
	Non-Aboriginal otitis-prone children	-0.059	0.620
	Aboriginal otitis-prone children	0.077	0.531

TABLE 3 | Correlation of antigen specific IgG RAI with age.

Antigen Specific	Group	Correlation Coefficient	P-value
ChimV4	Non-otitis-prone children	-0.337	0.059
	Non-Aboriginal otitis-prone children	0.230	0.052
	Aboriginal otitis-prone children	0.106	0.388
rsPilA	Non-otitis-prone children	0.318	0.076
	Non-Aboriginal otitis-prone children	0.391	0.001
	Aboriginal otitis-prone children	0.046	0.710
Protein D	Non-otitis-prone children	0.168	0.358
	Non-Aboriginal otitis-prone children	0.116	0.333
	Aboriginal otitis-prone children	-0.03	0.808
OMP26	Non-otitis-prone children	0.028	0.880
	Non-Aboriginal otitis-prone children	0.065	0.585
	Aboriginal otitis-prone children	0.110	0.370

levels below protective thresholds despite being high-quality. Previous studies assessing vaccine induced antibody against *Haemophilus influenzae* type B and *Streptococcus pneumoniae*, show that higher avidity antibodies are more protective through improved bactericidal activity or opsonisation (Schlesinger et al., 1992; McGhee et al., 1999). Together these data suggest that reduced amounts of these high avidity IgG may contribute to the chronicity of OM observed in this population, and this warrants further investigation.

OMP26 IgG titres were similar in all children within the cohort, however, non-otitis-prone children had significantly reduced RAI in comparison to otitis-prone children. Only one study has reported OMP26 IgG in sera of otitis-prone children, demonstrating titres to be higher than in non-otitis prone children (Ysebaert et al., 2019). When assessed for bactericidal activity, OMP26 IgG was not bactericidal to NTHi in either group (Pichichero et al., 2010; Khan et al., 2012). However, rat studies have shown that vaccination with OMP26 produces bactericidal antibodies (Ercoli et al., 2015; Singh et al., 2020). In addition, previous animal work has shown that responses to OMP26, and the subsequent protection afforded by these, differ based on natural infection or vaccination (Kyd et al., 2003). Therefore, while natural antibody responses highlight important antigens, they only form part of the pre-clinical data needed to support moving vaccine antigens into clinical trials and this is a limitation of our study.

Similar to previous reports in this cohort, no relationships were observed between age and IgG titres, nor was RAI related to age. This is likely due to the older age range included in this study and it would be expected that all have been exposed to NTHi. The lack of microbiological data in this cohort is another limitation, however several studies have demonstrated that PD IgG titres in healthy children peak by approximately 3 years of age and then stabilise (Hua et al., 2016; Igor et al., 2016). Together these data suggest that IgG titres in these children have reached their peak production from natural exposure and further increases would need to be elicited by carefully developed vaccines.

Historically, research into OM in Australian Aboriginal children has concentrated in remote communities even though most Aboriginal children live in urban areas (Statistics ABo, 2018). The focus on children living in remote areas was based on the assumption that children from remote communities have more severe disease than those in urban areas. However, it has been shown recently that Australian Aboriginal children from urban areas in Western Australia have similar rates of OM as those reported for remote communities (Swift et al., 2020); suggesting that environmental risk factors alone may not lead to increased susceptibility of Australian Aboriginal children to OM. This is supported by our data showing similar IgG titres for 3/4 NTHi antigens, with similar quality, being observed between locations. OMP26 specific IgG was reduced in children from urban areas in comparison to children from remote areas and this may reflect differences in levels of NTHi exposure (and NTHi strain diversity (Pickering et al., 2014) as well as environmental risk factors. These data suggest a vaccine containing PD, rsPilA and ChimV4 may benefit all Australian Aboriginal children.

TABLE 4 | Correlation of antigen specific IgG RAI and IgG titres.

Antigen Specific	Group	Correlation Coefficient	P-value
ChimV4	Non-otitis-prone children	0.110	0.556
	Non-Aboriginal otitis-prone children	0.158	0.184
	Aboriginal otitis-prone children	0.168	0.167
rsPilA	Non-otitis-prone children	0.157	0.399
	Non-Aboriginal otitis-prone children	0.175	0.141
	Aboriginal otitis-prone children	0.361	0.002
Protein D	Non-otitis-prone children	0.345	0.058
	Non-Aboriginal otitis-prone children	-0.040	0.741
	Aboriginal otitis-prone children	-0.157	0.197
OMP26	Non-otitis-prone children	0.217	0.242
	Non-Aboriginal otitis-prone children	0.146	0.220
	Aboriginal otitis-prone children	0.213	0.079

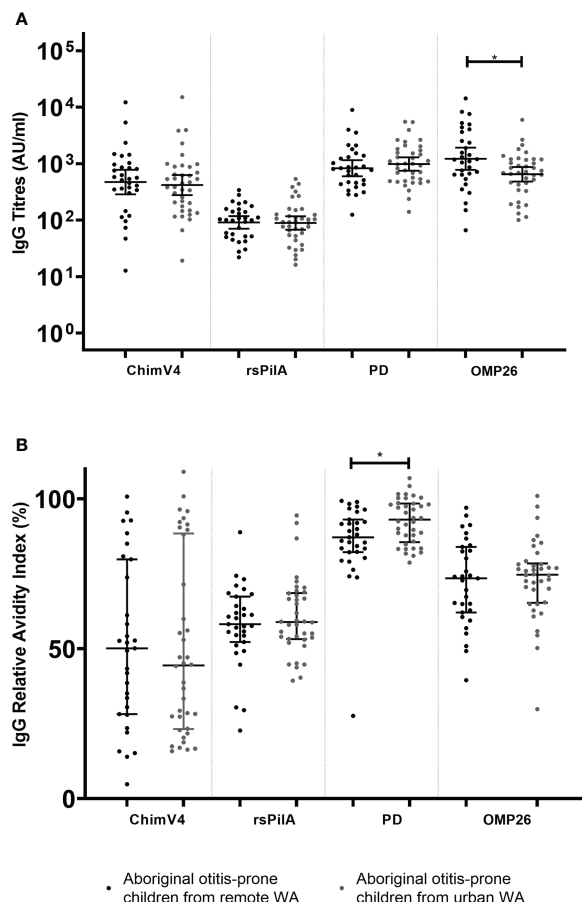

FIGURE 2 | Comparison of serum IgG titres and relative avidity indices (RAI) between Aboriginal otitis-prone children living in remote and urban Western Australia. **(A)** Comparison of serum IgG titres between remote and urban Aboriginal otitis-prone children. Levels of serum IgG against NTHi proteins are presented for each individual child, with the horizontal bars depicting the GMC ± 95%CI (not age adjusted). Statistical analyses were conducted on the log transformed GMC data, adjusted for age. **(B)** Comparison of serum IgG RAI between remote and urban Aboriginal otitis-prone children. RAI serum IgG against NTHi proteins are presented for each individual child, with the horizontal bars depicting the median ±Interquartile Range. ChimV4, chimeric protein V4 (rsPilA+ protein 5), rsPilA,recombinant soluble PilA; PD, Protein D; OMP26, outer membrane protein 26. *P ≤ 0.05.

In summary, we have shown that Australian Aboriginal children can produce high quality IgG to the important NTHi antigens, rsPilA and ChimV4, but at reduced levels. Boosting antibody titres through vaccination strategies using these antigens is therefore warranted to impact the chronicity and high burden of OM in this population.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Princess Margaret Hospital for Children, Armadale-Kelmscott Memorial Hospital Ethics Commitee, Osborne Park Hospital Ethics Committee, the Western Australian Aboriginal Health Ethics Committee, and Aboriginal community-controlled health services in the Kimberley and Eastern Goldfields regions.

AUTHOR CONTRIBUTIONS

SC and KC developed and conducted serological testing. SC analysed antibody data and drafted the manuscript. RT, SV, PR and HC were responsible for sample collection. AC, LN and LB provided proteins for assays and expert advice during assay development and manuscript preparation. ES, LK, RT, and PR oversaw sample selection, experimental conduct, analysis, data interpretation and manuscript preparation. PR, RT and HC obtained funding for study initiation and recruitment. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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STUDY PROTOCOL
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BIGDATA: A Protocol to Create and Extend a 25-Year Clinical Trial and Observational Data Asset to Address Key Knowledge Gaps in Otitis Media and Hearing Loss in Australian Aboriginal and Non-Aboriginal Children

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Introduction: Otitis media (OM) is a common childhood illness, often resolving without intervention and acute and long-term complications are rare. However, Australian Aboriginal and Torres Strait Islander infants and children experience a high burden of OM and are at high risk of complications (tympanic membrane perforation and chronic infections). Bacterial OM is commonly associated with *Streptococcus pneumoniae*, non-typeable *Haemophilus influenzae*, and *Moraxella catarrhalis*. BIGDATA is a data asset combining over 25 years of microbiology and OM surveillance research from the Ear Health Research Program at Menzies School of Health Research (Northern Territory, Australia), including 11 randomized controlled trials, four cohort studies, eight surveys in over 30 remote communities (including data from Western Australia), and five surveys of urban childcare centers including Aboriginal and Torres Strait Islander and non-Indigenous children. Outcome measures include clinical examinations (focusing on OM), antibiotic prescriptions, pneumococcal vaccination, modifiable risk factors such as smoking and household crowding, and nasopharyngeal and ear discharge microbiology including antimicrobial resistance testing.

Methods and Analysis: The initial series of projects are planned to address the following key knowledge gaps: (i) otitis media prevalence and severity over pre pneumococcal conjugate vaccines (PCVs) and three eras of increasing PCV valency; (ii) impact of increasing valency PCVs on nasopharyngeal carriage dynamics of pneumococcal serotypes, and antimicrobial resistance; (iii) impact of increasing valency PCVs on nasopharyngeal carriage dynamics and antimicrobial resistance of other otopathogens; and (iv) serotype specific differences between children with acute OM and OM with effusion or without OM. These data will be utilized to identify research gaps,

providing evidence-based prioritization for ongoing research.

Ethics and Dissemination: Data asset creation and priority analyses were approved by the Human Research Ethics Committee of Northern Territory Department of Health and Menzies School of Health Research (EC00153, 18-3281), the Child and Adolescent Health Service Human Research Ethics Committee and Western Australian Aboriginal Health Ethics Committee. Dissemination will be through peer review publication and conference presentations.

Keywords: otitis media, pneumococcal conjugate vaccines, *Streptococcus pneumoniae*, non-typeable Haemophilus influenzae, nasopharyngeal carriage, middle ear microbiology, antimicrobial resistance, indigenous

HIGHLIGHTS

- This expandable data asset created from over 25 years of research includes standardized assessments of the middle ear (video otoscopy and tympanometry), risk factors (household crowding, smoking, breastfeeding, living with young children), microbiological data from nasopharyngeal swabs and ear discharge (pneumococcal serotyping, antimicrobial resistance), and medical record review, and antibiotic use is an invaluable data resource.
- The planned analyses will comprehensively describe the OM trends and otopathogen carriage and resistance prevalence over the period 1992 to 2018, and describe factors increasing or decreasing OM and carriage risk, primarily the impact of three sequential pneumococcal conjugate vaccines on pneumococcal serotype replacement.
- This dataset includes research and health service data only where individual consent was provided, and does not describe other broader trends that may impact OM and carriage such as health policy change (medical management guidelines, workforce engagement, and community wide interventions like mass drug administrations). However, data-linkage and historical records can be used to link trends in OM and otopathogens carriage with policy change, workforce trends, housing stock, and public health interventions.
- Original eligibility criteria for participants varied across the studies according to the condition and outcomes of interest, therefore only surveillance and baseline screening data represent community prevalence of OM.
- Beyond the described priority analyses this resource will be used to improve our understanding of ear disease pathology and best practice treatment (from trials and administrative data) to reduce otitis media prevalence and severity in high-risk populations. This includes data linkage to assess educational outcomes, surgical intervention outcomes, and hearing aid fittings. As we are the only group providing pneumococcal serotype epidemiology data in Australia, we plan to develop a parsimonious surveillance modeling system, and will apply to funding to test this system and extend monitoring of pneumococcal serotype replacement and antimicrobial resistance as new extended valency pneumococcal conjugate vaccines are introduced to the Australian market.

Abbreviations: EHRP, Ear Health Research Program; OM, otitis media; RCT, randomized controlled trial; CCC, childcare center; NP, nasopharyngeal/nasopharynx; NVT, non-vaccine type; ED, ear discharge.

INTRODUCTION

Otitis media (OM) is a common childhood illness globally, with an estimated burden of more than 360 million episodes each year (1). OM is one of the primary reasons for presentations at healthcare facilities and for antibiotic prescriptions in children (2). There are varying disease pathologies, from episodic middle ear effusions to chronic drainage of purulent discharge through a perforation of the tympanic membrane. For most infants and children OM will resolve without treatment or intervention. However, some children are at high risk of more severe OM and long-term negative outcomes. Groups at higher risk for OM include children attending out of home childcare and Indigenous populations worldwide. Recognized risk factors for OM include age (with highest incidence between 6 and 11 months), exposure to tobacco and environmental smoke, reduced breast-feeding, and crowded living conditions (2).

Australia's Northern Territory (NT) faces some unique challenges. There is a vast geography with many widely dispersed small populations, with some residents living in very isolated areas. Multiple languages exist, and there is extensive population mobility linked to cultural and social traditions. Approximately 30% of the NT population are Aboriginal, compared to 3% Australia wide. Aboriginal people in the NT are generally younger, have lower educational attainment, have higher rates of chronic health problems, and live in more crowded housing than non-Aboriginal NT residents. 77% live in remote areas, contributing to difficulties in accessing health care and other services (3). The prevalence and severity of otitis media in these remote communities in northern and central Australia is extraordinarily high. Around 90% of children have some form of OM throughout their early years (including 15% with perforated tympanic membrane) (4–6).

All forms of OM are associated with hearing loss (7). The extreme prevalence of OM among Aboriginal children the NT makes this a significant public health problem. OM associated conductive hearing loss can impact the development of language skills and school preparedness when it occurs in children <4 years of age. However, there are limited audiology

data for Aboriginal children in this age group (8, 9). The relationship between OM disease status, hearing loss, and language development in this age group are still being established.

The burden of ear disease in NT Aboriginal children starts in the first weeks of life, driven by early, dense colonization of the nasopharynx with common bacterial respiratory pathogens including *Streptococcus pneumoniae*, non-typeable *Haemophilus influenzae* and *Moraxella catarrhalis* (10). *S. pneumoniae* causes considerable morbidity and mortality worldwide due to its ability to cause a range of mucosal and invasive diseases (including otitis media, sinusitis, conjunctivitis, pneumonia, meningitis and bacteremia) (11). The current pneumococcal conjugate vaccines (PCV) confer protection against up to 13 common serotypes (of the 100 known serotypes) causing invasive pneumococcal disease (IPD). The decrease in nasopharyngeal (NP) carriage of vaccine types (VT) among vaccinees has reduced pneumococcal transmission and rates of IPD in non-vaccinated groups as a result of herd effects (12). Near elimination of NP carriage by vaccine serotypes has also left a niche for non-vaccine types (non-VTs). Most populations have reported NP carriage replacement with non-VTs, as was observed in remote dwelling Australian Aboriginal children (13). Whilst non-VT serotypes are less likely to cause IPD than VTs, they continue to cause mucosal pneumococcal infections (including OM and bronchitis). Higher valency PCV trials are currently in progress (including15PCV, PCV20, PHiD-CV11, PHiD-CV12) (14–16). Carriage serotype epidemiology is needed for selection of vaccines as benefits will be population specific and driven by local serotype distribution and dynamics.

Objectives

The objective of the BIGDATA project is to use an expandable dataset created from otitis media and pneumococcal surveillance studies spanning decades of research to examine questions that could not previously be addressed using single studies. A series of planned analyses will be used to describe OM and NP carriage and identify research gaps to provide evidence-based prioritization for ongoing research. Looking forward, BIGDATA will be linked with health and education administrative data to understand mid-to long-term outcomes for children with and without OM in early childhood, and impacts of past health policies and programs on OM management and long-term outcomes.

METHODS AND ANALYSES

The Combined Dataset

The Ear Health Research Program (EHRP) at Menzies School of Health Research has been conducting research relating to OM and respiratory pathogen surveillance in children at high risk of ear disease, specifically Australian Aboriginal and/or Torres Strait Islander children living in remote settings, and children attending childcare in urban settings (both at-risk populations) for almost 30 years. A series of cross-sectional studies, longitudinal studies, and randomized controlled trials (RCTs) have been conducted since the early 1990s. This program of research represents a significant data asset relating to ear health states, risk factors, and bacterial pathogen carriage in the nasopharynx (and middle ears of children with tympanic membrane perforation). Currently, BIGDATA includes 3 birth cohort studies, 11 RCTs, 3 cohort studies, 8 surveillance surveys of remote community children (including children in from all over the NT and Kimberley, Goldfields, Pilbara, Gascoyne, and metropolitan area of Perth in Western Australia) and 5 surveillance surveys of urban children attending child care centers (CCC), spanning 1995 to 2018.

Data Sources, Management, and Amalgamation

A core group of investigators has maintained the EHRP research, ensuring funding, standardized methods for ear assessments and microbiology, long-term consistency of data collection, including cleaning and storage.

Ears were assessed using (video) pneumatic otoscopy or (video) otoscopy and tympanometry. Minimal change to diagnostic categories were made over the life of the program. Management of diagnostic categories of OM were made according to relevant guidelines at the time of each the study. Specialist referrals (audiology and ENT) were also made according to appropriate guidelines. In addition to otitis media diagnoses, detailed ear observations were recorded including tympanic membrane color, translucency, mobility, position (retraction, neutral or degree of bulging), perforation size and position.

Medical records of consented participants were also reviewed to collect additional data. This included non-study related clinician-diagnosed otitis media, all antibiotic prescriptions likely to influence otitis media and pneumococcal immunization data. Health data not related to OM or antibiotic use were not extracted.

Research nurses conducted standardized interview questionnaires with parents or caregivers regarding recognized risk factors. These included breastfeeding, smoking, family history of otitis media, and household occupancy. Observable general health indicators (runny nose, cough, visible skin problems) and weight and height were also collected as part of some studies. Original study data were collected on hard copy standardized forms for entry into secure electronic data storage systems.

Microbiological analysis of NP and ear discharge (ED) swabs for respiratory pathogens of interest used WHO-recommended standardized specimen collection, transport, storage, culture, identification and serotyping methods (Quellung) (17, 18) and are described in detail in **Supplementary Material**. Antibiotic sensitivity of bacterial isolates included disc diffusion predominantly by Calibrated Dichotomous Susceptibility (CDS) (19) and some E-test MIC determination.

Almost all studies collected a core set of clinical assessments and microbiological specimens and used the same standardized methods and data storage systems.

TABLE 1 | Summary of BIGDATA participants, enrolments, ear examinations and nasopharyngeal swab data.

	Remote Aboriginal Communities (*N*, %)	Urban Childcare Centers (*N*, %)
Individual children	4,692 (66.6)	2,358 (33.4)
Enrolments	8,323 (75.2)	2,751 (24.8)
Ear examinations (children)	10,989 (65.8)	5,720 (34.2)
Nasopharyngeal swabs	11,992 (63.8)	6,814 (36.2)

Some studies included adults, some audiology testing, some virology. All available clinical, microbiological, and risk factor data for children has been included in the combined dataset. Analyses of combined data for uncommon subgroups and outcomes will be undertaken where feasible to do so. Appropriate inclusion and exclusion criteria will be applied in each analysis.

All original data were subject to quality assurance by a process of searching for missing data, logic checks, and audits against primary data sources. Data from the original studies were assessed for variables common in definition. If a comparable variable existed in multiple studies it was included in the combined dataset. Coded data were checked using the original study data dictionary to ensure coding was consistent and standardized as necessary. Variables that were not collected in multiple studies or had inconsistent definitions were not included. We aim to continue building the data asset by adding data from pre-1995 studies, and current studies after completion (bolstering data from 2015 onwards).

Over time, some children represented in this dataset have participated in more than one trial or survey (multiple enrolments). Special attention was given to the demographic data, particularly HRN (hospital registration number) to identify any child enrolled in multiple studies and across regions in the NT. This process involved looking for exact matches, (given and family name, date of birth), and highly probable matches (allowing for minor errors in spelling and dates). The HRN is a unique identifier used across the NT and is assigned to a child at birth. This was done computationally initially and finalized by manual review. A generated unique identifier enables the HRN and other identifying fields to be removed to prevent reidentification.

The data from these studies were combined to create a dataset of all study visit dates for each individual child and associated data for that visit date. Summary data of the number of individual children, enrolments, ear examinations, nasopharyngeal swabs and ear discharge swabs are presented in **Table 1**. The age distribution is predominantly younger children, with a median age of 2.5 years at enrolment (range 0–17.9).

Original study designs, study enrolments, age profiles and NP microbiology and ear examination data inclusion are summarized in **Table 2**, additional information is included in **Supplementary Material**. Where data are published, publications are referenced in **Table 2**.

