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ORIGINAL RESEARCH

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Establishment of novel immortalized middle ear cell lines as models for otitis media

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Abstract

Objective: Otitis media (OM) is among the most frequently diagnosed pediatric diseases in the US. Despite the significant public health burden of OM and the contribution research in culture models has made to understanding its pathobiology, a singular immortalized human middle ear epithelial (MEE) cell line exists (HMEEC-1, adult-derived). We previously developed MEE cultures from pediatric patients with non-inflamed MEE (PCI), recurrent OM (ROM), or OM with effusion (OME) and demonstrated differences in their baseline inflammatory cytokine expression and response to stimulation with an OM-relevant pathogen lysate and cytokines. Herein, we sought to immortalize these cultures and assess retention of their phenotypes.

Methods: MEE cultures were immortalized via lentivirus encoding temperature-sensitive SV40 T antigen. Immortalized MEE lines and HMEEC-1 grown in monolayer were stimulated with non-typeable *Haemophilus influenzae* (NTHi) lysate. Gene expression (*TNFA*, *IL1B*, *IL6*, *IL8*, *MUC5AC*, and *MUC5B*) was assessed by qPCR.

Results: Similar to parental cultures, baseline cytokine expressions were higher in pediatric OM lines than in HMEEC-1 and PCI, and HMEEC-1 cells were less responsive to stimulation than pediatric lines.

Conclusion: Immortalized MEE lines retained the inflammatory expression and responsiveness of their tissues of origin and differences between non-OM versus OM and pediatric versus adult cultures, supporting their value as novel in vitro culture models for OM.

KEYWORDS

cell culture, culture model, epithelial lines, *Haemophilus influenzae*, middle ear, otitis media

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1 | INTRODUCTION

Otitis media (OM) is among the most frequently diagnosed pediatric diseases in the United States and accounts for up to 18% of all pediatric office visits.[1–3] OM is characterized by inflammation of the middle ear and can be further subdivided into acute otitis media (AOM) when presenting with acute inflammatory symptoms such as otalgia or fever, or otitis media with effusion (OME) when acute symptoms are absent but chronic middle ear fluid persists.[4,5] Acute episodes of OM are frequently preceded by viral respiratory tract infection and most commonly include the bacterial pathogens *Streptococcus pneumoniae*, non-typeable *Haemophilus influenzae* (NTHi), and *Moraxella catarrhalis*.[4,6] Up to 85% of children have an episode of OM by the age of three, with most cases resolving spontaneously[4]; however AOM may transmute to OME or become recurrent (ROM) or chronic (COM).

OM is a leading cause for childhood antibiotic prescriptions, ambulatory surgery, and hearing loss.[7–9] Risk factors for AOM include those related to host (e.g., age, sex, race/ethnicity, family history of AOM and genetic predisposition, craniofacial anomalies, atopy, immunodeficiency, adenoid hypertrophy, gastroesophageal reflux) and environment (e.g., day-care attendance, passive smoking, older siblings, use of pacifier, no breastfeeding, pollution, season).[10–14] Research to improve OM prevention and treatment continues to be a priority given its prevalence, economic and health burdens across societies and the potential for serious difficulties including hearing loss, developmental delay, and even death.[1,15,16] Despite the high prevalence, morbidity, and healthcare expenditures, there remains a lack of information regarding the cellular and molecular immunologic and inflammatory events that contribute to the pathogenesis of OM. Experimental models of OM are integral to investigation of these processes and have made substantial contributions to the understanding of OM pathobiology to date.[17–19] Immortalized cell culture models offer the benefits of reduced cost, labor, and complexity relative to animal models and facilitate experimental reproducibility and scaling. Inflammatory signaling initiated by the middle ear epithelium (MEE) is critical to the pathogenesis of OM and MEE cell culture models represent a valuable tool for its study.[20] Prior study supports the utility of in vitro models of OM, demonstrating that stimulation of MEE cells with NTHi pathogen lysate activates toll-like receptor (TLR) signaling to mediate downstream inflammatory pathways implicated in OM pathogenesis.[21,22] However, publicly available in vitro cell culture models for OM are currently limited to a single immortalized human middle ear epithelial cell line (HMEEC-1) derived from an adult patient.[23] Given that adults and children exhibit differences in innate immunity and inflammatory signaling originating from the MEE,[24–27] we recently cultured MEE of patients with and without OM and characterized their OM-relevant gene expressions and responses to cytokine and pathogen lysate stimulation relative to HMEEC-1.[28] Our findings corroborated differences between pediatric versus adult, and OM versus non-OM derived MEE and suggested that specific molecular phenotypes associated with OM are retained in primary cell culture making pediatric MEE cultures superior to adult-derived cell lines when assessing OM-associated molecular signaling. To overcome the limitation of the short lifespan of primary cultures and address the paucity of human MEE culture models for OM, we sought to immortalize these primary cultures and validate their retention of their OM-relevant phenotypes.

2 | MATERIALS AND METHODS

2.1 | Cell immortalization and culture

Previously described primary cultures[28] were derived from middle ear biopsies of a 4.4-year-old Black female without OM with bilateral profound sensorineural hearing loss undergoing cochlear implantation (PCI), a 2.7-year-old White female with OME and mild 40 dB hearing loss (OME1), a 3.9-year-old Black female with OME and mild unilateral 30-40 dB hearing loss (OME2), a 1.4-year-old White female with ROM and normal hearing (ROM1), and a 5.1-year-old White male with ROM and normal hearing (ROM2). Informed consent/assent was obtained for specimen collection, culture, and immortalization of cells from all subjects per Children's Wisconsin Institutional Review Board protocol 369996-9. Per the original description of biopsies from which primary cultures were developed,[28] OME was defined as the persistence of effusion for longer than 3 months with minimal constitutional symptoms. ROM was defined as three or more acute OM presentations within a 6-month period in which clinical evidence of OM and effusion resolved between episodes. Exclusion criteria for both groups included immunological abnormality, either intrinsic or pharmacological; anatomical or physiological defect of the ear; syndrome associated with OM (e.g., Down syndrome, cleft palate); sensorineural hearing loss; and chronic mastoiditis, cholesteatoma, or other OM complications except for conductive hearing loss. Control biopsy specimens were obtained from a patient in the same otolaryngology practice undergoing cochlear implantation with an age range matching the experimental group. Exclusion for the control group included history of OM defined as an average of one or fewer episodes per 6 months of life, no AOM within the prior 3 months, and no diagnoses of OME or middle ear pathology. Cells were cultured as previously described[28] in PneumaCult Ex Plus media (STEMCELL Technologies, Vancouver, BC Canada) supplemented with 1X antibiotic-antimycotic (ThermoFisher Scientific, Waltham, MA) in BioCoat collagen coated flaskware (Corning, Corning, NY) at 37°C/5% CO_2. Each was immortalized via lentivirus encoding temperature-sensitive mutant tsA58 SV40 Large T antigen (Applied Biological Materials Inc., Richmond, BC Canada); cells at 25% confluence in 12-well plates were exposed to lentivirus at MOI 2–5 with 6.7 μg/mL polybrene (Sigma Aldrich, St Louis, MO). After 24 h, lentivirus was replaced with normal growth media and cells returned to culture for 48 h at 37°C/5% CO_2. Thereafter cells were maintained at 33°C/5% CO_2 to permit temperature-dependent expression of SV40 T antigen. Immortalization was confirmed by continuous culture >20 passages. All experiments were performed on cells of passage >4 at 33°C.

2.2 | Cytokeratin immunofluorescence

Expression of cytokeratins (CK) characteristic of epithelial cells was assayed by immunofluorescent staining as previously described[29] using pan-cytokeratin and vimentin (mesenchymal marker) antibodies (Abcam, Cambridge UK), mouse immunoglobulin as a negative control (Santa Cruz Biotechnology, Dallas, TX), and Alexa Fluor 488-conjugated anti-mouse (ThermoFisher Scientific). Coverslips were mounted using Diamond Prolong Antifade Reagent with 4′,6-diamidino-2-phenylindole nuclear stain (DAPI; ThermoFisher Scientific).