As per the CONSIDER statement (37) the generation of the consolidated data asset and the planned primary analyses were subject to Ethics approval process, including review by the Aboriginal Ethics sub-committee. Use of the data for analyses beyond those specified will be subject to the same process. The leaders of this body of work have extensive experience of research with Aboriginal communities and people. These proposed analyses are part of a broader research program to reduce OM in Aboriginal children. All studies from which data have been drawn were preceded by extensive community consultation and engagement with relevant stakeholders within the communities. These studies have provided employment opportunities for Aboriginal people as researchers, and community-based employment in remote settings. Through recent training and education opportunities, Aboriginal youth have joined us in the laboratory, processing samples collected as part of this body of work. The ongoing engagement over almost 30 years of up to 50 Aboriginal communities in the NT demonstrates the importance of improving ear health outcomes for individuals and communities. The Menzies Child Health Division First Nations Reference Group, whose members are from around the NT, provides guidance, support and feedback to researchers to ensure research activities and dissemination are culturally appropriate. All research dissemination through peer-review publication and community-based feedback (oral and written) to stakeholders includes appropriate management to ensure individuals and communities are not identifiable unless written informed consent is provided.

Data Limitations

Whilst this resource represents a comprehensive collection, data for all described fields were not included in every study due to the variation in study designs. Original eligibility criteria for participants varied across the RCTs according to the condition (specific OM diagnosis) and outcomes of interest, such as intervention trials for treatment of acute OM or chronic suppurative OM. Data from these studies will be used when they meet inclusion criteria for each research question. Inclusion and exclusion criteria for unpublished data studies can be accessed upon reasonable request. This dataset only includes research and health service data where individual consent was provided. Currently community-level data relating to other factors that may impact OM and NP carriage, including government initiatives such as swimming pools, housing, trends in primary healthcare services and policy, or widespread antibiotic use or mass drug administration for trachoma are not included in BIGDATA. These data could potentially be linked in the future.

Research Questions and Hypotheses

This expandable dataset was constructed with the goal of describing long term population trends in prevalence of otitis media and nasopharyngeal carriage, to increase statistical power in analyses, and to compare clinical pathways (specialist services) and quality of life outcomes (vulnerability, school attendance

TABLE 2 | Summary of original studies.

Years data coll	Description	Study design (no. visits) (No. RAC/ CCC)	no. children (% Indigenous) Age—mean/median [range]	Procedures and micro	
1996–2001	Double-blinded randomized controlled trial of long-term amoxicillin vs. placebo for otitis media with effusion (20–27)	L (1–18) RCT (3)	125 (100%) 4.1/2.8 [0.2–19]	Ears NP ED	Spn Hi Mc Sa
2000	The clinical course of acute otitis media in high-risk Australian Aboriginal children: a longitudinal study (28)	L (1–16) Cohort (1)	31 (100%) 1.6 /1.1 [0.2–5.8]	Ears NP ED	Spn Hi Mc
2000–2001	Randomized Controlled Trial of Amoxycillin for Persistent Nasal Discharge in Rural and Remote Aboriginal children (29, 30)	L (1–7) RCT (1)	34 (100%) 59.6/61.4 [26.2–109]	Ears NP ED	Spn Hi
2001	Topical ciprofloxacin vs. topical framycetin-gramicidin-dexamethasone in Australian aboriginal children with recently treated chronic suppurative otitis media: a randomized controlled trial (31)	L (1–10) RCT (3)	97 (100%) 7.8/7.4 [1.1–15.2] years	Ears NP ED	Spn Hi Mc Sa Ps
2001	Otitis media in young Aboriginal children from remote communities in Northern and Central Australia: a cross-sectional survey (4)	X Surv (29)	709 (100%) 1.5/1.5 [0.05–3.4]	Ears	–
2001–2004	Longitudinal nasopharyngeal carriage study of Indigenous children receiving 7-valent pneumococcal conjugate vaccine (21, 23–27, 32–34)	L (1–22) Cohort (3)	97 (100%) 1.6/1.1 [0.2–8]	Ears NP ED	Spn Hi
2001	Childcare hygiene intervention study in 20 Darwin childcare centers (22, 25, 29, 30, 35, 36)	L (1–15) RCT (20)	456 (11%) 29/29 (7–54)	Ears NP ED	Spn Hi
2002–2004	Cross sectional surveillance of bacterial nasal carriage and antibiotic resistance in Indigenous children & adults from Tiwi communities (33, 39)	X Cohort (4)	545 (100%) 25 [0.8–80] **years**	NP	Spn
2003	Randomized controlled trial of surfactant vs ciprofloxacin or sofradex for resolving discharge in Indigenous children in a Tiwi community	L (1–4) RCT (1)	32 (100%) 8.3/7.9 [4.4–13.2] **years**	Ears NP ED	Spn Hi Pa
2003 PCV7	Cross sectional surveillance of OM and bacterial nasal carriage and antibiotic resistance in Indigenous children from 27 remote communities (13, 25, 33)	X Surv (27)	644 (100%) 18/18 [6-30]	NP ED	Spn Hi Mc
2003–2005	Double-blinded randomized controlled trial of azithromycin vs. amoxicillin for treatment of acute otitis media in 16 communities (25, 26, 35, 36, 40–43)	L (1-3) RCT (16)	320 (100%) 17.5/13 [6-72]	Ears NP ED	Spn Hi Mc
2003	Cross-sectional surveillance of bacterial nasal carriage and antibiotic resistance in children attending 18 urban childcare centers in Darwin and Alice Springs	X Surv (18)	303 (unknown%) 33.6/34 [2.6-66]	NP	Spn
2004	Cross-sectional surveillance of bacterial nasal carriage and antibiotic resistance in children attending 23 urban childcare centers in Darwin and Alice Springs (25)	X Surv (26)	473 (unknown%) 33.7/33.4 [3.7-83.8]	NP	Spn Hi Mc
2005	Cross sectional surveillance of OM and bacterial nasal carriage and antibiotic resistance in Indigenous children from 18 remote communities (13, 33)	X Surv (18)	819 (100%) 34/34 [0-72]	NP	Spn
2005	Cross-sectional surveillance of bacterial nasal carriage and antibiotic resistance in children attending 26 urban childcare centers in Darwin and Alice Springs	X Surv (23)	394 (11%) 35.5/36.4 [3.2-65.1]	NP	Spn
2006-2011	PneuMum: A randomized controlled trial of pneumococcal polysaccharide vaccination for	L (1-4) RCT	225 (100%) birth	Ears NP	Spn Hi

(Continued)

TABLE 2 | Continued

Years data coll	Description	Study design (no. visits) (No. RAC/ CCC)	no. children (% Indigenous) Age—mean/median [range]	Procedures and micro	
	Aboriginal and Torres Strait Islander mothers to protect their babies from ear disease (44–49)	(4)		ED	Mc Sa
2008-2013	A double blind placebo-controlled randomized clinical trial of the use of oral azithromycin in Aboriginal children between 6 months and 30 months of age presenting with asymptomatic acute otitis media without perforation	L (1-3) RCT (14)	149 (100%) 16.2/16.1 [5–30.7]	Ears NP ED	Spn Hi Mc Sa
2008–2011	High Nasopharyngeal Carriage of Non-Vaccine Serotypes in Western Australian Aboriginal People Following 10 Years of Pneumococcal Conjugate Vaccination (50)	X Surv (40)	1500 (100%) 0–101 **years**	NP ED	Spn Hi Mc Sa
2008	Cross sectional surveillance of OM and bacterial nasal carriage and antibiotic resistance in Indigenous children from 2 remote communities (5, 38, 51)	X Surv (2)	54 (100%) 19.6/20.8 [4.3–32.2]	Ears NP ED	Spn Hi Mc Sa
2009–2010	A randomized control trial of the effect of swimming in pools in Aboriginal children between 5 and 12 years of age presenting with tympanic membrane perforation (52)	L (1–2) RCT (2)	92 (100%) 8.8/8.7 [4.9–13.6] **years**	Ears NP ED	Spn Hi Sa Pa
2009	Cross sectional surveillance of OM and bacterial nasal carriage and antibiotic resistance in Indigenous children from 10 remote communities (5, 38, 51)	X Surv (10)	174 (100%) 17.1/17.3 [0.9–40.5]	Ears NP ED	Spn Hi Mc Sa
2010	Cross sectional surveillance of OM and bacterial nasal carriage and antibiotic resistance in Indigenous children from 21 remote communities (5, 6, 38, 51, 53)	X Surv (21)	253 (100%) 24.5/21.1 [2.7–70.2]	Ears NP ED	Spn Hi Mc Sa
2010	Cross-sectional surveillance of OM, bacterial nasal carriage and antibiotic resistance in children attending 24 urban childcare centers in Darwin & Alice Springs;	X Surv (24)	462 (100%) 33.9/35 [0.3–73.1]	Ears NP ED	Spn Hi Sa
2011	Mobile phones for advanced case management of chronic suppurative otitis media (CSOM) in remote Indigenous communities: a pilot randomized controlled trial (54)	L (1–2) RCT (1)	53 (100%) 7.5/7.9 [0.9–15.1] **years**	Ears ED	Spn Hi S a Pa
2011	Cross sectional surveillance of OM and bacterial nasal carriage and antibiotic resistance in Indigenous children from 19 remote communities (5, 6, 38, 51, 53, 55)	X Surv (19)	568 (100%) 37.6/33.9 [0.4–96.3]	Ears NP ED	Spn Hi Mc Sa
2012	Cross sectional surveillance of OM and bacterial nasal carriage and antibiotic resistance in Indigenous children from 12 remote communities (5, 6, 38, 51, 53, 55, 56)	X Surv (12)	276 (100%) 25.6/23.4 [0.7–76.2]	Ears NP ED	Spn Hi Mc Sa
2012	Cross-sectional surveillance of OM, bacterial nasal carriage and antibiotic resistance in children attending 33 urban childcare centers in Darwin and Alice Springs (53)	X Surv (33)	607 (100%) 34.1/34.8 [3.4–60.8]	Ears ED	Spn Hi Sa Ps
2013	Cross sectional surveillance of OM and bacterial nasal carriage and antibiotic resistance in Indigenous children from 14 remote communities (6, 51, 55)	X Surv (14)	371 (100%) 35.9/36.3 [0.16–82.9]	Ears NP ED	Spn Hi Mc Sa
2011–2018	A randomized controlled trial of pneumococcal conjugate vaccines Synflorix and Prevenar13 in sequence or alone in high-risk Indigenous infants (PREV-IX_COMBO): immunogenicity, carriage and otitis media outcomes (53, 56–58)	L (1-5) RCT (5)	172 (100%) 31.7/32 [25-40] **days**	Ears NP ED	Spn Hi Mc Sa

L, longitudinal (number visits); RCT, randomized controlled trial; X, cross-sectional; Surv, surveillance. RAC, Remote Aboriginal Community; CCC, Childcare centers; NP, nasopharyngeal; ED, ear discharge; Spn, S.pneumoniae; Hi, H.influenzae; Mc, M.catarrhalis; Sa, S.aureus; Pa, P.aeruginosa.

and performance) for children with or without early chronic otitis media. We aim to identify research gaps and inform and guide new research in ear and hearing health preventative and treatment options to improve outcomes for high risk populations. Priority analyses, which have appropriate Ethical approvals, are described below.

(i) Otitis media prevalence and severity (by diagnosis) including unilateral and bilateral disease trends, over time, during pre-pneumococcal conjugate vaccines (PCVs) and three increasing valency PCV eras (PCV7, PCV10, and PCV13), by region and other known predictors (risk factors).

Published surveillance to 2013 indicates a reduction in the prevalence of severe OM (CSOM or any TMP), however, prevalence of other forms of OM has increased such that overall OM prevalence remains unchanged and unacceptably high (4, 38).

Aims

To describe prevalence of OM and severe OM over time and explore factors associated with these outcomes.

Outcomes

Presence of OM and different OM diagnoses (proportions with 95% CIs) will be reported, per time period and region. Markers of severity (size of perforation, volume of discharge, bilateral disease) will be compared over time and vaccine eras. Regression analyses, corrected for repeated measures in the same participants where relevant, will be used to describe OM associations with modifiable risk factors, age, time, prior antibiotic use, gender and geographic region.

Multilevel mixed effects models will be used, utilizing fixed effects for antibiotics, vaccination, age, gender and year; and random effects for region.

(ii) Impact of increasing valency PCVs (PCV7, PCV10 and PCV13) on nasopharyngeal carriage dynamics of pneumococcal serotypes, and associated antibiotic resistance, and by region and other known predictors (risk factors).

PCV impact on nasopharyngeal carriage has been addressed in specific EHRP study publications, as the vaccines have been introduced (**Table 1**). However, a comprehensive descriptive analysis including all study data from the non-vaccine studies and pre-vaccine era has not been undertaken.

Aims

To describe prevalence of nasopharyngeal pneumococcal carriage, serotype-specific carriage, and resistance over time and explore associated risk predictors.

Outcomes

Using all available non-interventional data, we will describe pneumococcal carriage, including serotypes and antibiotic resistance over vaccine eras (pre-PCV, PCV7, PCV10 and PCV13), reporting children positive for pneumococcus, vaccine and non-vaccine serotypes (proportions with 95% CIs) by age, region and vaccine era for comparison (chi-squared test). Regression analyses for risk predictors will use the same approach as above.

(iii) Impact of increasing valency PCVs on nasopharyngeal carriage dynamics of non-pneumococcal respiratory pathogens and antibiotic resistance over time, by region, and other known predictors (risk factors).

Aims

To determine carriage prevalence, density and resistance of non-typeable *H. influenzae*, *M. catarrhalis,* and *S. aureus*, over the vaccine eras.

Outcomes

Proportions positive for carriage, proportions resistant to common antimicrobials (particularly β-lactams and macrolides), categorical carriage density and co-colonization will be reported and analyzed as per the pneumococcal/OM analyses.

(iv) Serotype specific differences in nasopharyngeal carriage between children with acute OM and diagnoses of OM with effusion or healthy ears and the impact of increasing valency PCVs on serotype dynamics and other known predictors (risk factors).

Aims

In a subset of children (completed primary immunization series, aged 6 month to 5 years) from BIGDATA, with both a nasopharyngeal swab and an ear examination of the same day, describe the vaccine and non-vaccine serotype carriage, and impact of increasing valency PCVs on the serotypes associated with a diagnosis of AOM or no AOM.

Outcomes

Proportions of children (with 95%CIs) positive for pneumococci, serotype specific carriage, and vaccine and non-vaccine type carriage will be compared (chi-square) in children with and without AOM. Regression analyses will describe associations with modifiable risk factors, age and time as described above.

Future Directions

Beyond these initial analyses, other projects are being proposed for these data. Building on the pneumococcal carriage analyses, we aim to develop an efficient remote community and childcare center sentinel surveillance model for pneumococcal serotypes to determine efficient surveillance methods to detect emerging pneumococcal serotypes and resistance, and to improve the efficiency of sampling (by age range, geographic region, and sampling frequency). Modeling methodology is yet to be finalized. Accuracy of surveillance models will be tested in cross-sectional prospective studies across the NT.

Where we have comprehensive monitoring of individual children, we intend to examine the relationship between persistent ear disease and hearing loss in infancy and early childhood with subsequent outcomes such as hearing aid use and ENT surgical procedures. We will also describe ongoing indicators of life trajectory by combining our data with government held administrative data through the SA-NT

Government data-linkage program. Data will be analyzed using multivariate regression approach with appropriate link function. Multivariate regression will be fitted between ear diagnoses and education and health outcomes, using children with healthy ears as the reference. Linking Health and Education administrative datasets to BIGDATA will facilitate the long term follow up of children with persistent OM diagnoses. This would enable an assessment of the impact of current treatments on perforations, and whether specialist services including hospitalization, ear surgery for grommets and tympanoplasties, and availability of hearing augmentation impact AEDC (Australian Early Development Census) and NAPLAN (National Assessment Program—Literacy and Numeracy) results, school attendance, maltreatment and later engagement with the justice system.

A cascade analysis approach will be used to identify which forms of OM receive more appropriate management (e.g., antibiotic prescribing guidelines, follow-up schedule, correct prescribing, audiology and specialist referrals met) and any management trends over time. We will also describe the region and age that contribute to better outcomes, and the potential impacts of vaccine and antibiotic prescribing guidelines. These detailed data will be used to identify critical timepoints where disease progression might be halted or reversed, and critical age groups for maximizing the benefits of hearing aids or surgery on language development, social development, and education outcomes.

We anticipate that these analyses will further support policies and programs aimed at reducing disadvantage attributed to early childhood ear and hearing problems. The analyses will also identify priority gaps in knowledge and lead to improved design of future studies aimed at eliminating the contribution that ear and hearing problems make to poor quality of life.

DISCUSSION

There are currently no ongoing NT or Australian surveillance studies of OM and NP carriage. Creating this combined data asset and extending content with new studies and linkage of administrative data increases its value for all Australians. While new research is vital, it is important that the data we already have is used effectively and thoroughly. We now have data spanning the early life of four cohorts of infants which will be re-analyzed to gain a clearer picture of OM in the first months of life. We would like to look at the relationship between NP carriage serotypes and those causing pediatric invasive pneumococcal disease as the conjugate vaccines are introduced. We intend to combine similar treatment arms across RCTs to determine the impact of different classes of antibiotics on resistance and take advantage of the increased statistical power compared to original studies. These data and analyses will also identify the research gaps are that can be the focus of prospective research studies.

As mentioned, we plan for the BIGDATA data asset to be expanded, both with additional EHRP study data, and through data linkage. Government initiatives in the region which impact health service delivery especially as relates to hearing services and ear surgery are of particular interest. Similarly, the changes in the primary healthcare workforce and service delivery (including outreach and tele-otology programs) are likely to be important. Programs such as the mass drug administrations of azithromycin for trachoma eradication (which have the potential to impact otopathogen carriage and antimicrobial resistance), can also be linked to BIGDATA in the future.

CONCLUSIONS

This combined dataset is a valuable research tool for understanding otitis media and nasopharyngeal pathogen carriage in at risk populations in the Northern Territory. This data asset and the described planned analyses will improve our understanding of ear disease pathology and best practice treatment to reduce otitis media prevalence and severity in high-risk populations. Our overall goal for the BIGDATA analyses is to improve hearing and long-term outcomes (such as education and employment) and to address the challenge of increasing antimicrobial resistance in the region.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Human Research Ethics Committee of Northern Territory Department of Health and Menzies School of Health Research. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AL conceived the idea for the creation of the combined dataset. AL, PM, and HS-V are senior investigators on nearly all the research projects. JB managed the data amalgamation and was responsible for the data and wrote this manuscript. All authors made intellectual contribution to the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fped.2022.804373/full#supplementary-material

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Microbiota Associated With Cholesteatoma Tissue in Chronic Suppurative Otitis Media

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Otitis media (OM), defined as infection or inflammation of the middle ear (ME), remains a major public health problem worldwide. Cholesteatoma is a non-cancerous, cyst-like lesion in the ME that may be acquired due to chronic OM and cause disabling complications. Surgery is required for treatment, with high rates of recurrence. Current antibiotic treatments have been largely targeted to previous culturable bacteria, which may lead to antibiotic resistance or treatment failures. For this study, our goal was to determine the microbiota of cholesteatoma tissue in comparison with other ME tissues in patients with long-standing chronic OM. ME samples including cholesteatoma, granulation tissue, ME mucosa and discharge were collected from patients undergoing tympanomastoidectomy surgery for chronic OM. Bacteria were profiled by 16S rRNA gene sequencing in 103 ME samples from 53 patients. Respiratory viruses were also screened in 115 specimens from 45 patients. Differences in bacterial profiles (beta-diversity) and the relative abundances of individual taxa were observed between cholesteatoma and ME sample-types. Additionally, patient age was associated with differences in overall microbiota composition while numerous individual taxa were differentially abundant across age quartiles. No viruses were identified in screened ME samples. Biodiversity was moderately lower in cholesteatoma and ME discharge compared to ME mucosal tissues. We also present overall bacterial profiles of ME tissues by sample-type, age, cholesteatoma diagnosis and quinolone use, including prevalent bacterial taxa. Our findings will be useful for fine-tuning treatment protocols for cholesteatoma and chronic OM in settings with limited health care resources.

Keywords: 16S rRNA, cholesteatoma, microbiome, middle ear, otitis media

INTRODUCTION

Otitis media (OM) or middle ear (ME) infection/inflammation remains a major public health problem worldwide, with an estimated 60% of hearing-impaired children under 5 years old having OM as the cause of hearing loss (GBD 2019 Hearing Loss Collaborators, 2021). Risk factors for OM include young age, lack of breastfeeding, allergies, upper respiratory infection, second-hand smoke exposure, low social status, daycare attendance, multiple siblings, and family history (Zhang et al., 2014; Brennan-Jones et al., 2015). OM may persist as recurrent acute (RA)OM or chronic (C)OM, for which treatment includes antibiotics and surgery. In the United States, annual health care use due to office visits, antibiotics, and surgeries for OM in children is estimated to cost >$5 billion (Suaya et al., 2018). Antibiotics are prescribed for 67% of children with OM (Hersh et al., 2016), inappropriately in 10-33% of cases, causing concern for antibiotic resistance (Fleming-Dutra et al., 2016; DeMuri et al., 2017). Globally, age-standardized rates for years lived with disability due to acute OM are still increasing (GBD 2019 Diseases and Injuries Collaborators, 2020). In children, OM can cause not only permanent hearing loss but also impairments in speech perception, auditory processing or phonological awareness while reading, thereby affecting academic performance (O'Niel et al., 2015; Khavarghazalani et al., 2016; Cai and McPherson, 2017; le Clercq et al., 2017; Carroll and Breadmore, 2018). If persisting to adulthood, COM, even with only mild-to-moderate hearing loss, is associated with poorer quality of life and mental health, particularly if revision surgeries were performed (Bakir et al., 2013). After tympanoplasty surgery, the 10-year recurrence rate of COM is 15-26% (Nardone et al., 2012).

Cholesteatoma is a non-cancerous cyst-like lesion in the ME that either develops congenitally (rare) or, more commonly, is acquired in COM. Cholesteatoma was estimated to develop in 10-24% of OM cases (O'Connor et al., 2009; Rosito et al., 2017). In a tertiary hospital setting in Colorado, out of 67,661 children seen over 10 years, 36.5% had OM and 12.0% were diagnosed with COM; of those with COM, 5.1% of children presented with cholesteatoma (unpublished data from COMPASS database). Prevalence is higher in adults: of 1,006 adults with OM, 24.4% had COM; of those with COM, 39.2% had cholesteatoma. These data show that while COM and cholesteatoma are prevalent in lower income countries such as the Philippines (Carrillo et al., 2007; Santos-Cortez et al., 2007), these disease entities are also significant health issues in high-income countries including the United States.

Cholesteatoma has a propensity for insidious growth and erosion of the ossicles or temporal bone that houses neural structures and may lead to disabling complications such as hearing loss, facial nerve palsy, vertigo, or intracranial extension. Surgery is required for treatment, with high rates of recurrence even after >10 years (Kuo et al., 2012; Nardone et al., 2012). Histologically, cholesteatoma is filled by keratin debris and lined by keratinized squamous epithelium (matrix), which is similar to the outer epidermal layer of the tympanic membrane (TM) but with Langerhans cells and basal cells (Lim and Saunders, 1972). Between the matrix and ME mucosa, there is a layer of loose connective tissue (*i.e.*, perimatrix) containing collagen fibers and fibrocytes, typically with inflammation between the perimatrix and ME mucosa (Lim and Saunders, 1972). Under the operating microscope, these tissues can be differentiated well: the cholesteatoma is the encapsulated collection of keratin debris within the ME space, while the hyperplastic, severely inflamed mucosa that is in contact with the cholesteatoma appears as granulation tissue that is distinct from normal-looking but infected ME mucosa (Saunders et al., 2011). Fifty years after its histology was resolved, there is still no consensus on how a cholesteatoma forms, except that the process is probably a hybrid of theories (Maniu et al., 2014; Kuo et al., 2015): [a] desquamated keratin accumulates in a retraction pocket formed by negative ME pressure; [b] squamous epithelium of the TM migrates to the ME; [c] ME mucosa transforms into keratinizing epithelium; [d] keratin-filled microcysts form within the basal layer of the TM and invade subepithelial tissue. Better understanding of cholesteatoma pathology will aid in implementation of standard classifications of lesions to guide management and predict surgical outcomes (James et al., 2019). It was suggested that an exaggerated inflammatory response causes cholesteatoma growth, proliferation, and bony erosion (Maniu et al., 2014; Kuo et al., 2015). Biofilm formation and aberrant gene expression are also associated with cholesteatomas (Chole and Faddis, 2002; Maniu et al., 2014; Kuo et al., 2015; Baschal et al., 2019).

Pseudomonas aeruginosa and *Staphylococcus aureus* are the most prevalent bacteria cultured from suppurative COM and cholesteatomatous OM (Ricciardiello et al., 2009). In previous bacterial profiling between different patients, the relative abundances of *Alloiococcus, Haemophilus* and *Clostridiales* were increased in cholesteatomas vs. healthy ME, non-cholesteatomatous OM, or tympanosclerotic plaques (Neeff et al., 2016; Kalcioglu et al., 2018). In another recent study of untreated cholesteatoma, no differences in bacterial or fungal profiles were found between cholesteatoma and ME mucosa (Weiss et al., 2019). In these previous microbiota studies, no biodiversity or relative taxa abundance estimates were significant, whether when comparing by case-control status or by sample-types, and when correcting for multiple testing [e.g. false discovery rate (FDR)] (Neeff et al., 2016; Minami et al., 2017; Kalcioglu et al., 2018; Weiss et al., 2019). On the other hand, in other studies up to 36% of cholesteatomas were reported as positive for human papillomavirus (HPV), a virus that can induce aggressive growth of tissue lesions such as papillomas or cancers (Bergmann et al., 1994; Franz et al., 2007).

In this study, we collected ME samples from Filipino patients undergoing tympanomastoidectomy for COM and submitted them for [1] broad-range bacterial 16S rRNA gene amplification and sequencing and [2] PCR screening for HPV and common viral otopathogens. Our goal was to determine if the microbiota of cholesteatoma tissue is different from other ME samples within the context of long-standing, insufficiently treated suppurative COM.

MATERIALS AND METHODS

Study Design

Prior to initiation, this study was approved by the University of the Philippines Manila Research Ethics Board (UPMREB 2015-238-01) and the Colorado Multiple Institutional Review Board (protocols 16-1525, 16-2673, and 17-1679). Informed consent was obtained from all study participants, including parents of minors. Patients who were diagnosed to have COM with or without cholesteatoma and scheduled for tympanomastoidectomy surgery at the Philippine General Hospital were recruited for study. Preparation for surgery was done according to standard procedures. No antiseptic or antibiotic wash or powder was directly applied to the middle ear before or during surgery. During surgery, different types of ME samples, namely cholesteatoma tissue, ME discharge (obtained by sterile Puritan applicator swab), ME mucosal tissue, and granulation tissue, were collected per patient. All tissues and discharge were collected by the surgeon upon identification under the operating microscope. Each ME sample was placed separately in an Oragene P117 kit (DNA Genotek, Ottawa, Ontario, Canada), which preserved microbial DNA/RNA while shipping to Colorado. Microbial DNA and RNA were isolated from all ME samples using the MasterPure Complete DNA and RNA Purification Kit (Lucigen, Middleton, WI, USA) for DNA samples and the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) for RNA samples, respectively.

Microbiota Profiling

Bacterial 16S rRNA genes were amplified from DNA samples using primers specific for the V1V2 region (27FYM 5'-AGAGTTTGATYMTGGCTCAG and 338R 5'-TGCTGCCT CCCGTAGGAGT), as previously described (Santos-Cortez et al., 2018; Bootpetch et al., 2020; Frank et al., 2020). All work was performed in either a biosafety level 2 hood or a HEPA-filtered PCR hood following 15 minutes of ultraviolet irradiation. Three to four negative reagent controls were included in each batch of DNA extraction, PCR, and sequencing to detect contaminants. PCR amplicons were normalized and pooled using a SequalPrep Normalization Plate Kit (Invitrogen/Thermo Fisher Scientific, Waltham, MA, USA) and pools quantified using a Qubit® 2.0 Fluorometer (Invitrogen). Paired-end sequencing was conducted on the Illumina Miseq platform using the 600 cycle v3 kit (San Diego, CA, USA). Illumina Miseq paired-end reads were aligned to human reference genome hg19 with bowtie2 and matching sequences discarded (Langmead and Salzberg, 2012). The remaining non-human paired-end sequences were demultiplexed then assembled using phrap (Ewing and Green, 1998; Ewing et al., 1998). Pairs that did not assemble were discarded. Assembled sequence ends were trimmed over a moving window of 5 nucleotides until average quality was met or exceeded 20. Trimmed sequences with more than 1 ambiguity or shorter than 250 nt were discarded. Potential chimeras identified with Uchime (usearch6.0.203_i86linux32) (Edgar et al., 2011) using the Schloss (Schloss and Westcott, 2011) Silva reference sequences were removed from subsequent analyses. Assembled sequences were aligned and classified with SINA (1.3.0-r23838) using the 418,497 bacterial sequences in Silva 115NR99 as reference configured to yield the Silva taxonomy (Pruesse et al., 2007; Pruesse et al., 2012; Quast et al., 2013). Taxonomic assignment by SINA used the lowest common ancestor approach with default parameters. Operational taxonomic units (OTUs) were produced by binning sequences with identical taxonomic assignments. This process generated a median of 71,960 sequence/sample (IQR: 17391-107146) for 103 ME samples. In contrast, 12 negative control samples yielded a median of 21.5 (IQR: 17-184.3) sequences per control sample.

Statistical Analysis

The software packages R v4.1.0 (Team, 2019) and Explicet v2.10.5 (Robertson et al., 2013) were used to analyze and visualize data. For microbiome analysis, differences in overall composition (i.e., beta-diversity) were assessed through permutational ANOVA [PERMANOVA (Anderson et al., 2011; Oksanen et al., 2019)] with the Bray-Curtis dissimilarity index. PERMANOVA p-values were inferred through 10^6 label permutations and FDR-corrected for multiple comparisons (Benjamini and Hochberg, 1995) when multiple pairwise tests were performed. Alpha-diversity indices (i.e., S_{obs}, Shannon H, Shannon H/Hmax) were assessed by linear regression modeling; p-values were FDR-adjusted when multiple pairwise tests were performed. PERMANOVA and linear regression were performed on the main outcome (sample-type) both as one-way tests and adjusting for covariates (patient ID, age, sex, cholesteatoma diagnosis, or medications) as noted in the text. Individual taxa differing between treatment groups were identified using the ANOVA-like differential expression (ALDEx2) R package (Fernandes et al., 2013; Fernandes et al., 2014). The distribution of taxa in each sequence library was estimated through 1000 Dirichlet Monte Carlo re-samplings of sequence count data. To account for the compositional nature of microbiome sequence data, datasets were then subjected to a center log-ratio transformation with all features used as the denominator. The aldex.glm module was used to assess between-group differences in taxon relative abundances while adjusting for patient covariates; both nominal p-values and FDR-corrected p-values (Benjamini and Hochberg, 1995) were inferred using this approach, as noted in the text and figures.

Viral Profiling

To determine whether viruses play a role in cholesteatoma, the ME samples were screened for presence of HPV DNA by qPCR using the PowerUp SYBR Green (Applied Biosystems/Thermo Fisher) reagent and a Bio-Rad instrument (Hercules, CA, USA). We also screened for eight common respiratory viruses that are known to be associated with OM, namely: two DNA viruses – human adenovirus (HAdV) and human bocavirus (HBoV); and six RNA viruses – rhinovirus (RV), enterovirus (EV), respiratory syncytial virus (RSV), coronavirus-229E/NL63/OC43 (hCoV), human metapneumovirus (hMPV), influenza A/B or InfA/InfB [**Table S1**; (Loeffelholz et al., 2011; Nokso-Koivisto et al., 2015)]. Microbial RNA was converted into cDNA using the Superscript IV Reverse Transcriptase (Thermo Fisher) with random hexamer primers. Control plasmids matched to specific

primers for each virus were used as positive control. Additionally, beta-actin was used as positive control and was successfully amplified in every sample.

RESULTS

Patient Characteristics

Microbial DNA was isolated from 118 specimens from 54 patients enrolled in this study. Out of 118 microbial DNA samples, bacteria were profiled in 103 (87.3%) samples from 53 patients by broad-range 16S rRNA gene PCR amplification and Illumina amplicon sequencing of the V1V2 variable region (**Table 1**). The Goods coverage index was ≥98% for each sample, indicating excellent depth of sequence coverage. The cohort with sequence data was 49% male and had a mean age of 36.8 years old (SD: ± 14.7 years; age range: 15-73 years). Sequence data was available from cholesteatoma tissue ("Chol"; N = 25), granulation tissue ("Gran"; N = 34), ME mucosal tissue ("MEMuc"; N = 17), and ME discharge ("MEDisc"; N = 27; **Table 1**). Almost all patients were diagnosed with suppurative COM, except for two with chronic adhesive OM. Thirty-six patients had cholesteatoma (67.9%, **Table 1**). In addition to hearing loss in all patients, additional complications due to OM were found in 13 (24.5%) patients, including one with brain abscess, six with post-auricular soft tissue abscess, and six patients with dizziness and/or damage to the semicircular canal(s). In one severe case, the patient had acute meningitis, facial paralysis and labyrinthitis. Nine (17%) patients had a previous history of tympanomastoid surgery for COM (i.e. 2-27 years prior to current surgery); of these nine, five patients had cholesteatoma on the same ear while one patient had the previous surgery on the opposite ear.

Microbial Diversity of Middle Ear Specimens

Initial univariable PERMANOVA tests found significant associations between overall microbiota composition (i.e., beta-diversity) and sample-type (p=0.006; **Figure 1A**), age (p=2.9e-05; **Figure 1B**), cholesteatoma diagnosis (p=1e-06; **Figure 1C**), quinolone use (p=0.0006; **Figure 1D**) and use of other broad-spectrum antibiotics (p=0.03; data not shown). The effects of these variables on ME microbiota were visualized through both principal component plots (left panels, **Figure 1**) and bar charts (right panels, **Figure 1**), the latter of which also show pairwise differences between different variable levels. In contrast, no differences in beta-diversity were observed in association with biological sex (p=0.20) or cephalosporin use (p=0.37). Only a few patients were positive for [a] use of local steroid as a component of antibiotic otic drops and [b] for *FUT2* or *SPINK5* variants (**Table 1**), which we have previously shown to be determinants of ME microbiota (Santos-Cortez et al., 2018; Frank et al., 2020; Elling et al., 2022); consequently, steroid use, *FUT2* genotype, and *SPINK5* genotype were excluded from all analyses.

Alpha-diversity indices were also influenced by sample-type, cholesteatoma diagnosis, and quinolone use in univariable analyses (**Figure 2**). Age had little, if any, effect on alpha-diversity. In general, microbial diversity was increased in MEMuc samples compared to Chol and MEDisc samples as measured by richness (Chao1) and Shannon diversity (H) indices, while Gran samples were intermediate between these extremes. Cholesteatoma diagnosis was associated with decreased richness (p=0.006), whereas patients treated with quinolone antibiotics exhibited significantly increased richness (p=0.04), evenness (p=0.0009), and Shannon diversity (p=3.0e-05).

Regardless of the sample-type collected, 16S rRNA gene sequence profiles (**Figure 1**) were dominated by a diverse set of bacteria belonging to the phyla Actinobacteria (e.g., *Corynebacterium*), Bacteroidetes (e.g., *Prevotella*), Firmicutes (e.g., *Bacillus, Streptococcus*), and Proteobacteria (e.g., *Stenotrophomonas*). Of the 157 taxa identified in the set of Chol samples, 18 taxa were observed in ≥75% of the Chol samples (**Figure S1**, **Table S2**), defining a core microbiota for cholesteatomas. Fourteen of these taxa were observed at ≥75% prevalence in all four sample-types (**Figure S1**), while 30 taxa were observed at ≥50% prevalence in all sample-types (data not shown). The highly prevalent, shared taxa consisted of a variety of commensal and potentially pathogenic bacteria from genera such as *Corynebacterium, Propionibacterium, Prevotella, Staphylococcus, Streptococcus, Haemophilus*, and *Pseudomonas.*

We next compared the relative abundances of individual taxa across sample-types and age quartiles (**Figure S2**). Consistent with the beta-diversity results, few taxa were differentially abundant between Chol and the other sample-types, even when relatively lax criteria were used to designate features of

TABLE 1 | Clinical information and collected middle ear (ME) samples from 53 Filipino patients with chronic otitis media.

Variable	Cholesteatoma Tissue	Granulation Tissue	ME Mucosa	ME Discharge	All Samples	All Patients
N	25	34	17	27	103	53
Age (mean ± SD; years)	32.4 ± 16.6	34.1 ± 12.9	35.2 ± 12.6	35.1 ± 17.1	34.1 ± 14.8	36.8 ± 14.7
Sex (%male)	13/25 (52.0)	17/34 (50.0)	9/17 (52.9)	14/27 (51.9)	53/103 (51.5)	26/53 (49.1)
Cholesteatoma (%)	25/25 (100.0)	22/34 (64.7)	11/17 (64.7)	19/27 (70.4)	77/103 (74.8)	36/53 (67.9)
Quinolone (%)	10/22 (45.5)	12/28 (42.9)	7/15 (46.7)	12/24 (50.0)	41/89 (46.1)	19/46 (41.3)
Cephalosporin (%)	18/22 (81.8)	26/28 (92.9)	12/15 (80.0)	20/24 (83.3)	76/89 (85.4)	40/46 (87.0)
Other broad-spectrum antibiotics (%)	4/22 (18.2)	4/28 (14.3)	3/15 (20.0)	4/24 (16.7)	15/89 (16.9)	5/46 (10.9)
Steroid (%)	2/22 (9.1)	1/28 (3.6)	0/15 (0)	2/24 (8.3)	5/89 (5.6)	2/46 (4.3)
FUT2 variant (%)	1/18 (5.6)	2/24 (8.3)	1/8 (12.5)	1/20 (5.0)	5/70 (7.1)	2/39 (5.1)
SPINK5 variant (%)	2/17 (11.8)	1/23 (4.3)	1/11 (9.1)	2/21 (9.5)	6/72 (8.3)	2/38 (5.3)

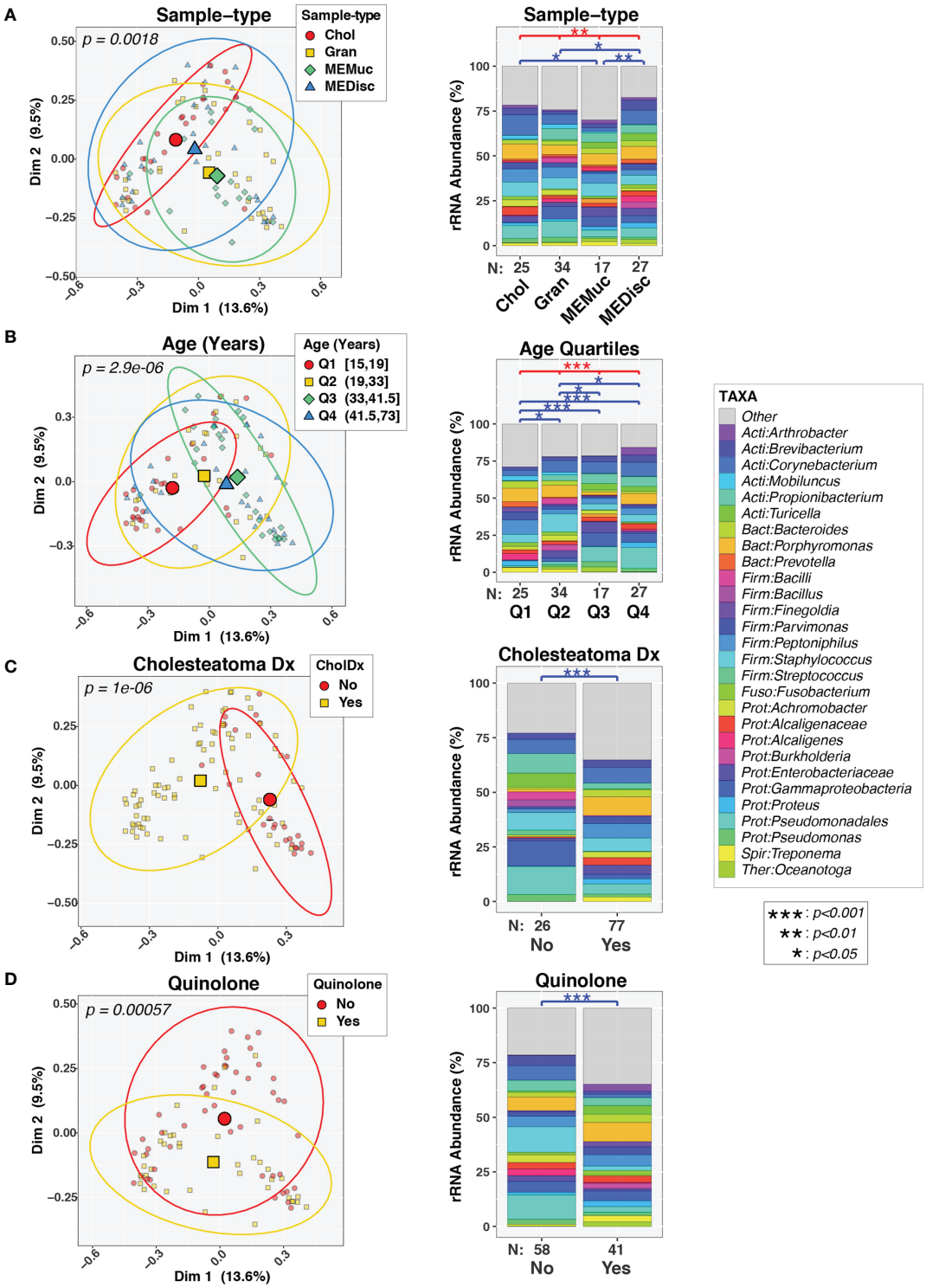

FIGURE 1 | Variability in composition of middle ear microbiota. Pairs of plots show beta-diversity across sample-types **(A)**, age quartiles **(B)**, cholesteatoma diagnosis **(C)**, or quinolone use **(D)**. The left column displays principal coordinates analysis (PCoA) plots of the first two PC axes, color- and symbol-coded by the indicated variables. *Smaller symbols* designate individual subjects while *larger symbols* represent group means along both PC axes. Ellipses designate 90% confidence level for a multivariate t-distribution. The right column displays barcharts summarizing the mean relative abundances of predominant taxa (>2%RA) in each group; rarer taxa are grouped into the "Other" category. Results of PERMANOVA tests are indicated above barcharts. *Red lines/symbols* indicate p-values across all 4 groups. *Blue lines/symbols* indicate FDR-corrected p-values for pairwise comparisons.