2.3 | Cytogenetics analysis

Chromosomes of metaphase cells were counted and analyzed using G-banding (Wisconsin Diagnostics Laboratories, Milwaukee, WI).

2.4 | Clonogenic soft agar assay

Cells were used for soft agar assay to investigate retention of the noncancer cell characteristic of anchorage-dependent growth. As previously described,[29] six-well plates were prepared with 0.5% agarose in 50% normal growth media on which 2500 cells in 0.3% agarose and 75% normal growth media was overlaid in duplicate wells and cultured at 37°C and 5% CO_2 for 4 weeks and fed as needed with normal growth media to prevent drying. Controls included wells absent cells (negative control) and A549 lung adenocarcinoma cells (American Tissue Type Collection, Manassas, VA), known to form colonies (positive control). Colonies were stained with 0.005% crystal violet (Sigma Aldrich).

2.5 | Cell treatment

Cells were treated or mock-treated in triplicate with 5 µg/mL whole cell lysate (WCL) of NTHi, prepared as previously described,[30] in normal growth media. RNA was harvested directly from culture plates using TRIzol reagent (ThermoFisher Scientific).

2.6 | Real-time quantitative polymerase chain reaction

RNA was reverse transcribed with Superscript IV VILO (ThermoFisher Scientific) and analyzed using TaqMan Gene Expression (ThermoFisher Scientific) assays for *HPRT1* (housekeeping gene), *TNFA*, *IL1B*, *IL6*, *IL8*, *MUC5AC*, and *MUC5B* in quadruplicate reactions along with a reference sample on each plate.