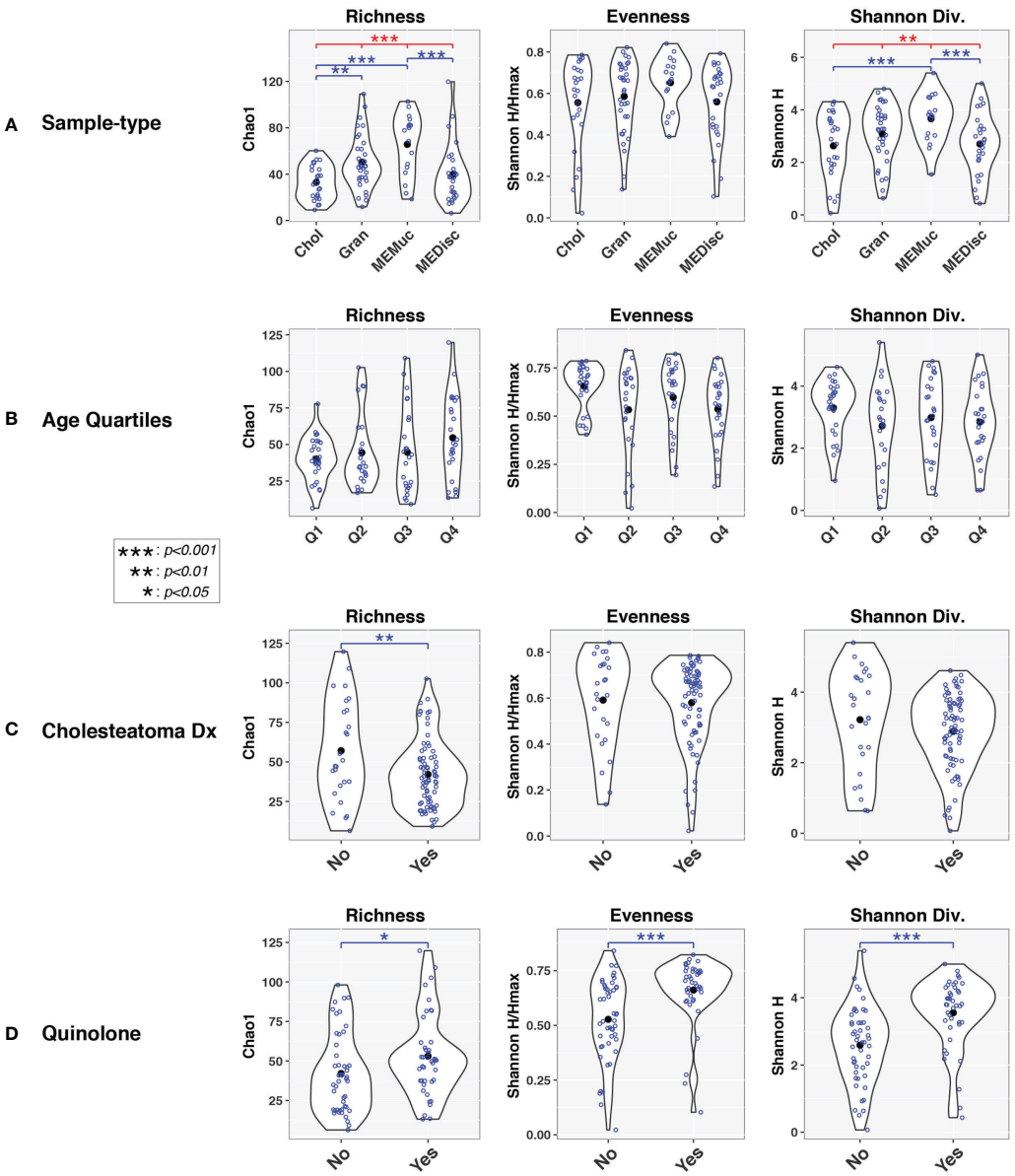

FIGURE 2 | Alpha-diversity indices vary by sample-type and age. *Violin plots* show within-group distributions of values across sample-types **(A)**, age quartiles **(B)**, cholesteatoma diagnosis **(C)**, or quinolone use **(D)**. *Open blue circles* indicate individual samples while *filled black circles* designate group means. Results of linear mixed-effects tests are indicated above violin plots. *Red lines/symbols* indicate p-values across all four groups. *Blue lines/symbols* indicate p-values for pairwise comparisons with false discovery rate correction.

interest (nominal-p≤0.1, fold-change≥1.5). No taxa had FDR-corrected p-values≤0.1 in pairwise comparisons of sample-types. In contrast, even when using more stringent cutoffs (i.e., FDR-corrected-p≤0.05, fold-change≥1.5), numerous taxa differed in relative abundance between subjects in the first (Q1) and fourth (Q4) age quartiles (**Figure 3**). Younger age (Q1) was associated with elevated levels of diverse Firmicutes, along with *Mycoplasma* and *Prevotella*, while older subjects (Q4) harbored elevated levels of Proteobacteria (*e.g.*, pseudomonads, comamonads) and *Propionibacterium*.

In a multi-variable PERMANOVA model (**Table S3B**), age (p=0.02), cholesteatoma diagnosis (p=3.6e-05), and quinolone use (p=0.0009) remained significant after adjusting for all other variables, while sample-type approached significance (p=0.09). Use of other broad-spectrum antibiotics was no longer significant (p=0.34) in this analysis (data not shown). Based on these findings, the following sections examine the complex interrelationships between cholesteatoma diagnosis, quinolone use, sample-type, and age in influencing ME microbiota. Although sample-type had less apparent effect than the other

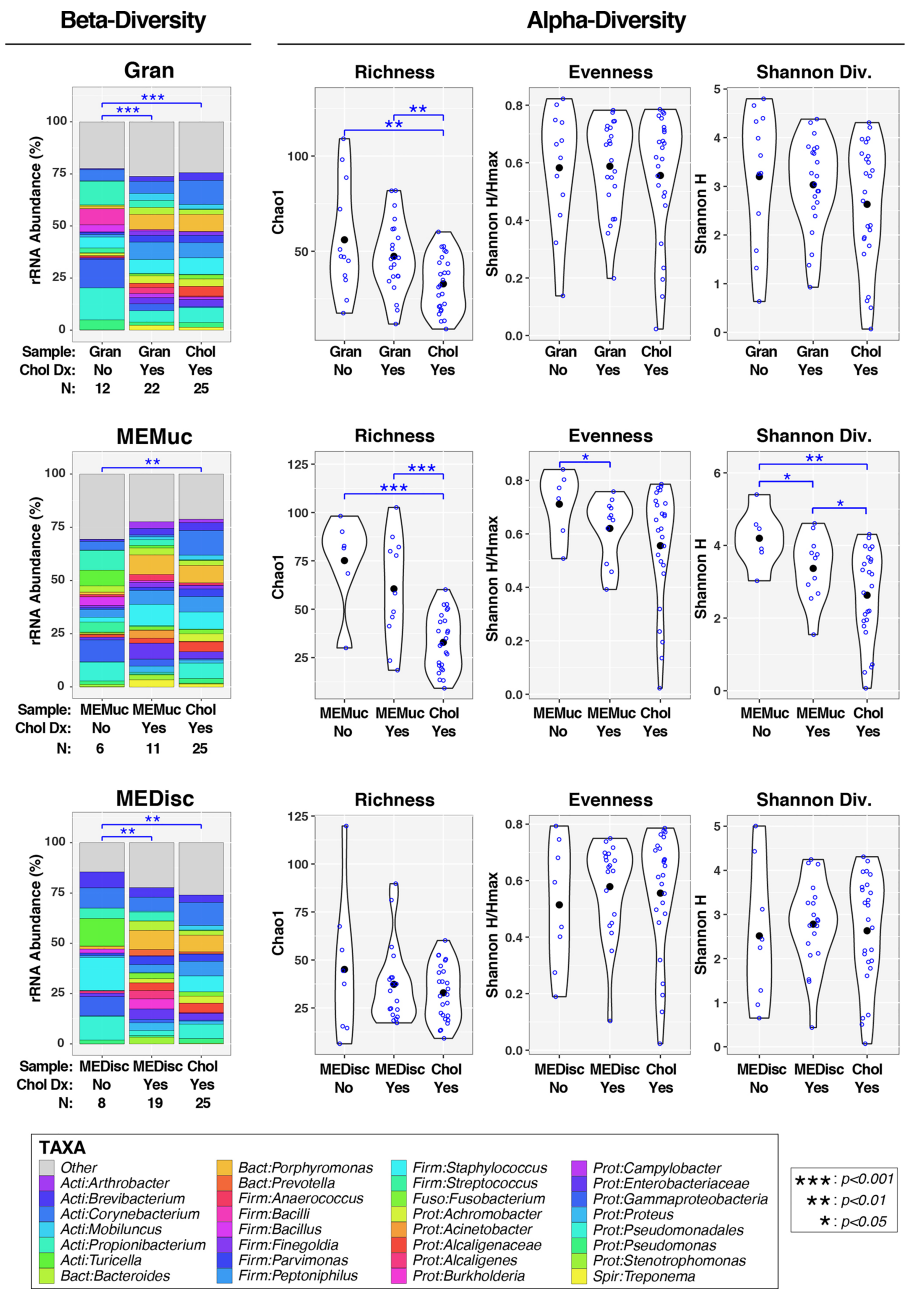

FIGURE 3 | Association of cholesteatoma diagnosis with middle ear microbiota. Each panel compares microbiota for either Gran, MEMuc, or MEDisc samples between patients without and with cholesteatoma diagnosis; for comparison, each panel includes Chol samples from patients with cholesteatoma. The left column (under the heading "Beta-Diversity") displays barcharts summarizing the mean relative abundances of predominant taxa (>2%RA) in each comparison group; rarer taxa are grouped into the "Other" category. Results of PERMANOVA tests (adjusted for age and quinolone use) are indicated above barcharts. *Blue lines/symbols* indicate FDR-corrected p-values for pairwise comparisons. The right columns (under the heading "Alpha-Diversity") display violin plots for each comparison group. *Open blue circles* indicate individual samples while *filled black circles* designate group means. Results of linear mixed-effects tests (adjusted for age and quinolone use) are indicated above violin plots. *Blue lines/symbols* indicate p-values for pairwise comparisons with false discovery rate correction.

factors in driving differences between ME microbiotas, we nevertheless stratified analyses by sample-type. This was because the practical goals of this study were to determine 1) whether choice of sample-type influences microbiome results and 2) whether the effects of medications or other factors on ME microbiota differ by sample-type. Indeed, age, cholesteatoma diagnosis, and quinolone use all varied in their associations with ME microbiota across sample-types (**Table S3**).

Effects of Cholesteatoma on ME Microbiota

We next assessed how cholesteatoma diagnosis affected microbiota in the different ME sample-types. In addition, we sought to determine whether microbiota of cholesteatoma tissue samples (Chol) differed from the other sample-types (Gran, MEMuc, MEDisc) when analyses were stratified by cholesteatoma diagnosis. In PERMANOVA analyses of beta-diversity that adjusted for age and quinolone use (left panels, **Figure 3**), both Gran (p=0.0008) and MEDisc (p=0.002) samples differed significantly between patients with and without cholesteatoma diagnosis, while no differences were observed in MEMuc samples (p=0.17). Similarly, Chol samples (by default from patients with cholesteatoma diagnosis) also differed significantly from Gran (p=9e-06), MEMuc (p=0.002), and MEDisc (p=0.002) samples from patients without cholesteatoma diagnosis. In contrast, Chol samples did not differ significantly from Gran, MEMuc, or MEDisc subjects with cholesteatoma diagnosis.

In analyses of alpha-diversity (middle and right panels, **Figure 3**), subjects with and without cholesteatoma diagnosis exhibited few differences in Gran, MEMuc, or MEDisc samples; only MEMuc samples from cholesteatoma patients had lower evenness (p=0.05) and Shannon Diversity (p=0.03) compared with non-cholesteatoma patients. Regardless of cholesteatoma diagnosis, Chol tissue samples exhibited decreased richness compared to both Gran and MEMuc samples, while Shannon Diversity was also lower in Chol tissues compared with MEMuc.

Next, we sought to identify individual taxa differing by cholesteatoma diagnosis and sample-type (**Figures 4**, **S3**). Likely due to the relatively small number of subjects per comparison group, few taxa differed significantly in relative abundance following FDR correction of p-values; consequently, we report exploratory results using less stringent cutoffs (nominal-p≤0.05, fold-change≥1.5). Multiple taxa differed between subjects with and without cholesteatoma diagnosis in both Gran and MEDisc comparisons (**Figure 4**), whereas only one taxon (*Leptotrichia*) met the cutoff criteria for MEMuc. Cholesteatoma diagnosis was associated with increased abundances of the genera *Campylobacter, Peptococcus, Porphyromonas*, and *Prevotella* in both Gran and MEDisc samples. Conversely, the genera *Bacillus* and *Propionibacterium* were enriched in both Gran and MEDisc samples of subjects without cholesteatoma. Finally, among subjects with cholesteatoma diagnosis, only three taxa differed significantly between Chol tissue and MEMuc, while no taxa were differentially abundant between Chol tissues and either Gran or MEDisc (**Figure S3**).

Effects of Quinolone Antibiotic Use on ME Microbiota

Our initial analyses (**Figure 1**) indicated that quinolone use was significantly and independently associated with altered ME microbiota (beta-diversity) after adjusting for other covariates (age, cholesteatoma diagnosis, sample-type). The effects of quinolone use were observed in subjects both with [p(adjusted) =0.001; adjusted for age and sample-type] and without [p (adjusted)= 0.01; adjusted for age and sample-type] cholesteatoma diagnosis (**Figures S4B**). PCoA analysis (**Figure S4A**) suggested that quinolone use magnified the differences in microbiota observed between subjects with and without cholesteatoma diagnosis. Fewer taxa were differentially abundant in subjects without cholesteatoma (n=3 taxa) than those with cholesteatoma (n=25 taxa), perhaps reflecting the differences in sample size between these groups (**Figures S4C, D**). In cholesteatoma patients, quinolones reduced the abundances of *Corynebacterium, Staphylococcus*, and diverse Proteobacteria (e.g., *Haemophilus, Enterobacter*), with concomitant increases in Firmicutes (e.g., *Peptococcus, Enterococcus, Lachnospiraceae*)

Analyses stratified by sample-type (**Figure S5**, **Table S3**) documented that quinolone use had significant effects in Gran [p(adjusted)=0.055] and MEMuc samples [p(adjusted)=0.02], respectively, adjusted for age and cholesteatoma diagnosis), but not Chol [p(adjusted)=0.13] or MEDisc [p(adjusted)=0.24] samples. PCoA plots (**Figure S5A**) provided further evidence that quinolone use had a larger effect on ME microbiota than sample-type. Finally, despite the significant differences in beta-diversity (**Figure S5B**), few taxa were differentially abundant in association with quinolone use in either Gran (n=5 taxa) or MEMuc (n=2 taxa) samples.

No Viruses Identified in ME Tissues from Chronic OM Patients

For viral studies, 115 ME samples (23 cholesteatoma, 22 mucosal tissue, 35 granulation tissue, 35 discharge/swabs) from 45 patients with COM were screened for nine viruses. No viral presence was detected in any of the screened ME samples.

DISCUSSION

In this study, we examined the bacterial and viral microbiotas of cholesteatoma tissue and other ME specimens that were collected from Filipino patients undergoing tympanomastoidectomy surgery for long-standing COM. No viruses were identified in any ME sample, indicating that COM with or without cholesteatoma is primarily a bacterial disease. There was strong overlap in the bacteria found between sample-types whether from the same or across different patients (**Figure S1**). Considering that multiple samples were taken from a limited field at the same ME surgery for each ear, the similarity in community composition is somewhat expected. Nonetheless, we found that the cholesteatoma tissues had moderately less biodiversity than did ME mucosal samples as indicated by richness and Shannon diversity indices (**Figure 2**). Likewise, ME discharge had less alpha-diversity than ME mucosal samples, while granulation tissue had higher richness than cholesteatoma samples (**Figure 2**).

Overall, our results indicate that factors such as quinolone use and cholesteatoma diagnosis had much more substantial effects on ME microbiota composition than did the particular sample-type collected (**Figures 1**, **3**, **S4, S5**). For example, granulation tissues and ME discharge from patients with cholesteatoma diagnosis differed substantially from those with suppurative

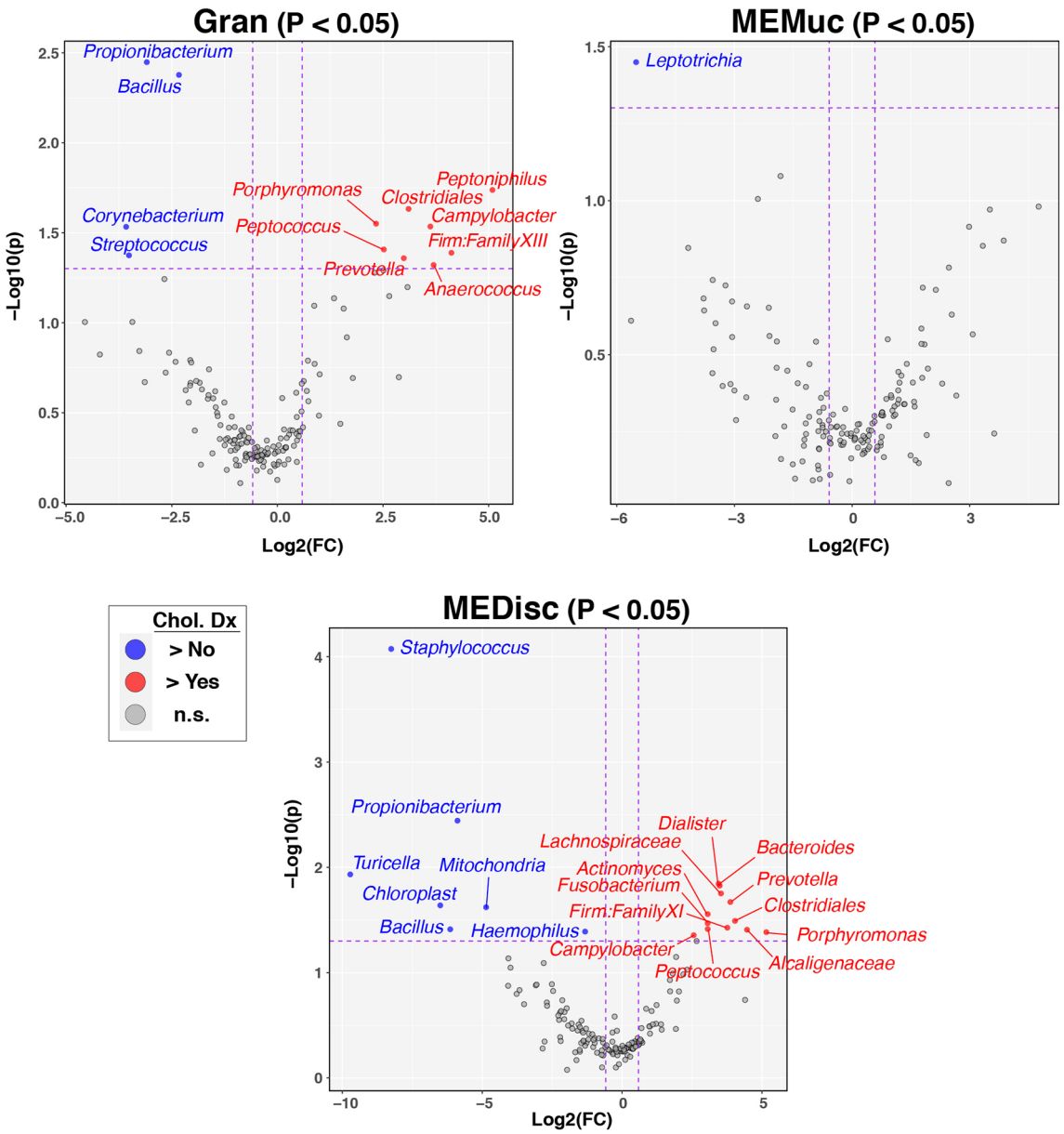

FIGURE 4 | Individual taxa differing between patients with and without cholesteatoma diagnosis. Between-group differences in the relative abundance of individual bacterial taxa were identified using the ANOVA-like differential expression (ALDEx2) test, which considers the compositional nature of microbiota datasets. The three panels show results stratified by sample-type (Gran, MEMuc, or MEDisc). *Vertical dashed lines* indicate fold-change cutoffs≥1.5. *Horizontal dashed lines* show p-value cutoffs for comparisons (nominal $p \leq 0.05$). *Blue circles in the upper left quadrants* denote taxa enriched in patients without cholesteatoma diagnosis while *red circles in the upper right quadrants* denote taxa enriched in patients with cholesteatoma diagnosis.

COM without cholesteatoma (**Figure 3**). Overall, these findings suggest that the microbiota of middle ears with cholesteatoma have a different profile than middle ears with suppurative COM only, which may necessitate a change in approach to treatment.

Differences in biodiversity also were documented across ages of patients with COM (**Figures 1**, **2**), with greater diversity in patients >33 years old compared to teenagers (**Figure 1**; Q1 vs Q3 and Q4). To place this finding within context, the Philippine General Hospital serves a large, indigent population from different regions of the country (Santos-Cortez et al., 2007), many of whom may not have had access to specialists in their locality. Thus, the differences by age bracket may, in fact, be reflective of the duration of ME infection, rather than chronological age *per se*. If the study participants did have some form of antibiotic or surgical treatment prior to sample collection, a large majority is assumed to have received sporadic

treatment since the initial infection. By usual protocol, at initial consult, the patients are prescribed antibiotic otic drops (e.g., Ofloxacin, Neomycin-Polymixin B with or without steroids). However due to limited facilities and long waitlists, it may take months from the initial consult before surgery is performed. This situation is common among populations with limited access to otologic health care, particularly in lower-income countries or marginalized groups. Taken together, the differences that we observed across age quartiles are likely to reflect [1] long-standing, untreated or inadequately treated OM, and [2] recent antibiotic use on top of a potentially long history of antibiotic prescription (local or oral) and high likelihood of antibiotic resistance (Xu et al., 2020).

In addition to quinolone, other antibiotics may also have indirectly influenced our findings on ME bacterial profiles. Notably *Pseudomonas*, which is a common target of antibiotic otic drops, had greater relative abundance in older patients (**Figure S2**). This finding may indicate antibiotic resistance and/or extensive formation of treatment-resistant biofilms due to long-standing infection. On the other hand, the most abundant taxa in cholesteatoma tissues were *Corynebacterium*, *Porphyromonas*, and *Staphylococcus*, with 11.5%, 8.3%, and 8.0% mean abundances (**Table S1**, **Figure 1**). *Staphylococcus* is often isolated from COM samples, but may be resistant to penicillin, macrolides or quinolones (Xu et al., 2020).

Corynebacterium is also commonly identified in COM but is typically viewed as a commensal, even potentially otoprotective in acute OM (Lappan et al., 2018; Jorissen et al., 2021). In contrast, *Porphyromonas*, *Fusobacterium* and *Campylobacter* (**Figure 1**, **Figure 3**) are less commonly isolated from patients with COM or cholesteatoma (Brook, 1995; Yusuf et al., 2015). *Porphyromonas* and *Fusobacterium* are more common in other anaerobic infections of the head and neck, such as within the oral cavity, oropharynx, and sinuses (Brook, 2011; Yusuf et al., 2015). However, recent 16S rRNA sequencing has identified these bacteria more often in COM cases, indicating the utility of sequencing towards greater understanding of the microbial communities in COM (Santos-Cortez et al., 2016; Johnston et al., 2019). Similar to anaerobic infections of the sinuses and head and neck tissues, the presence of *Porphyromonas* or *Fusobacterium* is likely due to seeding of necrotic or damaged tissue by anaerobic bacterial pathogens after failure of antibiotic treatment (Brook, 2011). These anaerobes may also facilitate biofilm formation (Wang et al., 2018). Identification of these bacteria in cholesteatoma samples that may not be reached by and/or are resistant to the usual otic drops suggest an important opportunity to administer intravenous or oral antibiotics with proper bacterial coverage (e.g., clindamycin, metronidazole, chloramphenicol) immediately after cholesteatoma removal. When the post-surgical ear dressing or packing is removed, instillation of antibiotic drops according to culture and sensitivity test results will help minimize risk of COM recurrence. Local information on antimicrobial susceptibility patterns, which may vary considerably within a few years (del Rosario et al., 1990; Abes et al., 1998; Ayson et al., 2006; Suzuki et al., 2015; Suzuki et al., 2020), will need to be updated regularly and is necessary to efficiently treat COM and cholesteatoma, particularly for the patients described here who lack regular access to health care. Also at the local level, the range of ototopical prescriptions might need to be expanded in order to include antibiotics with proper coverage of target bacteria for post-surgical treatment of cholesteatoma, e.g. chloramphenicol, clindamycin, bacitracin, gramicidin, sulphacetamide, or rifampicin otic drops (van Dongen et al., 2015; Brennan-Jones et al., 2020; de Jong et al., 2020).

There are several limitations of this study. The limited sample size allowed us to identify significant differences in alpha- and beta-diversity but may have been too limited to comprehensively identify differentially abundant taxa, particularly after FDR-correction. The differences in the surgical findings per patient also did not allow us to collect all four sample-types from each patient and reduced the number of samples for paired analyses. Because of restricted access to the ME and to the patients included in this study, all analyses are cross-sectional rather than longitudinal. Lastly, short-read 16S rRNA sequencing limits phylogenetic resolution and, therefore, we took the conservative approach of classifying sequences only to the genus-level. Furthermore, microorganisms such as archaea, fungi, and viruses were not targeted for broad-range sequencing. If feasible, given the low microbial biomasses observed in these specimens, future metagenomic sequence analyses will increase the phylogenetic depth and breadth of these studies. Bacterial species/strain identification will be more helpful in future patients whose ME samples may be submitted for culture and testing of antibiotic sensitivity for the taxa identified in this study.

While having a control population with no history of OM is desirable, we purposefully did not aspire to collect samples from healthy controls, (e.g., from patients undergoing cochlear implantation). There is ongoing debate whether the healthy ME is sterile (Jervis-Bardy et al., 2019; Lee et al., 2021). In our own pilot study of ME swabs from healthy ME of cochlear implant patients with no previous history of OM, no microbial DNA was isolated in 4 out of 5 patients. In one patient, DNA was amplified and submitted to sequencing; upon review of clinical history, this patient had a history of tympanomastoidectomy on the ear opposite to the sampling site, suggesting that the sampled ear was also seeded by previous ME infection, and thus not accurately described as a "healthy control". Our data therefore support the sterile state of the healthy ME (Jervis-Bardy et al., 2019), precluding any comparison with the microbiota of our patients with COM.

To summarize, in this cohort of patients with long-standing COM, we found that age, cholesteatoma diagnosis, and quinolone use were significantly and independently associated with ME bacterial profiles. Biodiversity was moderately lower in cholesteatoma and ME discharge compared to ME mucosal tissues. Cholesteatoma also had less biodiversity than granulation tissue samples. The findings from this study will be useful in guiding surgical or medical treatment protocols for cholesteatoma and COM, especially in settings with limited health care resources. On a more practical level, these findings indicate that the details of patients' medical histories and

demographic factors are more critical for studies of ME microbiota than the choice or availability of particular types of ME specimens.

DATA AVAILABILITY STATEMENT

Demultiplexed 16S rRNA paired-end sequence data and associated metadata were deposited in the NCBI Sequence Read Archive under Bioproject ID PRJNA748418.

ETHICS STATEMENT

The study was approved by the University of the Philippines Manila Research Ethics Board (UPMREB 2015-238-01) and the Colorado Multiple Institutional Review Board (protocols 16-1525, 16-2673, and 17-1679). All adult participants and parents of minors provided informed consent.

AUTHOR CONTRIBUTIONS

Conceptualization – RS-C. Methodology and resources – DF, JM, KV, JS, KD, AM, EY, HD, RP, JL, JA, BG, KM, CR, GI, AC, S-LL, ET, NS, TY, CC, and RS-C. Formal analysis and investigation – DF, TB, JK, CR, S-LL, and RSC. Original draft preparation – DF, TB, and RS-C. Manuscript editing – all authors. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.746428/full#supplementary-material

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Effect of Angiogenesis and Lymphangiogenesis in Diesel Exhaust Particles Inhalation in Mouse Model of LPS Induced Acute Otitis Media

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Lymphangiogenesis and angiogenesis might have significant involvement in the pathogenesis of otitis media with effusion. This study investigated the effect of diesel exhaust particles (DEP) on inflammation and lymphangiogenesis in a mouse model of acute otitis media (AOM). BALB/c mice were injected with LPS and exposed to 100 μg/m^3 DEP. The mice were divided into four groups: control (no stimulation), AOM, AOM + DEP, and DEP + AOM.

The effects of DEP inhalation pre- and post-DEP induction were estimated based on measurements of the auditory brainstem response, mRNA levels of lymphangiogenesis-related genes and cytokines, and histology of the middle ear. Cell viability of human middle ear epithelial cells decreased in a dose-response manner at 24 and 48 hours post-DEP exposure. DEP alone did not induce AOM. AOM-induced mice with pre- or post-DEP exposure showed thickened middle ear mucosa and increased expression of TNF-α and IL1-β mRNA levels compared to the control group, but increased serum IL-1β levels were not found in the AOM + Post DEP. The mRNA expression of TLR4, VEGFA, VEGFAC, and VEGFR3 was increased by pre-AOM DEP exposure.

The expression of VEFGA protein was stronger in the AOM + Post DEP group than in any other group. The expression of CD31 and CD45 markers in the mouse middle ear tissue was higher in the Pre DEP + AOM group than in the AOM group. This result implies that pre-exposure to DEP more strongly increases inflammation and lymphangiogenesis in a mouse model of acute otitis media.

Keywords: diesel exhaust particles, lipopolysaccharides, otitis media, lymphangiogenesis, angiogenesis

INTRODUCTION

Otitis media (OM) is one of the most common infections in young children. It is a common cause of physician visits and hearing loss in children (Rovers, 2008; Monasta et al., 2012). For those under 18 years of age, OM ranked the fifth highest in terms of direct medical spending in the United States. The costs related to OM are estimated at about $3.2 billion (Soni, 2014). OM disturbs the development of language and speech in children, and negatively affects the central auditory nervous system and auditory processing (Colella-Santos et al., 2019). In addition, it induces sleep disturbances, loss of appetite, and behavioral problems. OM deteriorates the quality of life of children and parents (Rovers, 2008; Homøe et al., 2020), and OM-related hearing loss is associated with poorer early academic achievement (Su et al., 2020).

Several studies have suggested that traffic-related air pollution is associated with the development of OM in children (Brauer et al., 2006; Kennedy et al., 2018). Kennedy et al. reported that exposure to air pollution from motor vehicles during the first year of life is associated with OM, bronchiolitis, and pneumonia (Kennedy et al., 2018). Brauer et al. showed that exposure to higher levels of PM2.5, elemental carbon, and NO_2, as traffic-related air pollutants, were associated with the development of OM in children younger than 2 years old (Brauer et al., 2006). Diesel exhaust particles (DEP) are considered to be the major component of traffic-related air pollution. A meta-analysis showed that the incidence of OM is correlated with higher levels of exposure to particulate matter (PM), which has similar characteristics to those of DEP. The correlation was strong, especially for younger children, and a stronger relationship was found for PM2.5 than for PM10 (Lee et al., 2020). *In vivo* and *in vitro* studies have demonstrated that DEP could contribute to the development of OM. DEP decreased cell viability in human middle ear epithelial cell lines and increased the inflammatory cytokines and mucin gene expression (Song et al., 2012). Growing evidence indicates that DEP is associated with the development of OM. However, when and how DEP exposure affects the middle ear has yet to be clarified.

It is known that DEP exposure induces angiogenesis. Xu et al. reported that DEP exposure significantly increased the expression of vascular endothelial growth factor (VEFG) and hypoxia-inducible factor (HIF)-1 alpha, while decreasing prolylhydroxylase (PHD) 2 expression. DEP exposure increased the vessel volume, blood flow, capillary tube formation, and sprouting *in vivo* and *in vitro* (Xu et al., 2009). Lymphangiogenesis, which refers to the process of lymphatic vessel formation from existing lymph vessels, has a significant role in cancer metastasis, organ graft rejection, and lymphedema (Yamakawa et al., 2018). Angiogenesis is an essential process of organ growth and repair that constitutes an important therapeutic target for cancer, cardiovascular disease, macular generation, and wound healing (Ferrara and Kerbel, 2005).

Lymphangiogenesis and angiogenesis play a significant role in the pathogenesis of OM with effusion (OME) (London and Gurgel, 2014). Jung et al. first detected VEGF in the middle ear fluid of OME patients and middle ear mucosa of chronic otitis media patients (Jung et al., 1999). VEGFR inhibitors have been found to moderate angiogenesis and lymphangiogenesis in the inflamed middle ear mucosa and improve OM (Cheeseman et al., 2011). Our previous study showed that micro-particles increased VEGFA expression in human middle epithelial cells, as increased VEGFA was detected in transcriptome analysis and validated by quantitative real-time polymerase chain reaction (qRT-PCR) (Song et al., 2013). Li et al. reported that VEGF plays a role in the acute phase of OME. The expression of HIF-1α mRNA was found to be correlated with the expression of VEGF and VEGFR-1 mRNA in an LPS-induced OME model (Li and Ye, 2021). It has been reported that the levels of VEGFA and TGF-β cytokines in adenoids with exudative otitis media were higher than in conditions of adenoid hypertrophy alone, prompting the suggestion that VEGFA and TGF-β could be used as additional and objective tests to confirm the clinical diagnosis of OME caused by a bacterial pathogen (Zelazowska-Rutkowska et al., 2020). In addition to its role in OM, VEGF plays an essential role in cochlear function and hearing, the latter of which is exemplified by its association with sensorineural hearing loss (London and Gurgel, 2014). However, the mechanism of DEP exposure-induced lymphangiogenesis and angiogenesis in OM has yet to be well defined. Therefore, the aim of this study was to investigate the effect of DEP on inflammation and lymphangiogenesis in a mouse model of acute OM (AOM). In addition, we exposed mice to DEP pre- and post-infection to investigate the preconditioning effect. To our best knowledge, this is the first study to compare the preconditioning effect of DEP in OM.

RESULTS

Exposure of HMEECs to DEP Resulted in Dose-Dependent Reductions in Cell Viability

Figure 1 shows the results when various concentrations of DEP (0, 20, 40, 80, 160, and 320 μg/mL) were added to HMEECs for 24 hr and 48 hr, and cell viability was estimated using the CCK-8 assay; When HMEECs were exposed to DEP, there was no significant difference in cell viability after 24 hr at 40 μg/mL, but a significant difference was found after DEP exposure for 24 and 48 hr at 80 μg/mL. Moreover, as time passed, cell viability significantly decreased compared to the control (both $p < 0.05$, **Figure 1**). The results showed that DEP induced gradual decreases in cell viability in a dose- and time-dependent manner. Thus, HMEECs were stimulated with a final DEP concentration of 80 μg/mL (**Figures 1**, **2A**).

Effects of DEP Inhalation on the Severity of Symptoms in a Mouse Model of AOM

The severity of symptoms in the mouse model of AOM was measured the day after the last exposure to DEP (**Figure 2B**). Histopathological examinations revealed typical pathological features of AOM in LPS-induced mice, and the DEP-exposed group exhibited more significant symptoms than the control mice. As shown in **Figure 3**, DEP exposure induced pathologic changes of the tympanic membrane, as assessed by otoscopy. Furthermore, the observed TEM images revealed the presence of

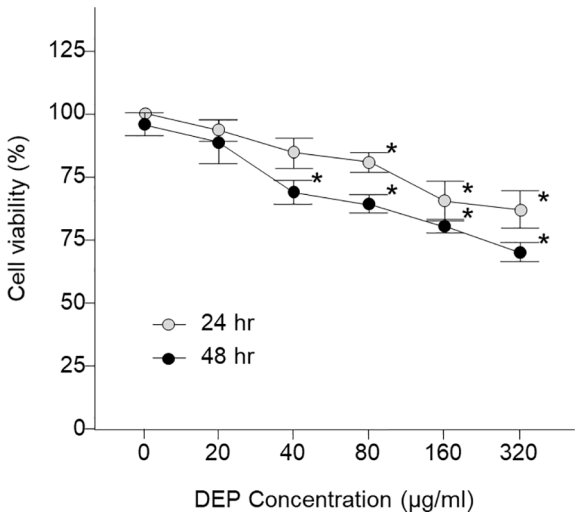

FIGURE 1 | Viability of LPS- and DEP-exposed HMEECs assessed by the CCK-8 assay and study design of the *in vitro* model. LPS and DEP exposure decreased cell viability in HMEECs. *p < 0.05 compared with the 24 hr and 48 hr normal control groups. LPS, lipopolysaccharide; DEP, diesel exhaust particles; HMEECs, human middle ear epithelial cells.

particles inside eustachian tube cells connected to middle ear tissues (**Figure 2C**). The tympanic membranes of mice in the control group showed clear structures, but this was not the case for the LPS- and DEP-treated groups (**Figure 3A**). The location shown in the mucosa in **Figure 3B**, near the eustachian tube and in a similar position in each group, was used to measure the mucosa thickness of the middle ear. The thickness of the mucosa significantly increased in the LPS- and DEP-treated groups compared to the control group. In particular, the thickness of the mucosa increased significantly in the Pre-DEP + AOM group relative to the AOM group ($p < 0.05$, **Figures 3B, C**). The LPS- and DEP-exposed mice exhibited a significant increase in the symptom score and had effusion and changes in the tympanic membrane structure compared with the control group. The effusion and structural changes of the tympanic membrane were exacerbated in the Pre-DEP + AOM group, but the symptom score showed no significant increase in the AOM group ($p < 0.05$, **Figure 3D**). Hearing in the LPS- and DEP-treated mice was evaluated by auditory brainstem response (ABR) threshold analysis. Before exposure, the Pre-DEP + AOM group showed that the ABR thresholds for click, 4 kHz, and 16 kHz increased after exposure. However, the increases were not statistically significant (**Figures 3E–G**). The average hearing levels in the control, AOM, and AOM + Post-DEP

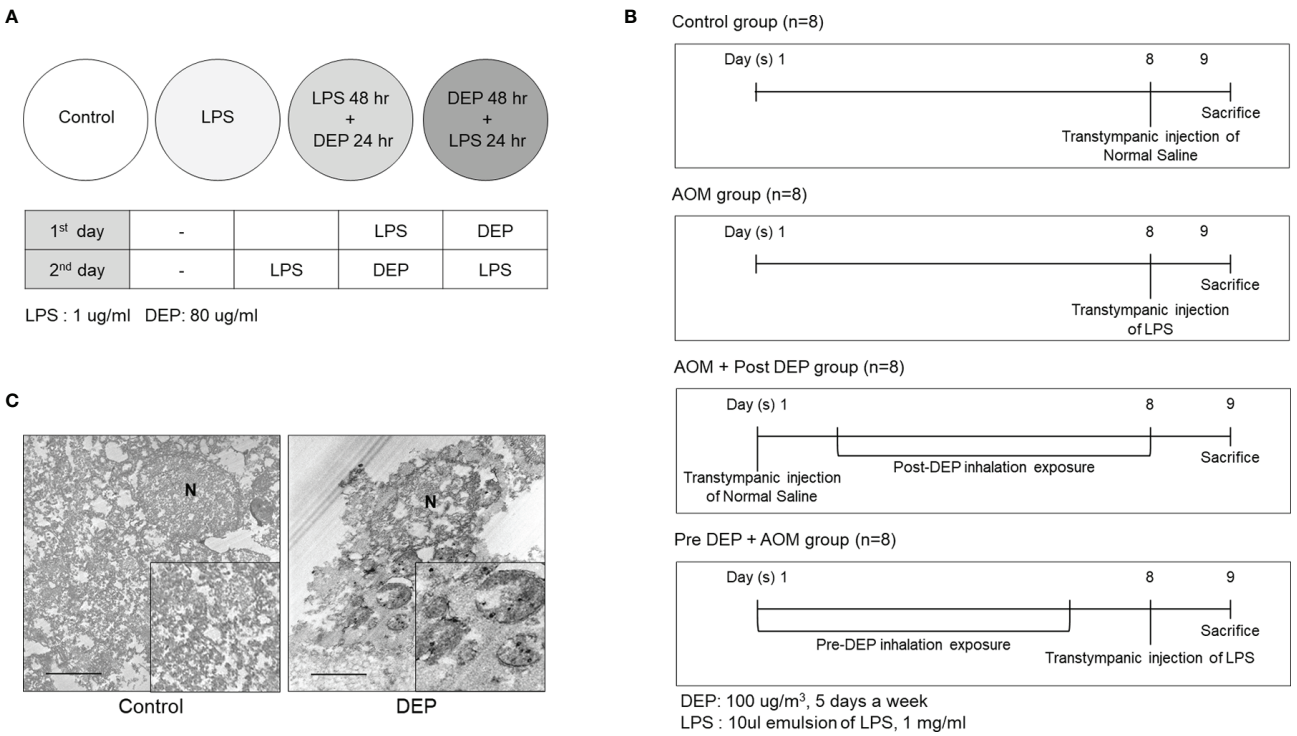

FIGURE 2 | Study design and DEP inhalation in a mouse model of acute otitis media induced by transtympanic LPS injection. **(A)** HMEECs were treated with a cytotoxic dose of LPS (1 μg/mL) and DEP (80 μg/mL) for 24 hr and 48 hr, or both in different sequences or simultaneously as indicated. **(B)** Experimental protocol for DEP exposure in a mouse model of AOM (n = 13 in each group) (DEP 100 μg/m^3; 10 μL emulsion of 1 mg/mL LPS). **(C)** The DEP were observed in transmission electron microscopy images inside eustachian tube cells connected to tissues only among mice that inhaled 100 μg/m^3 DEP for 1 hr a day for 5 days a week (N, nucleus; scale bar, 1 μm; magnification ×30,000). LPS, lipopolysaccharide; DEP, diesel exhaust particles; HMEECs, human middle ear epithelial cells; AOM, acute otitis media.