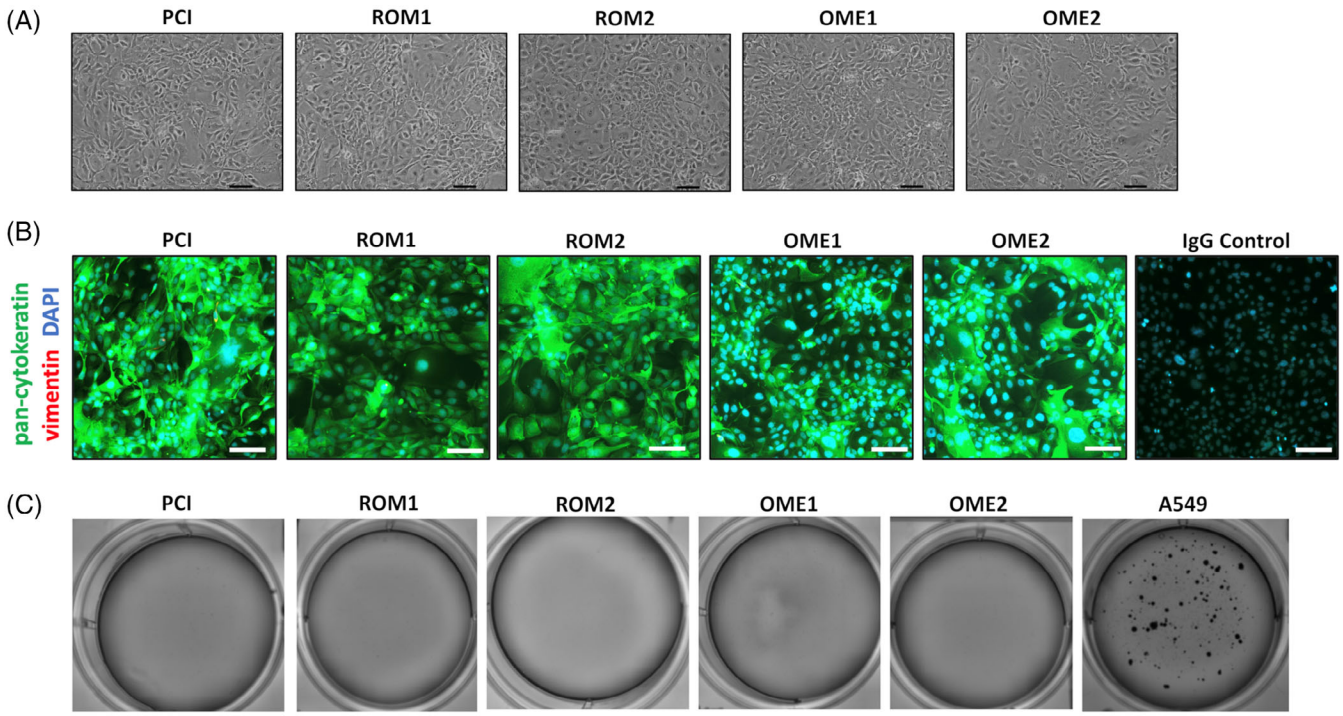

FIGURE 1 (A) Representative brightfield images of MEE cells in culture. Scale bars equal 100 µM. (B) Representative immunofluorescent images showing MEE lines stained with a pan-cytokeratin epithelial marker and vimentin, a mesenchymal marker. Images were taken at constant exposure for biomarkers of interest. Scale bars equal 100 µm. (C) Representative images of MEE cells grown in a soft agar to assess transformation. All soft agar assays were performed in duplicate. The A549 lung cancer line was used as a positive control. MEE, middle ear epithelial.

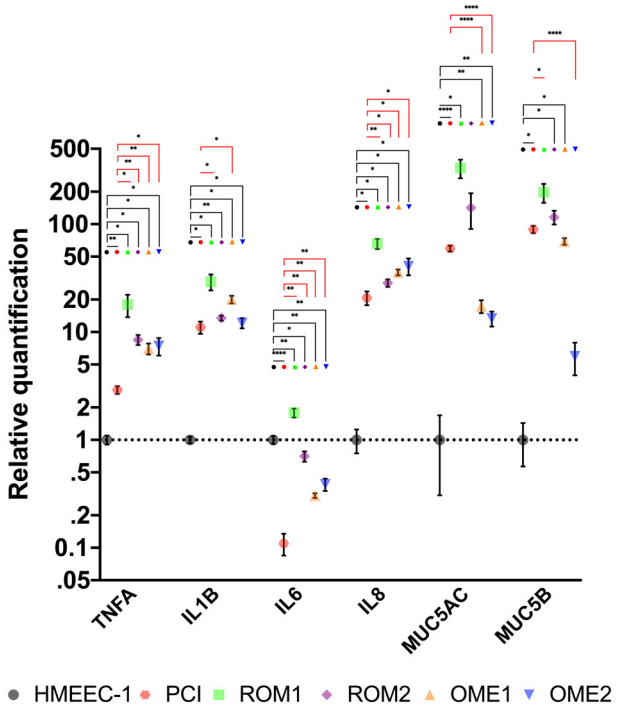

FIGURE 2 Baseline expression of genes of interest in untreated cells. The five immortalized pediatric MEE cell lines and the previously established HMEEC-1 (adult-derived) were grown in triplicate and gene expression of key cytokines and mucins was analyzed by RT-qPCR. The mean and standard deviation of gene expression relative to HMEEC-1 is shown. Pairwise comparisons of all novel MEE lines to HMEEC-1 (black brackets) and all OM lines to the non-OM line PCI (red brackets) were performed and statistically significant differences are indicated (* = $p < .05$, ** = $p < .01$, *** = $p < .001$, **** = $p < .0001$). MEE, middle ear epithelial; OM, otitis media.

2.7 | Statistical methods

For qPCR data, RQ's were calculated using the delta–delta method. The student's *t*-test was used to compare groups and the step-down Bonferroni method was used to adjust for multiple comparisons. $p < .05$ was considered as statistically significant. RQ means and standard deviations were normalized relative to HMEEC-1 for each gene for baseline gene expression and relative to the untreated control for each gene in each cell line for NTHi stimulation.