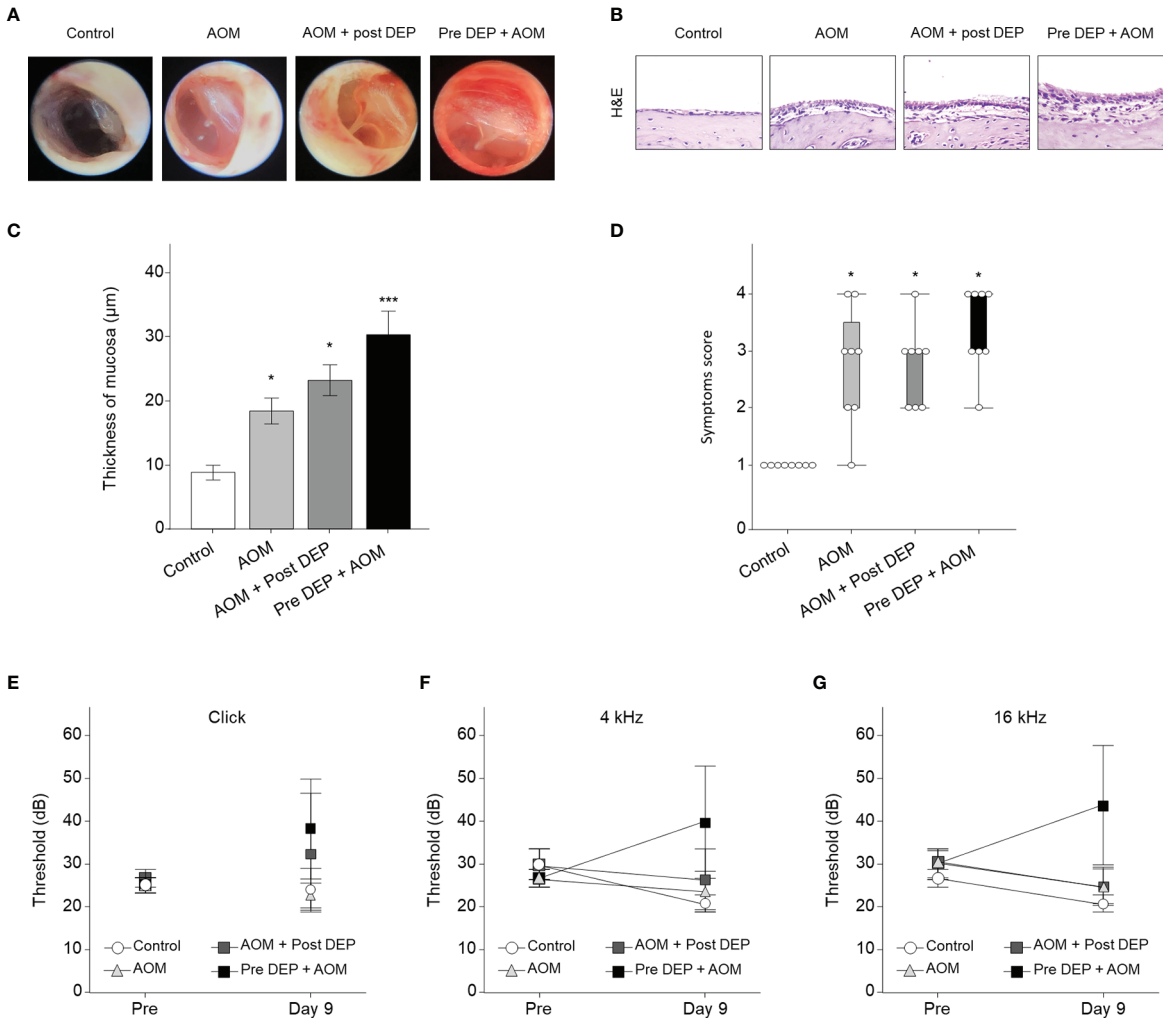

FIGURE 3 | Acute otitis media symptoms and H&E staining analysis of the middle ear in LPS + DEP exposure mouse. **(A)** Otoscope images of the tympanic membrane before sacrifice. **(B)** H&E staining of middle ear pathology. **(C)** The thickness of the middle ear epithelium is higher in the DEP + LPS exposure group. **(D)** The symptom score is higher in the acute otitis media group. **(E–G)** Auditory brainstem response threshold changes in the mouse model. The same location shown in each slide was chosen (H&E, magnification ×400). *p < 0.05, compared with the control group; ***p < 0.05 compared to the AOM group. LPS, lipopolysaccharide; DEP, diesel exhaust particles; H&E, hematoxylin and eosin; AOM, acute otitis media.

groups showed virtually no change, while a non-significant increase was observed in the Pre-DEP + AOM group (**Figures 3E–G**). The LPS- and DEP-exposed mice exhibited a higher symptom score than the control group, and this was exacerbated in the Pre-DEP + AOM group, but the differences were not significant. These results provide support for the possibility that DEP inhalation exacerbates signs and symptoms.

DEP Triggered IL-1β and TNF-α mRNA Expression in the Mouse Middle Ear and HMEECs

Cytokines play a key role in the pathogenesis of AOM-related mRNA expression of IL-1β and TNF-α in HMEECs and the mouse middle ear. The levels of mRNA encoding IL-1β and TNF-α changed following DEP treatment of HMEECs and the mouse middle ear, as assessed by qRT-PCR. The LPS-exposed groups exhibited greater IL-1β and TNF-α mRNA expression than the control group, and the expression of these cytokines was also higher in the DEP 48 hr + LPS 24 hr group and the Pre-DEP + AOM group than in the LPS 48 hr + DEP 24 hr and AOM + Post-DEP group ($p<0.05$, **Figures 4A–F**). Although the Pre-DEP + AOM group in the *in vivo* component of the present study showed significantly higher cytokine mRNA levels than the AOM group, no significant differences were found in comparisons with the LPS 24 hr group in the *in vitro* component of the study (**Figures 4A–D**). Serum levels of IL-1β and TNF-α changed following DEP treatment in mice, as assessed by ELISA. The LPS and DEP-exposed group exhibited higher IL-1β and TNF-α mRNA levels than the control group (**Figures 4E, F**), but increased serum IL-1β levels were not found

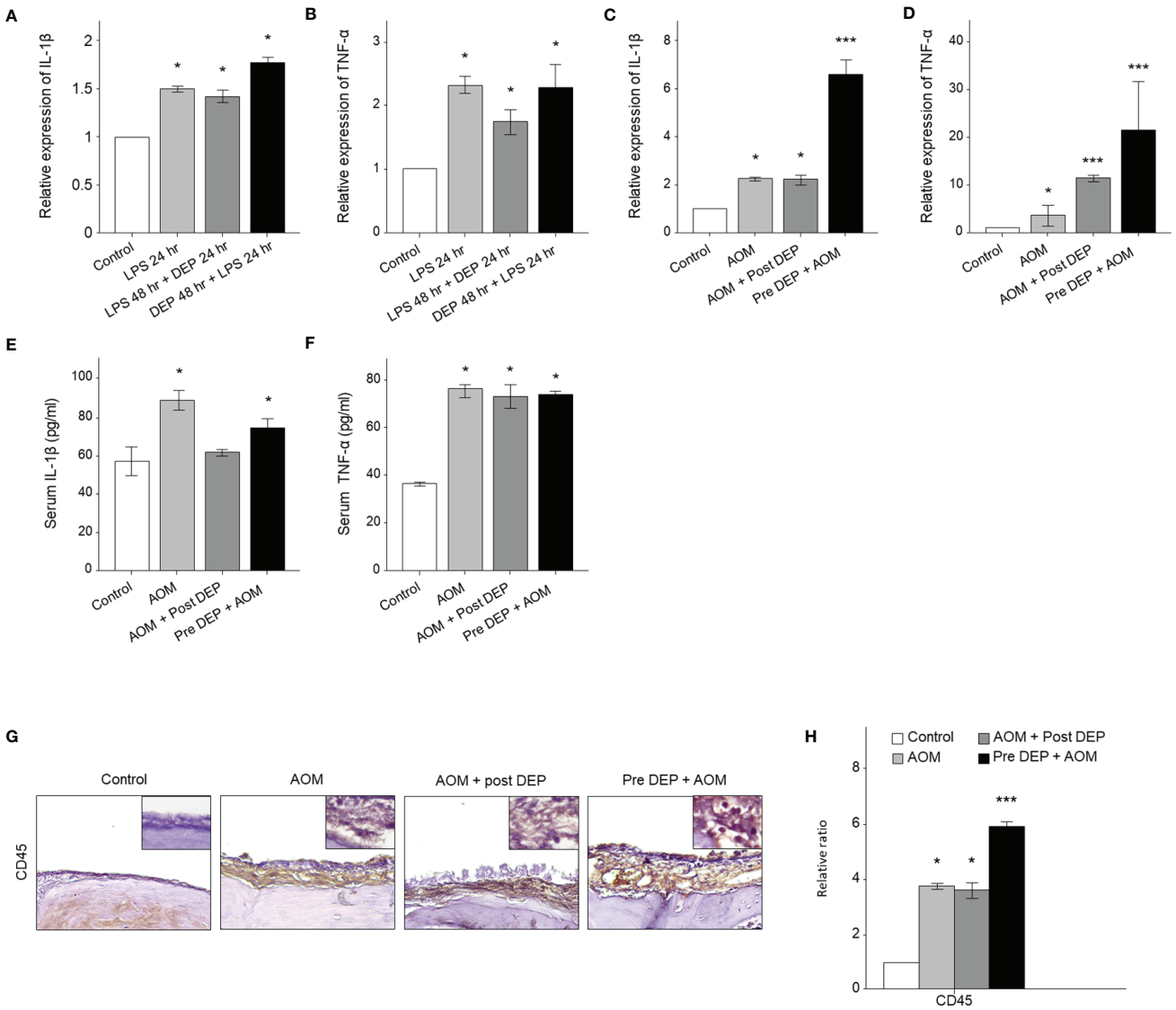

FIGURE 4 | Analysis of cytokine expression in LPS + DEP-exposed mice and HMEECs. The qRT-PCR analysis of mRNA levels of **(A)** IL-1β and **(B)** TNF-α in LPS in HMEECs. The mRNA levels of **(C)** IL-1β and **(D)** TNF-α a mouse model of acute otitis media. The cytokines in the serum of mice were assessed *via* ELISA kits, including **(E)** IL-1β, **(F)** TNF-α. **(G)** Immunohistochemical staining of mouse middle ear tissue using antibodies against CD45 (Microvessel; black arrow, magnification ×400). **(H)** Quantitation of the CD45 staining intensity. *p < 0.05, compared with the control group; ***p < 0.05 compared to the LPS 24 hr and AOM group. LPS, lipopolysaccharide; DEP, diesel exhaust particles; HMEECs, human middle ear epithelial cells; AOM, acute otitis media.

in the AOM + Post-DEP group (**Figure 4E**). The immunohistochemistry results showed that CD45, a marker of immune cell infiltration, increased in the middle ear (**Figures 4G, H**).

Effect of DEP Inhalation on Angiogenesis and Lymphangiogenesis Signaling Pathways in the Mouse Middle Ear and HMEECs

The mRNA levels of angiogenesis- and lymphangiogenesis-related factors in the mouse middle ear and HMEECs were evaluated by qRT-PCR and immunohistochemistry (**Figure 5**). In DEP 48 hr + LPS 24 hr group in the *in vitro* component of the study and in the Pre-DEP + AOM group in the *in vivo* component of the study, TRLR4, VEGFA, VEGFC, VEGFR1, and VEGFR3 mRNA levels were higher in the control group. However, VEGFA mRNA levels were not significantly increased in the LPS 24 hr, LPS 48 hr + DEP 24 hr groups. Significantly higher TLR4 and VEGFC mRNA levels were found in the DEP 48 hr + LPS 24 hr group than in the LPS 24 hr group ($p < 0.05$, **Figures 5A, C**). Moreover, VEGFA and VEGFR3 mRNA levels were significantly higher in the Pre-DEP + AOM group than in

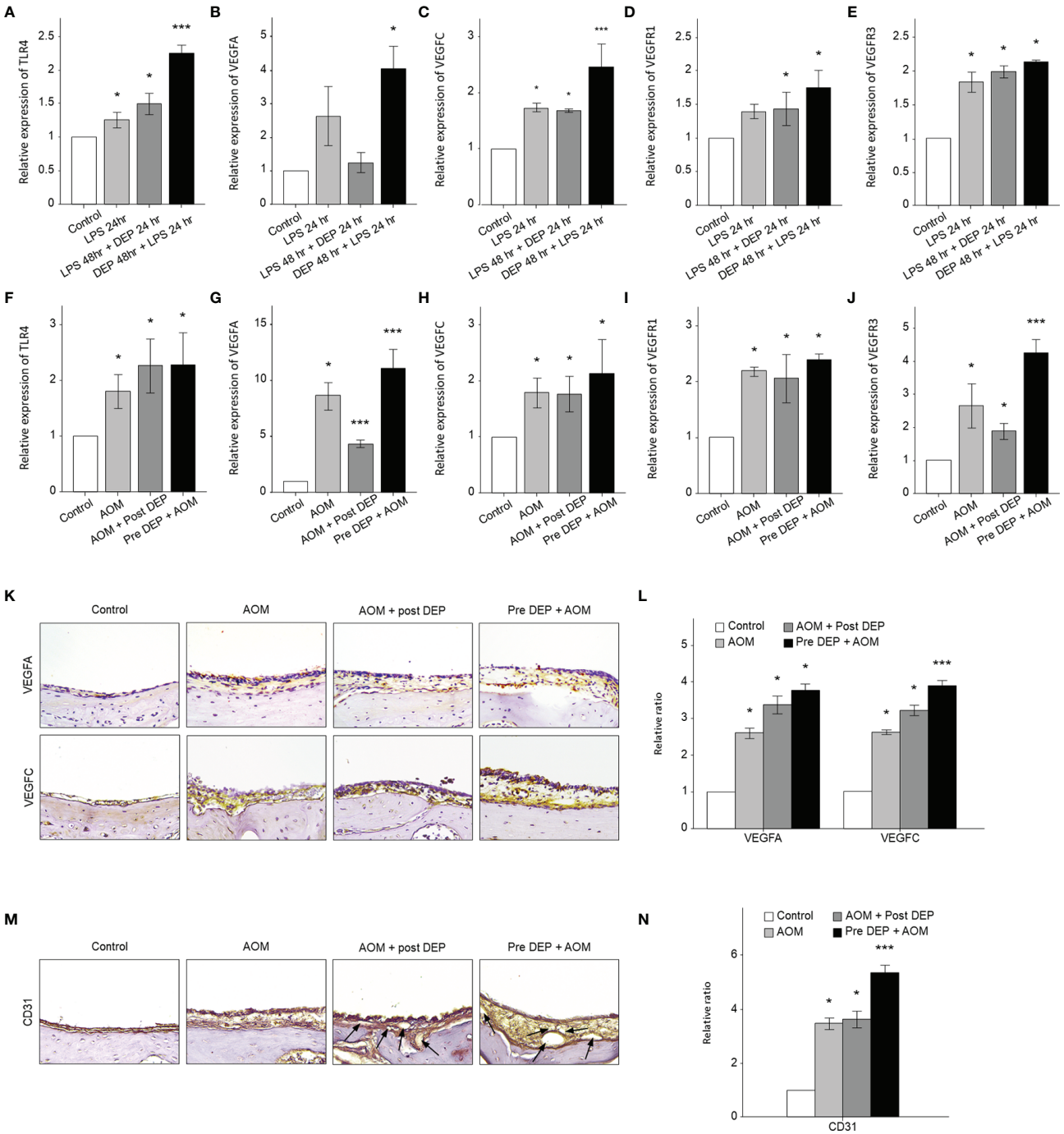

FIGURE 5 | Analysis of the expression of angiogenesis and lymphangiogenesis-related genes in LPS + DEP-exposed mice and HMEECs. The results show the mRNA levels of **(A)** TLR4 and **(B)** VEGFA, **(C)** VEGFC, **(D)** VEGFR1, and **(E)** VEGFR3 in LPS-exposed HMEECs. The mRNA levels of **(F)** TLR4 and **(G)** VEGFA, **(H)** VEGFC, **(I)** VEGFR1, **(J)** VEGFR3 in LPS in a mouse model of AOM. Immunohistochemical (IHC) staining of mouse middle ear tissue using antibodies against **(K)** VEGFA, VEGFC, and **(M)** CD31 (IHC, magnification ×400). The same location shown in each slide was chosen (IHC, magnification ×400). **(L, N)** Quantitation of the CD45 staining intensity. *$p < 0.05$, compared with the control group; ***$p < 0.05$ compared to the LPS 24 hr and AOM group. LPS, lipopolysaccharide; DEP, diesel exhaust particles; HMEECs, human middle ear epithelial cells; AOM, acute otitis media.

the AOM group ($p < 0.05$, **Figures 5G, J**). The expression of the angiogenesis and lymphangiogenesis markers VEGFA and VEGFC in the mouse middle ear tissue was significantly higher in the AOM and DEP-treated groups than in the control group (**Figures 5K, L**). The AOM + Post-DEP and Pre-DEP + AOM groups showed higher VEGFA expression than the AOM group. The expression of CD31, a marker of lymphangiogenesis and angiogenesis marker, in the mouse middle ear tissue was higher in the AOM and DEP-treated groups than in the control group (**Figures 5M, N**). The Pre-DEP + AOM group showed higher CD31 expression than the AOM group, showing that CD31 expression was augmented by DEP treatment.

DISCUSSION

This study demonstrated that DEP increased VEGF expression in HMEEC lines and an AOM mouse model with pre- or post-AOM DEP exposure (**Figure 6**). Pre-AOM DEP exposure more strongly aggravated the lymphangiogenesis and angiogenesis processes in otitis media.

Li et al. reported that VEGF plays a role in the acute phase of OME. The expression of HIF-1α mRNA was found to be correlated with the expression of VEGF and VEGFR-1 mRNA in an LPS-induced OME model (Li and Ye, 2021). It has been reported that the levels of VEGFA and TGF-β cytokines in adenoids with exudative otitis media were higher than in conditions of adenoid hypertrophy alone, prompting the suggestion that VEGFA and TGF-β could be used as additional and objective tests to confirm the clinical diagnosis of OME caused by a bacterial pathogen (Zelazowska-Rutkowska et al., 2020). In addition to its role in OM, VEGF plays an essential role in cochlear function and hearing, the latter of which is exemplified by its association with sensorineural hearing loss (London and Gurgel, 2014).