3 | RESULTS

All five immortalized pediatric MEE cell lines exhibited characteristic keratinocyte morphology in tissue culture (Figure 1A) and expressed cytokeratins with minimal expression of vimentin (a mesenchymal marker), suggesting epithelial phenotype and limited presence of fibroblasts (Figure 1B). Karyotypic analysis demonstrated genetic abnormalities in immortalized pediatric MEE cell lines comparable to those previously reported and observed herein in HMEEC-1[23] (Supporting Information 1). Immortalized pediatric MEE lines did not form colonies in soft agar assay, indicating their anchorage-dependence and non-transformed/non-cancer phenotype (Figure 1C).

All immortalized pediatric cell lines, including the non-OM line PCI, expressed higher levels of OM-relevant genes *TNFA*, *IL1B*, *IL8*, *MUC5AC*, and *MUC5B* relative to HMEEC-1 (Figure 2; Supporting Information 2.1) consistent with the pattern observed in primary culture.[28] Immortalized pediatric lines (with the exception of ROM1) expressed lower levels of *IL6* than HMEEC-1. Relative to the non-OM pediatric line PCI, OM lines frequently expressed higher levels of cytokines: OM lines expressed up to 6-fold higher levels of *TNFA* and up to 16-fold higher levels of *IL6*. While lines derived from patients with ROM (ROM1 and ROM2) expressed higher levels of mucins *MUC5AC* and *MUC5B* relative to PCI, mucin expression in the lines from patients with OME (OME1 and OME2) were lower than that of PCI.

Across most cytokines, the pediatric lines mounted a greater response to pathogen lysate (NTHi WCL) stimulation than HMEEC-1 (Figure 3; Supporting Information 2.2). PCI, ROM2, and OME2 generally demonstrated the greatest changes in expression of OM-relevant genes, with statistically significant 8-fold or greater induction of *TNFA*, *IL6*, and *IL8* following 2–4 h of pathogen lysate stimulation. Conversely, *MUC5AC* and *MUC5B* expression generally decreased following NTHi WCL exposure, frequently decreasing at 2 h stimulation, and rebounding to or above baseline levels at 4 h.

4 | DISCUSSION

The innate immune response of the middle ear mucosa and MEE cells play a key role in OM pathogenesis.[31] Inflammatory mediators present in the middle ear during OM promote extensive remodeling of the middle ear mucosa during AOM and perpetuate its progression to OME characterized by proliferation of mucin-secreting goblet cells and thickening of the pseudostratified epithelium.[32,33] MEE culture models have shown the ability to recapitulate this cytokine response and thus represent an invaluable tool for OM research.[32,34] In this study, we successfully immortalized five MEE primary cultures from pediatric patients with varying OM severity (ROM, OME) or absent OM.[28] To assess whether immortalized lines retained OM-relevant phenotypic characteristics of their tissue of origin we evaluated expression of six key genes (*TNFA*, *IL1B*, *IL6*, *IL8*, *MUC5AC*, and *MUC5B*) with a known role in OM pathology to evaluate the baseline inflammatory profile and response to NTHi stimulation.[32,35,36] *TNFA*, *IL1B*, and *IL6*, and *IL8* encode pro-inflammatory cytokines that potentiate a range of downstream effects including activation of adaptive immune cells, recruitment of neutrophils, further production of cytokines, antibody and mucin production, and histamine release.[32] These cytokines are thought to be responsible for many of the inflammatory changes induced by pathogenic organisms during OM as well as delayed recovery, recurrence, and persistence or chronicity of OME.[32,37–39] *MUC5AC* and *MUC5B* encode mucin 5AC and 5B, large secreted mucins which are hypersecreted during OM and associated

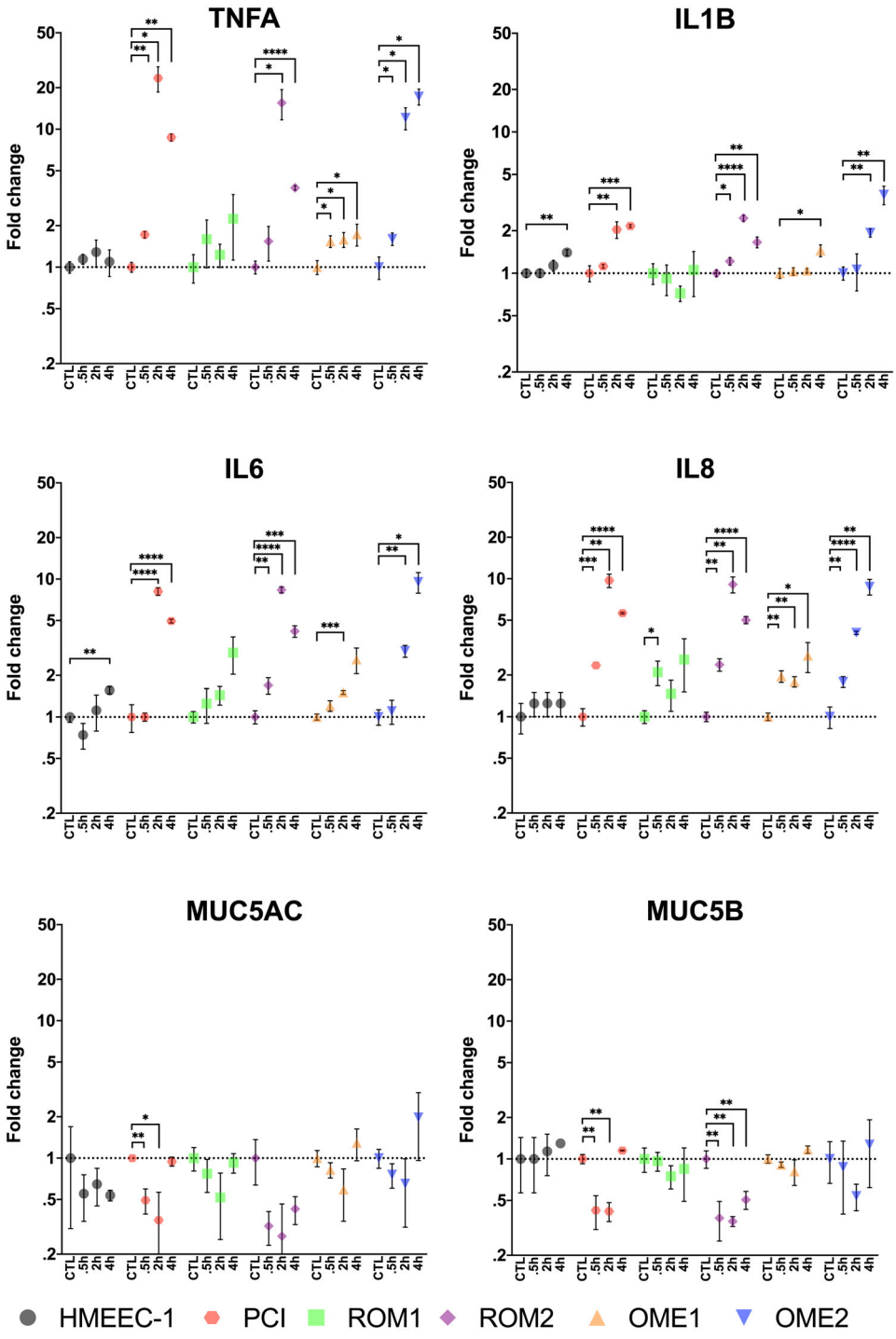

FIGURE 3 Cell lines were stimulated with 5 μg/mL whole cell lysate (WCL) of non-typeable *Haemophilus influenzae* (NTHi) for 0.5, 2, or 4 h. Relative expression of genes of interest compared to a mock-treated control is shown (mean ± SD). All experiments were performed in triplicate. Statistically significant changes in gene expression are indicated (* = $p < .05$, ** = $p < .01$, *** = $p < .001$, **** = $p < .0001$).