In this study, we observed increased vascular formation in the Pre-DEP + AOM group in the animal model and found that LPS increased VEGFA expression both *in vivo* and *in vitro*. Grilli et al. reported that diesel exposure induced the secretion of VEGF and other inflammation biomarkers in transcriptional profiling of human bronchial epithelial cells (Grilli et al., 2018). It is known that VEGF expression is increased after LPS, TNF-α, and IL-1β stimulation (Koyama et al., 2002). Our results demonstrate that VEGF and cytokine levels increased in mice and epithelial cells exposed to DEP or LPS, suggesting that DEP may trigger lymphangiogenesis, angiogenesis, and immune cell infiltration in AOM. The middle ear mucosa has low secretory ability. Vascular leakage is an important process in OM (Rynnel-Dagöö and Agren, 2000). In addition, VEGFR inhibitors moderated angiogenesis and lymphangiogenesis in inflamed middle ear mucosa (Cheeseman et al., 2011). VEGFA, which binds VEGFR1/Flt-1 tyrosine kinase and VEGFR2/Flt-2 tyrosine kinase, is the main inducer of the growth of blood vessels and plays a key role in neurons (Ferrara, 2004). The VEGFA and VEGFC/VEGFR3 pathway is significantly involved in lymphangiogenesis and angiogenesis. Lymphangiogenesis is an

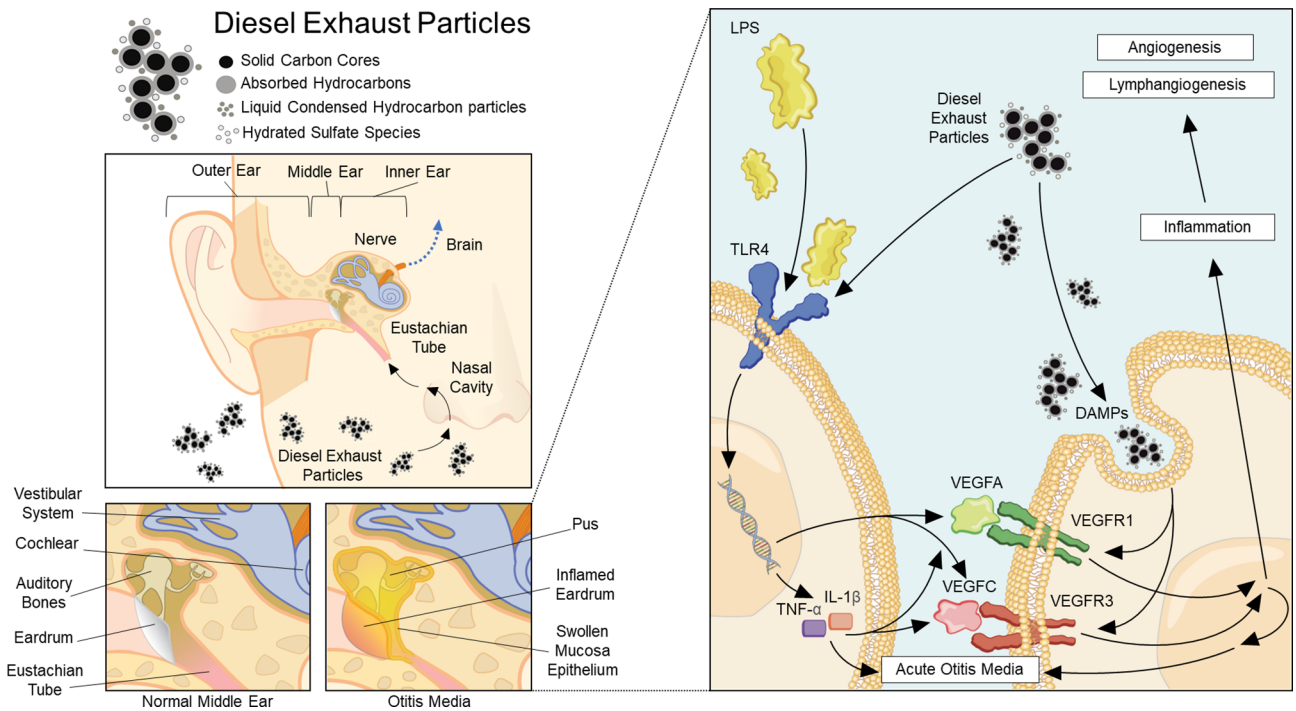

FIGURE 6 | Summary of the pathway of diesel exhaust particle-induced angiogenesis and lymphangiogenesis in acute otitis media.

important process not only in lymphatic development and disease, but also in cancer treatment and in neurological and cardiovascular diseases (Secker and Harvey, 2021). VEGFR3 is the major receptor for VEGFC. Sweat et al. reported that VEGFC treatment increased blood vessel sprouting and lymphatic sprouting in a rat mesentery culture model (Sweat et al., 2014). It has been reported that VEGFC regulates the proliferation, migration, and survival of lymphatic endothelial cells by VEGFR3 through activating the ERK, AKT, and JNK pathways (Salameh et al., 2005).

Previous studies have shown that DEP and PM decrease cell viability and increase inflammation in the middle ear (Song et al., 2012; Park et al., 2014). We observed the same findings in this experiment. DEP decreased cell viability in a dose- and time-dependent manner in HMEECs. LPS administration, preceded or followed by DEP exposure, induced AOM in a mouse model and showed increased mucosal thickening in the middle ear. Although DEP induced the expression of inflammatory cytokines such as IL-1β and TNF-α *in vivo* and *in vitro*, we did not find a synergistic effect on the expression of TNF-α and Il-1β in the conditions of LPS administration preceded or followed by DEP exposure in HMEECs. Interestingly, however, we found a strong synergistic effect in the Pre-DEP + AOM group in the animal model. The pre-exposed DEP group showed higher levels of inflammatory cytokines and inflammatory cells than the post-DEP exposure group, as well as thicker mucosa. Previous research has shown that PM exerts adverse health effects on the lung and brain, in which fine particles can induce systemic inflammation (Clifford et al., 2018; Kim et al., 2021). However, we did not observe a systemic inflammatory response through inhalation of PM in a mouse model of acute otitis media. A possible explanation for this finding is that LPS may induce the inflammatory response in the middle ear and around the eustachian tube, which may hinder the DEP from reaching the middle ear. In addition, DEP could change the immune response of the middle ear after LPS injection. For instance, it has been reported that macrophage regulation of the inflammatory response to viral infection could be changed by exposure to PM (Becker and Soukup, 1999; Kaan and Hegele, 2003).

In this study, we found different expression patterns of inflammatory cytokines and VEGFs between the *in vivo* and *in vitro* models. These differences may arise from the stimulation time and dose and anatomical characteristics of the middle ear and eustachian tube. *In vitro*, mRNA expression was measured within 24 hr after the last stimulation at 80 μg/mL because cell viability was significantly changed after this point. We investigated the lymphangiogenesis response 1 day after stimulation. *In vivo*, the inflammatory response rapidly increased 1 day after stimulation, peaked around 3 days, and gradually resolved within 14 days (Park et al., 2014). It is known that VEGF and its receptors play a role in the acute phase of OME rather than the chronic or recurrent stage (Ye and Li, 2019). VEGF mRNA expression was detected within 1 hr after instillation, dramatically increased from 6 hours to 1 day, and then progressively decreased by day 7 (Jung et al., 1999).

We used 100 μg/m^3 DEP for 1 hr a day in the experiment. The 2021 World Health Organization air quality guidelines recommend aiming for annual mean concentrations of PM_{10} not exceeding 15 μg/m^3 and 24-hour concentrations not exceeding 45 μg/m^3 (World Health Organization, 2021). A level of 100 μg/m^3 would be associated with an approximately 2.5% increase in daily mortality (World Health Organization, 2006).

This study showed the increased expression of VEGFC *in vitro*, but not *in vivo*. The mRNA expression of VEGFR3 was higher in the Pre-DEP + AOM group than in the AOM-only or Post-DEP + AOM groups in the animal model. Prior research has established that the expression of Toll-like receptors is increased in AOM, OME, COM, and cholesteatoma in the middle ear. TLR4 recognizes bacterial LPS and endogenous ligands, and induces interleukin expression *via* MyD88 (myeloid differentiation factor 88) and interferon through Toll-like-receptor adaptor molecules (Jung et al., 2021). The expression of TLR2, TLR24, and TLR9 was found to be higher in the middle ear fluid of AOM patients (Kaur et al., 2015). It has been found that DEP increased the expression of mucin genes through the TLR4-mediated activation of the ERK 1/2, p38 MAPK, and NF-κB signaling pathways (Na et al., 2019l). PM increased TLR4 expression in a lung injury animal model. Lee et al. suggest that the TLR4-MyD88 pathway could be a potential therapeutic target for diesel-induced lung injury (Lee et al., 2020).

Future research will be needed to investigate changes in the permeability of blood and lymphatic vessels. A controlled experimental design in humans will confirm the effects of DEP on lymphangiogenesis and angiogenesis in OM.

MATERIALS AND METHODS

Diesel Exhaust Particles

Standard Reference Material 2975 from the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA), collected from an industrial forklift truck, was used to generate DEP. DEP preparation was performed following a previously reported protocol (Kim et al., 2016). Briefly, a dry agglomerated DEP powder containing 15 g of a mixed phase (anatase and rutile) was used according to the manufacturer's instructions. After sonication, the DEP suspension was filtered through a 0.22-μm filter (Millipore, MA, USA).

HMEEC Culture and Stimulation with DEP

Human middle ear epithelial cells (HMEECs) (kindly provided by Dr. David J. Lim, House Ear Institutes, LA, USA) (Chun et al., 2002) were grown in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Walkersville, MD, USA) and bronchial epithelial basal medium (Lonza) (1:1) at 37°C in 5% CO_2. The medium was replaced every 48 hr until the cells reached 80% confluence. The lipopolysaccharide (LPS) dose was 1 μg/mL, while DEP concentrations of 0, 20, 40, 80, 160, and 320 μg/mL were used. Cell viability measurements were made using a Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto,

Japan) according to the manufacturer's protocol (**Figure 1**). HMEECs were then stimulated in several ways: 1) LPS 1 μg/mL only for 24 hr (LPS); 2) LPS 1 μg/mL for 48 hr and DEP 80 μg/mL for 24 hr (LPS 48 hr + DEP 24 hr); 3) DEP 80 μg/mL for 48 hr and LPS 1 μg/mL for 24 hr (DEP 48 hr + LPS 24 hr) (**Figure 2A**).

HMEEC Viability Assays

The viability of HMEECs was measured using a CCK-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions (**Figure 1**). The solution was added to each well and incubated for 2 hr at 37°C in 5% CO_2. The mixture was then shaken at room temperature for 5 minutes. Absorbance at 450 nm was measured with a microplate reader.

Experimental Design of Animals

Female BALB/c mice aged 6 to 7 weeks were purchased from The Koatech (Pyeongtaek, Korea). The mice were injected with LPS and exposed to 100 $\mu g/m^3$ DEP. BALB/c mice (n = 52) were divided into four groups (n = 13 per group): (1) control, (2) AOM, (3) AOM induction followed by DEP exposure, and (4) DEP exposure followed by AOM induction (**Figure 2B**). Lipopolysaccharide (LPS) extracted from *Pseudomonas aeruginosa* (Sigma Aldrich, St Louis, MO, USA) was dissolved in normal saline and used at a concentration of 1 mg/mL. AOM was induced by a transtympanic injection of a 10 μL emulsion of LPS 1 mg/mL into the middle ear (Tong et al., 2010). The control group received a transtympanic injection of an equal volume of normal saline, and the non-DEP groups were exposed to ambient air through nebulizer idling. The DEP inhalation protocol followed the procedure reported previously (Kim et al., 2016). Briefly, the mice in the DEP inhalation group were exposed to 100 $\mu g/m^3$ DEP for 1 hr a day for 5 days a week in a closed-system chamber attached to an ultrasonic nebulizer (NE-U780, Omron Corporation, Tokyo, Japan). On sacrifice day, otoscopy was performed and measurements were made of the ABR, mRNA levels of lymphangiogenesis-related genes and cytokines, and histology of the middle ear. This study was approved by Seoul National University Hospital Institutional Animal Care and Use Committee [IACUC No. 20-0154-S1A1(1)].

Transmission Electron Microscopy

The middle ear tissues were fixed with a 4% formaldehyde and 2.5% glutaraldehyde buffer. They were then dehydrated with graded ethanol and embedded in Araldite/Epon. Thin sections were contrasted with uranyl acetate and lead citrate. Observations and photographic records were obtained with transmission electron microscopy (TEM) (JEM-1604; JEOL, Tokyo, Japan) at 80 kV.

Symptom Score of Acute Otitis Media

To explore the effects of DEP and LPS, otoscopy was used to measure transtympanic structure and effusion 24 hr after the discontinuation of LPS or DEP. Based on the AOM symptom score of effusion and structures measured 24 hr after the last exposure, AOM symptom severity was graded as follows: 1 = normal tympanic membrane; 2 = partial effusion; 3 = effusion without accompanying structural changes of the tympanic membrane; 4 = concomitant structural changes of the tympanic membrane with effusion.

Auditory Brainstem Response

Audiometry was measured using a SmartEP (Intelligent Hearing Systems, Miami, FL, USA) and high-frequency transducers (HFT9911-20-0035) as previously described (Zheng et al., 1999). All mice were anesthetized through intraperitoneal injection of tiletamine/zolazepam (Zoletil) (Virbac, AH, France) (30 mg/kg) and placed in a small sound-attenuating chamber. The sound stimuli applied were clicks at 4 and 16 kHz. The procedures were similar to those previously described (Park et al., 2020). Mice were tested and monitored until recovery.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted using TRIzol (Invitrogen, CA, USA) according to the manufacturer's instructions. Total RNA was reverse-transcribed into cDNA using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen) at 42°C for 50 minutes and heat-inactivated at 70°C for 15 minutes. qRT-PCR was performed using the Applied Biosystems 7500 (Stratagene, La Jolla, CA, USA) with the QuantiTect SYBR Green PCR kit (Qiagen, Mississauga, ON, Canada) (Moon et al., 2015). The levels of mRNA expression were normalized to the housekeeping gene peptidyl-prolyl cis-trans isomerase A (PPIA). The sequences of primers of IL-1β, TNF-α, TLR4, VEGFA, VEGFC, VEGFR1, VEGF3, and PPIA are listed in **Table 1**.

Enzyme-Linked Immunosorbent Assay

Serum IL-1β and TNF-α levels were quantified using an enzyme-linked immunosorbent assay (ELISA) assay kit (Koma Biotech Inc., Seoul, South Korea) according to the manufacturer's instructions. Serum extracts were loaded onto the ELISA plate. The absorbance was measured at 450 nm on a microplate reader.

Immunohistochemical Analysis

Paraffin sections, hematoxylin, eosin, and immunohistochemistry staining methods in tissue were conducted as previously described (Kim et al., 2018). Mice middle ear paraffin block sections were deparaffinized and rehydrated. Non-specific binding was blocked using normal goat serum. We used antibodies for CD45 (1:100, Santa Cruz Biotechnology, Dallas, TX, USA), VEGFA (1:100, Abcam, Cambridge, UK), VEGFC (1:250, Santa Cruz), and CD31 (1:100, Santa Cruz). The following day, the sections were incubated with an ABC kit (Vector Laboratories, Burlingame, CA, USA). The color reaction was developed using a liquid DAB substrate kit (Vector). Stained middle ear tissue data were quantified using ImageJ (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

The data were entered twice into the SPSS statistical software package (ver. 20.0; IBM Corp., Armonk, NY, USA). All data are expressed as means ± standard deviation (SD) or SEM. Group differences were compared using the two-sample t-test, Mann–

TABLE 1 | The sequence of human and mouse primers in qRT-PCR.

Gene	Forward Sequence (5`-3`)	Reverse Sequence (5`-3`)
Human		
PPIA	TCCTGGCATCTTGTCCAT	TGCTGGTCTTGCCATTCCT
TLR4	CCAAGAACCTGGACCTGAGC	TCTGGATGGGGTTTCCTGTC
IL-1β	ACAGATGAAGTGCTCCTTCCA	GTCGGAGATTCGTAGCTGGAT
TNF-α	AGACGCCACATCCCCTGACAA	AGACGGCGATGCGGCTGATG
VEGFA	ACTTCTGGGCTGTTCTCG	TCCTCTTCCTTCTCTTCTTCC
VEGFC	ATGTTTTCCTCGGATGCTGGA	CATTGGCTGGGGAAGAGTTT
VEGFR1	CTTGGATTTTACTGCGGACAG	GGGGACACCATTAGCATGAC
VEGFR3	CTGGACCGAGTTTGTGGAGG	CACATAGAAGTAGATGAGCCG
Mouse		
PPIA	GGCAAATGCTGGACCAAA	CATTCCTGGACCCAAAACG
TLR4	TTTATTCAGAGCCGTTGGTG	CAGAGGATTGTCCTCCCATT
IL-1β	TGCCACCTTTTGACAGTGATG	TGGATGCTCTCATCAGGACAG
TNF-α	CTGAACTTCGGGGTGATCGG	GTGGTTTGCTACGACGTGGG
VEGFA	CAGGCTGCTGTAACGATGAA	CTATGTGCTGGCTTTGGTCA
VEGFC	TTTGCCAATCACACTTCCTGC	ACACTGTGGTAATGTTGCTGG
VEGFR1	AAATAAGCACACCACGC	ACCTGCTGTTTTCGATGTTTC
VEGFR3	CCCGAGAGCATCTTTGATAAG	GAAGAGCCTGGAGTCTTAGT

Whitney test, or the Pearson χ^2 test for normally distributed, skewed, and categorical data, respectively. One-way ANOVA or two-way ANOVA were used for multiple data. A p-value < 0.05 was considered to indicate statistical significance.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Seoul National University Hospital Institutional Animal Care and Use Committee [IACUC No. 20-0154-S1A1(1).

AUTHOR CONTRIBUTIONS

All authors were involved in discussing and drafting the article. B-GK, MP designed the experiment and wrote the manuscript. B-GK performed the animal model experiments and analyzed the data. M-GK, DC, B-GK performed the *in vitro* experiments, collected the samples, and assisted in interpreting the data. A-SJ, M-WS, JL, SO assisted in interpreting the data, provided contributions in the conception or design. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2022.824575/full#supplementary-material

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An in-depth discussion of cholesteatoma, middle ear Inflammation, and langerhans cell histiocytosis of the temporal bone, based on diagnostic results

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Objective: This study aimed to conduct an in-depth investigation of the learning framework used for deriving diagnostic results of temporal bone diseases, including cholesteatoma and Langerhans cell histiocytosis (LCH). In addition, middle ear inflammation (MEI) was diagnosed by CT scanning of the temporal bone in pediatric patients.

Design: A total of 119 patients were included in this retrospective study; among them, 40 patients had MEI, 38 patients had histology-proven cholesteatoma, and 41 patients had histology-proven LCH of the temporal bone. Each of the 119 patients was matched with one-third of the disease labels. The study included otologists and radiologists, and the reference criteria were histopathology results (70% of cases for training and 30% of cases for validation). A multilayer perceptron artificial neural network (VGG16_BN) was employed and classified, based on radiometrics. This framework structure was compared and analyzed by clinical experts according to CT images and performance.

Results: The deep learning framework results vs. a physician's diagnosis, respectively, in multiclassification tasks, were as follows. Receiver operating characteristic (ROC) (cholesteatoma): (0.98 vs. 0.91), LCH (0.99 vs. 0.98), and MEI (0.99 vs. 0.85). Accuracy (cholesteatoma): (0.99 vs. 0.89), LCH (0.99 vs. 0.97), and MEI (0.99 vs. 0.89). Sensitivity (cholesteatoma): (0.96 vs. 0.97), LCH (0.99 vs. 0.98), and MEI (1 vs. 0.69). Specificity (cholesteatoma): (1 vs. 0.89), LCH (0.99 vs. 0.97), and MEI (0.99 vs. 0.89).

Conclusion: This article presents a research and learning framework for the diagnosis of cholesteatoma, MEI, and temporal bone LCH in children, based on CT scans. The research framework performed better than the clinical experts.

KEYWORDS

PyTorch, cholesteatoma, langerhans cell histiocytosis, computed tomography (CT) scan, deep learning

Introduction

Middle ear inflammation (MEI) is a common condition among children. The incidence of otitis media is 8.1% (1), and imaging is not typically required in cases of MEI. However, for patients with MEI who present with hearing loss, and where the tympanic membrane cannot be visualized, imaging may be required (2). The specific imaging features will vary depending on the form.

The incidence of congenital cholesteatoma of the middle ear, once thought to be relatively rare, is on the rise and presently accounts for 2–5% of all the cholesteatomas (3). Langerhans cell histiocytosis (LCH) is a relatively rare condition that primarily affects children.

Soft tissue sarcoma and rhabdomyosarcoma are the most common among infants and young children. The head and neck are relatively common parts of the body involved in this condition; the percentage of cases with temporal bone involvement is approximately 7%.

Currently, high-resolution CT (HRCT) is typically used for the diagnosis and preoperative examination of suspected temporal bone tumors in clinical patients (4). Chronic otitis media and temporal bone tumors are more prone to erosion around the bone structure, which may require surgical treatment. Cholesteatoma is generally manifested in the location of the tympanic sinus (5). These two entities may appear in different positions of the tympanic chamber, which will also limit HRCT and differentiation between them.

Techniques that may be helpful for diagnosing tumors in the temporal bone include diffusion-weighted imaging or contrast-enhanced MRI; however, these methods may also affect the diagnostic results due to the high level of protein fluid present in inflammatory tissue and cases of otitis media (6).

Early stage/small tumors may not always be easily identifiable. In the medical field, a final accurate diagnosis must currently include an analysis of surgical tissue histopathology. Accurate preoperative differentiation between the two above-noted diseases is of significant clinical importance because most cases of temporal bone tumors require surgical treatment; chronic otitis media, on the other hand, can typically be treated conservatively (7). In addition, one of the key factors that can influence the choice of surgical methods and techniques is the available preoperative information concerning underlying diseases. Enhancing the imaging modality for identifying temporal bone tumors and otitis media can, thus, have a significant impact on clinical medicine, and efforts should be made to achieve this.

Radiomics is a method that is applied in the field of medicine that uses data representation algorithms to extract a large number of features from medical images. These features are known as "radiographic" data and can be used to observe several disease characteristics that are invisible to the naked eye. A radiology approach considers that the signal intensity patterns in medical images are closely related to the genetic, molecular, and biological characteristics of the tissue. These methods include T-scans and MRI scans.

The focus of the current study was to develop a deep learning framework for the automatic CT scan diagnosis of temporal bone diseases in children. To date, the radiographic features that are used to assess the utility of temporal bone disease have been rarely studied in the context of imaging. In this context, the current study aimed to provide a better analysis of these image scans to provide a more accurate diagnosis. The performance of artificial intelligence (AI) network model was tested on a separate dataset. The study also compared clinical specialists, including otologists, otolaryngologists, and radiologists.

Methods and materials

Study participants

The Institutional Review Committee of Children's Hospital of Fudan University approved the current study. In procedural terms, a search of the clinical database identified 161 children who had undergone middle ear surgery between May 2013 and October 2020. Medical records were rigorously checked to exclude patients who had received CT scans and were subsequently diagnosed with acute otitis media or inner ear disease, and who lacked temporal bone. A final group of 119 patients was included in the collected retrospective data.

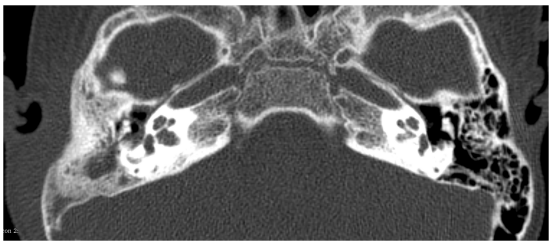

FIGURE 1
LCH of the temporal bone. The bone has not been destroyed.

Computed tomography imaging

All the registered patients required one or more CT scans of the temporal bone, and a total of 119 scans were available for analysis. These temporal bone scans were obtained using a 128-channel multidetector Somatom Definition Edge with z-Sharp^{TM} Technology CT scanners. The procedure for conducting each scan was as follows. A full scan was required for each patient from the lower edge of the external auditory canal to the upper edge of the petrous bone. The collimation was set at 128 mm × 0.625 mm, the field of view was 220 mm × 220 mm, the spacing was 0.8 mm, the matrix size was 512 × 512, the voltage was 120 kV, 240 mass, and the axial section was 0.625 mm. The scans were performed on sections where lesions were present, and the number of axial CT sections per scan ranged from 30 to 50. The total number of scans performed was 2,588. All the images were downloaded from the doctor's workstation and saved in the prescribed 224 × 224-pixel jpg format for subsequent analysis (**Figure 1**).

Clinical labeling

The distribution of clinical labels for each ear was first arranged according to each patient's diagnostic record. All the participants' medical records were examined independently by two experienced otolaryngologists and radiologists, who, concurrently, resolved any differences that arose to reach a consensus. The facts that were selected in the training and testing sessions for the classification network were reflected in these clinical labels. On closer examination of these labels, the three most common types of cases were found to be "cholesteatoma," "MEI," and "LCH." These three categories were based on web-based training and required in-depth learning to achieve adequate performance. The present article will summarize the distribution of clinical labels at a later stage. The labels for ear surgery were allocated according to pathology.

In temporal bone CT images, the lesion area accounted for a relatively small proportion of the overall image. During the training of a deep learning network, as the number of layers increased, additional lesion information was at risk of becoming lost in the image (8). Consequently, the current authors adopted a neural network with fewer layers.

A convolutional neural network known as VGG16 (9) is an architecture with 16 layers of weights and 3 × 3 filters. After the convolutional layer, there are two interconnected layers, followed by the soft maximum output. The VGG16_BN network adds a batch normalization function to the VGG16 network. The matrix is then added to the network and outputted as three categories (see **Figure 2**). It has about 138 million network parameters.

Layer (type)	Output Shape	Param #
Conv2d-1	[-1, 64, 224, 224]	1,792
BatchNorm2d-2	[-1, 64, 224, 224]	128
ReLU-3	[-1, 64, 224, 224]	0
Conv2d-4	[-1, 64, 224, 224]	36,928
BatchNorm2d-5	[-1, 64, 224, 224]	128
ReLU-6	[-1, 64, 224, 224]	0
MaxPool2d-7	[-1, 64, 112, 112]	0
Conv2d-8	[-1, 128, 112, 112]	73,856
BatchNorm2d-9	[-1, 128, 112, 112]	256
ReLU-10	[-1, 128, 112, 112]	0
Conv2d-11	[-1, 128, 112, 112]	147,584
BatchNorm2d-12	[-1, 128, 112, 112]	256
ReLU-13	[-1, 128, 112, 112]	0
MaxPool2d-14	[-1, 128, 56, 56]	0
Conv2d-15	[-1, 256, 56, 56]	295,168
BatchNorm2d-16	[-1, 256, 56, 56]	512
ReLU-17	[-1, 256, 56, 56]	0
Conv2d-18	[-1, 256, 56, 56]	590,080
BatchNorm2d-19	[-1, 256, 56, 56]	512
ReLU-20	[-1, 256, 56, 56]	0
Conv2d-21	[-1, 256, 56, 56]	590,080
BatchNorm2d-22	[-1, 256, 56, 56]	512
ReLU-23	[-1, 256, 56, 56]	0
MaxPool2d-24	[-1, 256, 28, 28]	0
Conv2d-25	[-1, 512, 28, 28]	1,180,160
BatchNorm2d-26	[-1, 512, 28, 28]	1,024
ReLU-27	[-1, 512, 28, 28]	0
Conv2d-28	[-1, 512, 28, 28]	2,359,808
BatchNorm2d-29	[-1, 512, 28, 28]	1,024
ReLU-30	[-1, 512, 28, 28]	0
Conv2d-31	[-1, 512, 28, 28]	2,359,808
BatchNorm2d-32	[-1, 512, 28, 28]	1,024
ReLU-33	[-1, 512, 28, 28]	0
MaxPool2d-34	[-1, 512, 14, 14]	0
Conv2d-35	[-1, 512, 14, 14]	2,359,808
BatchNorm2d-36	[-1, 512, 14, 14]	1,024
ReLU-37	[-1, 512, 14, 14]	0
Conv2d-38	[-1, 512, 14, 14]	2,359,808
BatchNorm2d-39	[-1, 512, 14, 14]	1,024
ReLU-40	[-1, 512, 14, 14]	0
Conv2d-41	[-1, 512, 14, 14]	2,359,808
BatchNorm2d-42	[-1, 512, 14, 14]	1,024
ReLU-43	[-1, 512, 14, 14]	0
MaxPool2d-44	[-1, 512, 7, 7]	0
AdaptiveAvgPool2d-45	[-1, 512, 7, 7]	0
Linear-46	[-1, 4096]	102,764,544
ReLU-47	[-1, 4096]	0
Dropout-48	[-1, 4096]	0
Linear-49	[-1, 4096]	16,781,312
ReLU-50	[-1, 4096]	0
Dropout-51	[-1, 4096]	0
Linear-52	[-1, 3]	4,097,000

Total params: 138,365,992
Trainable params: 138,365,992
Non-trainable params: 0

Input size (MB): 0.57
Forward/backward pass size (MB): 322.14
Params size (MB): 527.82
Estimated Total Size (MB): 850.54

FIGURE 2
VGG16_BN structure diagram.

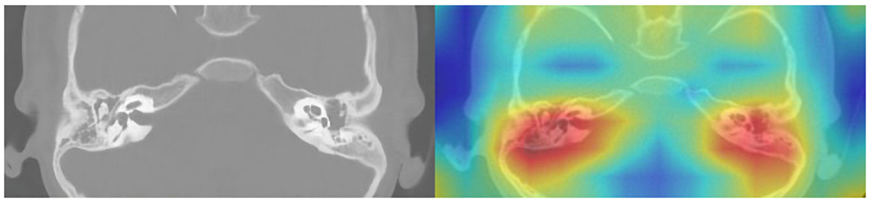

FIGURE 3
CT of the temporal bone with cholesteatoma: Original image **(left)** and heat map **(right)**.

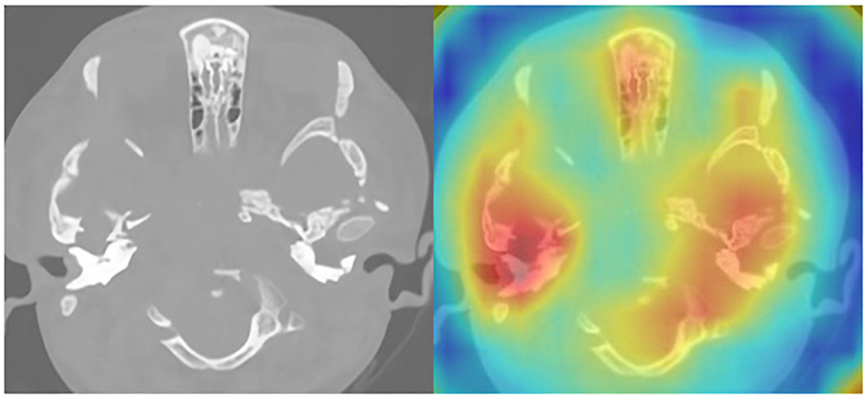

FIGURE 4
CT of the temporal bone with LCH: Original image **(left)** and heat map **(right)**.

Data enhancement technology of the classification task

To ensure the efficiency of deep learning, all the images required a mirror image, minor rotation, cropping, scaling, and side-length moving to increase the training data for the classification task. The implementation model of the data volume enhancement was carried out in real-time by the classification network. Finally, the Bicubic interpolation algorithm was used to scale all the images to 512 × 512-pixel size.

Classification task of the classification network

Based on the basic VGG16_BN classification network model, the Softmax function acted as its final classification layer. The probability distribution of cholesteatoma otitis media was trained using the PyTorch application in Python. A random selection of 85% of the dataset (n = 2,070) was used during the validation process, and five different parameters were included. The duration value of the training course was set to 100 (from the initial 0.01-bit speed). The remaining 15% of the data (n = 388) were stored and could be used to evaluate the performance of the model after the training was complete. Clinical experts could also use these datasets to assess performance.

Professional performance evaluation

Four clinical specialists were selected to evaluate the diagnostic performance of their test datasets. Two of the specialists were registered otologists with 15 years of combined experience, and the remaining two specialists were radiologists trained in ear diseases with 16 years of combined experience. Each of the four experts could only select one of the three categories, based on the CT images, to diagnose a case.

Analyzing and compiling statistics

To accurately evaluate the diagnostic ability of the AI model, the performances of the deep learning algorithm and clinical experts during data testing were summarized using the confusion matrix method, i.e., binarizing all the labels of the AI model and drawing the receiver operating characteristic (ROC) curve. The prediction probability was applied to measure the area under the ROC curve of the deep learning algorithm, and

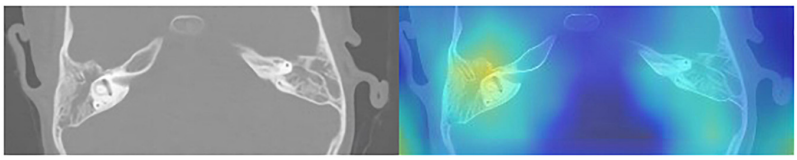

FIGURE 5
CT of the temporal bone with MEI: Original image **(left)** and heat map **(right)**.

a 95% CI was estimated using the Tak Long Estate Algorithm. Python and Excel were used to conduct all the statistical analyses (alpha level, 0.05).

Results

The model that was adopted in the current study is used in discrete networks. For multivariate classification tasks, the test results were satisfactory when the dataset was tested for the classification of cholesteatoma, LCH, and MEI. Based on the total weight parameters included in the pretraining, the heat map was created according to gradient-weighted class activation mapping. The heat map indicated the differences related to where the computer focused the imaging scan, in which the orange area was significant (**Figures 3–5**). The area under the curve for this network was 0.99 (see **Figure 6**). The network derived a better conclusion than the four experts who were included in the clinical trial.

Classification network and clinical experts' performance

The training accuracy was 99%, and the validation accuracy was 98.7% at its most balanced time. The confusion matrix is shown in **Table 1**.

The overall accuracy of the network was high (98.7 vs. 88.5%) for the three task categories, and the network achieved a higher F1 score (98.7 vs. 88.2%) compared to the clinical experts. These results are shown in **Table 2**.

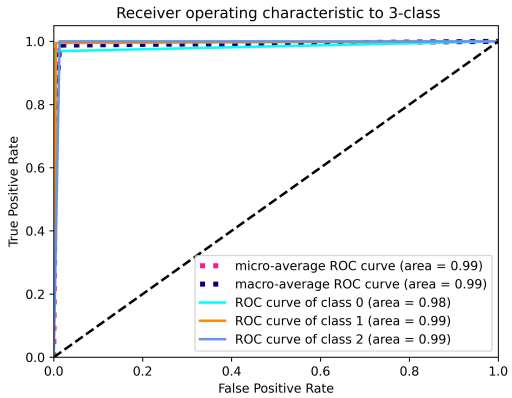

FIGURE 6
Deep learning framework detection of cholesteatoma (class 0), LCH (class 1), and MEI (class 2).

TABLE 1 Multi-classification confusion matrix for deep learning.

	Predict cholesteatoma	LCH	MEI
Cholesteatoma	185	2	4
LCH	0	190	1
MEI	0	0	191

TABLE 2 Confusion matrix comparison of AI vs. clinical experts.

	Precision	Recall	F1 score	Accuracy
Clinical experts	0.906	0.885	0.882	0.885
vgg16_bn	0.988	0.987	0.987	0.987

Discussion

With the rapid development of deep learning technology and the use of big data to develop the training of these networks, healthcare AI technology has undergone rapid development. Radiomics analysis of different types of imaging data has yielded valuable results that can be used to isolate different tumor entities throughout the human body. In some areas that rely on medical imaging, particularly radiology and pathology, the use of machine learning methods to perform a variety of medical tasks (and to a higher quality than physicians) has been very satisfactory. For example, machine learning is currently used in CT scanning to detect abnormalities in the head, classify masses in the liver, and predict amyotrophic lateral sclerosis. In the fields of neurology and otology, many leading figures have applied basic machine learning techniques for predicting the hearing results of patients experiencing sudden hearing impairment (9), or to make predictions regarding the hearing and language perception of children with cochlear implants (10).

The current article is the first study to apply a deep learning network to temporal bone tumors in children,

based on CT images. The performance of the adopted model was excellent, and its sensitivity and specificity were superior to those of medical and clinical experts. These advantages, as well as the absolute consistency and high efficiency of AI in decision-making, indicated that the clinical application of AI in the diagnosis of temporal bone tumors, based on CT scans, represents broad development prospects.

In the field of deep learning, overfitting is a classic problem, particularly when the number of samples is limited or the training steps are extensive.

In response to the problem of overfitting, the following methods can be used to avoid problems and improve the versatility of a model. Using data enhancement technology, the application of this technology can greatly increase the number of images that can be used for network training. A network model can use this method to achieve 98, 99, and 99% accuracy in the three classification tasks (training, verifying, and testing datasets, respectively).

The accurate diagnosis of otitis media is a challenging task. The key factor for a correct diagnosis in this regard is using guidelines and practices that are currently implemented by the country in which the research is conducted. This includes accurately confirming the length of time of infection and determining each TM that expands or contracts.

Acquired cholesteatoma has a history dating back three centuries, but the nature of this disease remains unconfirmed. If cholesteatoma is not detected and treated in time, it can become very dangerous by rapidly expanding and invading the internal structure of the temporal bone, causing various intra- and extracranial complications.

As shown in **Figure 1**, according to a CT scan, a diagnosis of otitis media may be assumed. However, the patient in question actually suffered from LCH, the diagnosis of which is histology-based.

It is difficult to identify a temporal mass using the naked eye without the presence of bone damage. With this model, an early diagnosis can be made before the temporal bone is entirely destroyed.

Following further analysis and inspection of misclassified cases, the network logic can be observed. Clinical experts and AI models may attempt to classify both the typical and non-challenging cases. However, in some situations, neither may be able to correctly classify these cases. When this happens, CT scanning will be required to highlight atypical and extremely subtle patterns. Although the strategy that was applied in the AI classification tasks was not clear, when classifying cases of cholesteatoma, LCH, and MEI, the higher recall rate that was obtained reflected that AI was in the CT image. When reading abnormal patterns, there was a high level of sensitivity. This ability is of great value in terms of providing a first step in the screening and diagnosis process because it minimizes the risk of overlooked cases. However, when clinical experts correctly identified more of the normal ears, they proved their greater particularity. Accordingly, to a large extent, they can help eliminate case results that have been misclassified and analyzed by AI to achieve the goal with minimal labor costs.

One of the most important obstacles to the broad application of deep learning in medicine is the inability to understand the basic principles of algorithmic conclusions. In a recent research experiment (11), the researchers constructed a framework based on algorithmic components that could generate a tissue image in a digital optical coherence tomography scan using a segmented network while concurrently creating a classification network; this network was then used for establishing a diagnosis and for providing treatment recommendations. This workflow divided the decision-making process into two distinct steps, i.e., explaining the initial scan, and establishing a judgment, based on the findings of the first step. In the current study, the authors did not design an automatic separation framework. Tumors may not always be confined to the mastoid process and, as such, may involve other parts of the temporal bone. The clinician should, thus, inspect and extract particular regions of interest.

The present study showed that an automated system, developed using deep learning methods, could be useful for identifying otitis media. Various CT imaging features were quantitatively evaluated by applying image processing methods, including segmentation and image feature extraction. The deep learning classifiers used image features to distinguish patients with temporal bone tumors from those with otitis media. This study was designed to examine the value of an automatic method, based on deep learning, for determining patient candidates for tympanotomy.

Employing deep learning methods may expand the number of candidates for tympanotomy. The findings of this study concurred with recent research on a deep learning approach (12), in which machine-learning models were developed to classify patients with chronic otitis media and cholesteatoma. The classifier and an optimal area that was less than 0.99, sensitivity was 0.97, and specificity was 0.80. The accuracy rate of the AI model was approximately 90%, but the average accuracy rate of clinical experts was relatively low (approximately 73.8%).

In the research process, the AI model obtained very good results. The accuracy of the model classification (99%) in the presence of otitis media and temporal bone tumors was 0.99, which was equivalent to/better than the results reported in studies based on ear mirroring, which ranged from 80 to 85.6% (13, 14).

The deep learning model presented in the current article has several possible advantages. The system used a computerized method to quantitatively evaluate various CT parameters. Multiple image features may enhance the ability of the model to predict pathological tissue. Pretrained deep learning models may significantly reduce the time required to determine whether symptom onset had been within the therapeutic time

window. These characteristics may enable an otolaryngologist to determine whether patients require tympanotomy or another type of intervention.

The following limitations are present in the current study and must be noted. First, only moderate datasets were available for inclusion, and these temporal tumor samples were too small to support creating a complete classifier, or there was a slight imbalance. Therefore, based on the currently known properties of deep learning techniques, the AI model presented herein can be used in two-dimensional CT images. Two-dimensional CT images are less detailed than their three-dimensional (3D) conunterparts because the latter includes the temporal bone and the middle ear region. Accordingly, for the validated AI model, using 3D images will improve the model's diagnostic accuracy. The above algorithm is also consistent with the CT scan explained by the clinician.

All the study participants were sourced from the same clinical center. Further verification resulting from multicenter research settings and different patient populations is warranted. Furthermore, the scientific data employed in the current article had been optimized and, accordingly, showed only the current population. As such, the results of this study should be interpreted with care.

Compared with the conclusions derived by the deep learning model, the outcomes derived by clinicians may vary too much for the former to be considered the gold standard. Additional validation using a large database is, thus, necessary.

Conclusion

The purpose of this study was to establish a deep learning research framework for the diagnosis of LCH of the temporal bone, cholesteatoma, and MEI, based on CT scanning of the temporal bone in children. An in-depth research framework, based on CT scanning of the temporal bone in children, was proposed for the in-depth study of temporal bone fractures, cholesteatoma, and confirming a temporal lobe tumor diagnosis. The results of the study also showed that the clinical application of AI in CT imaging presented good development prospects. In the future, temporal bone research, as well as 3D CT imaging technology, will be used to expand this method to the larger-scale treatment of temporal bone diseases.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by the Children's Hospital of Fudan University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

Author contributions

BD and Z-MX: conception and design of the research. BD, L-LP, W-XC, and Z-WQ: acquisition of data. BD, L-LP, and W-XC: analysis and interpretation of the data. BD, Z-WQ, and Z-MX: statistical analysis. BD: writing of the manuscript. Z-MX: critical revision of the manuscript for intellectual content. All authors read and approved the final draft.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer HJ declared a shared parent affiliation with the authors to the handling editor at the time of review.

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