with viscous, difficult to clear effusion, biofilm formation, disease recurrence, and chronicity.[22,35,40–43]

Consistent with the pattern previously observed in pediatric primary cultures relative to HMEEC-1,[28] baseline expressions of OM-relevant cytokines and mucins was higher in the immortalized pediatric MEE lines (including non-OM derived PCI) relative to adult-derived HMEEC-1, with the exception of *IL6*. This finding is in agreement with differences in innate immunity observed in adults relative to children predisposing the latter toward excessive inflammation.[24–27,44–46] However, due to differences in immortalization methodology and passage history, comparisons between the previously established adult-derived HMEEC-1 and our novel pediatric lines must be treated with caution. Similarly consistent with findings in the primary cultures, the immortalized pediatric OM lines expressed significantly higher baseline expressions of OM-relevant cytokines (*TNFA*, *IL1B*, *IL6*, and *IL8*) than the immortalized non-OM line PCI. While baseline mucin expressions herein were also generally higher in immortalized ROM lines relative to PCI consistent with the pattern observed in primary cultures for all OM lines except ROM1 (exclusively *MUC5B* was examined in primary cultures), *MUC5AC* and *5B* were depleted in

immortalized OME lines relative to PCI. *MUC5AC* and *5B* expression would be anticipated to be high in OME-derived lines and, accordingly, baseline levels of *MUC5B* were highest in primary OME cultures in the previous study.[28] The change in *MUC5B* baseline expression in OME cultures following immortalization may reflect changes incurred by the immortalization process, prolonged culture, or absence of a necessary stimulating factor. Chronic OME is thought to be related to retained bacterial antigens which produce chronic local stimulation of the immune system contributing to persistence of ME inflammation and effusion.[47] A study of MEE from ears with chronic OME demonstrated that 40% retained endotoxin.[48] It is possible that depletion of such antigens through continuous culture of immortalized pediatric MEE cells herein led to MUC5B depletion in OME-derived primary cultures. Depleted mucin expression may also represent a limitation of monolayer culture conditions relative to air-liquid interface (ALI) culture. In vitro study of mucin response frequently employ ALI for optimal mucin mRNA detection and/or corresponding detection of mucin secretion, hence future experiments are planned in our laboratory to investigate mucin gene expression and responsiveness in the pediatric MEE lines grown under ALI conditions. Overall, analyses of baseline expressions of OM-relevant genes demonstrate patterns similar to that found in primary culture and supports retention of phenotypic characteristics related to patient age and OM diagnosis.

In addition to preservation of baseline gene expression profiles, the capacity to mount an inflammatory response following pathogen lysate stimulation is an important characteristic of an OM cell culture model. As a critical mediator of the host innate immune system, epithelial cells recognize and respond to invading bacteria through interaction with pathogen-associated molecular patterns on a variety of bacteria via epithelial expressed TLRs. It has previously been demonstrated that epithelial cells recognize NTHi antigens via TLR2 leading to activation of NF-κB and subsequent upregulation of several key inflammatory mediators including IL-1β, IL-8, and TNF-α.[34,49–51] Consistent with the pattern observed in pediatric MEE primary cultures relative to HMEEC-1,[28] immortalized pediatric lines generally exhibited greater responses in OM-relevant genes to NTHi WCL exposure than did HMEEC-1. Greater responsiveness in pediatric MEE cells relative to HMEEC-1 was observed even for *IL6* which did not demonstrate higher baseline expression in pediatric cultures relative to adult HMEEC-1 in the current study or prior study of primary cultures. Also consistent with the previous study, OM lines which exhibited high baseline expression yielded lower response to stimulation presumably because expression was already near a maximal level. Herein, ROM1, which exhibited the highest baseline expression of OM-relevant genes, was generally the least responsive among immortalized pediatric lines to pathogen lysate stimulation. The capacity of these novel immortalized pediatric MEE lines to mount a robust response to pathogen lysate challenge supports their value as models for OM with the potential to facilitate mechanistic study of the inflammatory response originating within the MEE during OM.

While the immortalization and characterization of the cell lines presented in this study represents an important advance in in vitro model systems for studying OM, several limitations and areas for future work should be noted. First, as highlighted above the characterization in this study was conducted in monolayer culture only. Future studies growing these cell lines in air-liquid interface (ALI) culture are planned to investigate their characteristics and inflammatory response including mucin production in a more physiologically representative system. Second, six genes were chosen to characterize the baseline profile and inflammatory response of our lines. These genes were selected for investigation due to their known roles in inflammation and OM pathogenesis as discussed above. However, many other genes are likely crucial in OM, including both those with well-established roles in the MEE inflammatory response and those whose functions require further elucidation. While we observed in this study that differences between the previously established adult HMEEC-1 line and our pediatric lines and between non-OM and OM lines were preserved following immortalization across the six genes we studied, limited inferences into the preservation of the OME compared to the ROM phenotype could be drawn. Future RNA sequencing followed by principal component analysis will allow more in-depth characterization of these lines and their retention of characteristics of their tissues of origin. Third, analysis of inflammatory markers in this study was limited to transcriptional analysis. Future studies will investigate cytokine and mucin production of these cell lines at the protein level, both in monolayer and ALI culture. Fourth, this study employed NTHi whole pathogen lysate to induce an inflammatory response. This is a well-established method for studying OM in vitro[21,22] and likely recapitulates important elements of naturally-occurring OM in humans, as innate immune defenses including lysozyme, lactoferrin, PLUNC (palate, lung, and nasal epithelium clone), and defensins trigger pathogen lysis exposing the ME to intracellular bacterial components.[52,53] However, the use of pathogen lysate is not able to fully capture the complex pathogen-host interaction that takes place during OM. To better study this relationship, current efforts are underway in our group to coculture these novel lines with live OM bacteria. Finally, efforts were made to collect tissues and create cell lines from patients varying in age, sex, and racial background due to the known effects of these differences on OM risk. However, our five new lines were all derived from non-Hispanic Black and White patients between 1.4 and 5.1 years of age, and only one patient was male. While they represent a notable expansion in the donor diversity of publicly available MEE cell lines, further efforts are needed to create new lines that represent the full diversity of OM patients and allow more in-depth study of risk factors for OM.

5 | CONCLUSION

Despite its high prevalence, morbidity, and healthcare expenditures, there remains a lack of information regarding the cellular and molecular immunologic and inflammatory events that contribute to the pathogenesis of OM. Inflammatory signaling initiated by the MEE is critical to the pathogenesis of OM and MEE cell culture models represent valuable tools for its study. Publicly available human MEE cell lines are currently limited to a single line derived from an adult without

OM. Herein, we immortalized primary MEE cultures of pediatric patients with and without OM and with various disease severity (ROM, OME). These lines represent the first immortalized MEE lines derived from pediatric patients, the primary population affected by OM, and were derived from patients of varying age and racial background, factors known to influence risk of OM. In accord with differences observed in adult and pediatric innate immunity thought to predispose children toward greater risk of excessive inflammatory response and consistent with phenotypes observed previously in the corresponding primary cultures, the novel immortalized pediatric MEE lines exhibit higher baseline expression and response of OM-relevant cytokines relative to the currently available adult MEE line supporting their superiority as models for pediatric OM. These cell lines hold promise for future basic and translational research as valuable new in vitro models for the study of OM.

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CONFLICT OF INTEREST STATEMENT

Kerschner, Johnston, Khampang, and Samuels are Inventors on licensing agreements for the described cell lines with Applied Biological Materials Inc., Richmond, BC, Canada and American Type Culture Collection, Manassas, VA.

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Additional supporting information can be found online in the Supporting Information section at the end of this article.

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