

Differences in innate immune response gene regulation in the middle ear of children who are otitis prone and in those not otitis prone

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ABSTRACT

Objective: Acute otitis media (AOM) causes an inflammatory response in the middle ear. We assessed differences in innate immune responses involved in bacterial defense at onset of AOM in children who were stringently defined as otitis prone (sOP) and children not otitis prone (NOP).

Study Design: Innate immune genes analysis from middle ear fluid (MEF) samples of children.

Methods: Genes of toll-like receptors (TLR), nod-like and retinoic acid-inducible gene-I-like receptors, downstream effectors important for inflammation and apoptosis, including cytokines and chemokines, were studied from MEF samples by using a real-time polymerase chain reaction array. Protein levels of differentially regulated genes were measured by Luminex.

Results: Gene expression in MEF among children who were sOP was significantly different in upregulation of interleukin 8, secretory leukocyte peptidase inhibitor, and chemokine (C-C motif) ligand 3, and in downregulation of interferon regulatory factor 7 and its related signaling molecules interferon alpha, Toll-like receptor adaptor molecule 2, chemokine (C-C motif) ligand 5, and mitogen-activated protein kinase 8 compared with children who were NOP. Differences in innate gene regulation were similar when AOM was caused by *Streptococcus pneumoniae* or nontypeable *Haemophilus influenzae*.

Conclusion: Innate-immune response genes are differentially regulated in children who were sOP compared with children with NOP.

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Children who experience recurrent episodes of acute otitis media (AOM) become classified as otitis prone (OP)^{1,2} after they experience three AOM events within a 6-month time span or four AOM events within 12 months.³ Previously, we published data that showed children who were OP and stringently defined as OP (sOP) based on tympanocentesis confirmation of all clinical diagnoses had lower mucosal and serum antibody responses against *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae* antigens.^{4–7} We also found fewer B-cell memory and CD4 T-cell memory responses after AOM infections in children who were sOP.^{8–11}

The innate immune system plays a fundamental role in the prevention of AOM. Our group studied the innate immune responses in the blood and nasopharynx at the onset of AOM in children who were sOP compared with children who were not otitis prone (NOP).^{9,12–14} Differential expression of innate immunity genes and the mechanisms that regulate innate immunity in chronic rhinosinusitis have also been described previously.^{15,16} Here, to our knowledge, for the first time, we assessed differences in innate immunity in the middle ear of children who were sOP and children who were NOP. Real-time polymerase chain reaction (PCR) array was used to study the expression of different genes that mainly involve human antibacterial defense. The array included genes involved in bacterial-activated pattern recognition receptor (PRR) signal transduction, encoding downstream effectors important for inflammation and apoptosis, and encoding immune cell-expressed antimicrobial peptides. Such gene expression profiles provide insights into the molecular and cellular changes that occur during AOM and may provide avenues to novel therapeutic treatments or novel biomarkers for disease diagnosis.

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METHODS

Study Population

Our group is in the ninth year of a prospective, longitudinal study of the immunologic dysfunction in children who are OP, funded by the National Institutes of Deafness and Communication Disorders (R0108671). Subjects included in these experiments were children who experienced an AOM episode. Children were enrolled from a middle-class, suburban, sociodemographic population in Rochester, New York. The details of the study design were previously described.¹⁷ The study was approved by the Rochester General Hospital institutional review board. Children received all doses of a pneumococcal conjugate vaccine according to the U.S. schedule with either pneumococcal conjugate vaccine 7 or pneumococcal conjugate vaccine 13, depending on the date of enrollment. A diagnosis of AOM was performed by validated otoscopists (J.C., M.P.) and was based on signs of inflammation (bulging) of the tympanic membrane and presence of middle ear fluid (MEF) documented by tympanocentesis. Written informed consent was obtained from parents of the children at study entry. Tympanocentesis was performed for all AOM episodes. Children who were sOP were defined as those who experienced three or more episodes of AOM within 6 months or four or more episodes within a year. Each MEF was cultured for otopathogens by using standard culture techniques. MEF sampling procedures, microbiology, and organism identification have been previously described.^{18–20} Analysis of data reported here are from 24 children who were aged matched at 12 ± 1 months old into two groups; children who were sOP ($n = 12$) and children who were NOP ($n = 12$). Subject selection included only those with either *S. pneumoniae* ($n = 12$) or nontypeable *H. influenzae* ($n = 12$) culture positive MEFs.

Isolation of RNA from MEF Samples

MEF samples varied in quantity from 50 to 250 μL , and the entire sample was added to 500 μL of phosphate buffered saline solution (pH 7.4). After culturing a small portion of MEF, the remainder was centrifuged for 10 minutes at 4°C; 1 mL of TRIzol reagent (Sigma-Aldrich, St. Louis, MO) was added to the MEF pellet after removal of the supernatant, mixed by pipetting, and left at room temperature for 5 minutes. TRIzol-containing MEF samples were stored at -80°C for

further processing of DNA and RNA isolation. RNA isolation was performed as described previously.²¹ To remove traces of DNA, a DNA digestion step was performed by using deoxyribonuclease I enzyme from Qiagen (Hilden, Germany). After RNA isolation, ribonuclease inhibitor was added to each sample to avoid degradation. The quantity of RNA was determined by Nanodrop (Thermo Scientific, Wilmington, DE). Criteria for inclusion in downstream reverse transcriptase (RT-PCR) applications was based on an optical density of wavelength 260/280 of >2.0.

Complementary DNA Synthesis and Real-Time PCR Array

First-strand complementary DNA (cDNA) was generated for each RNA sample isolated from MEF by using a RT² first-strand kit (Qiagen) by following the manufacturer's instructions. An RT² Profiler PCR array was performed in 96-well plates to measure expression of 84 genes involved in antibacterial responses (catalog PAHS-148Z; Qiagen). The array included genes involved in bacterial-activated pattern recognition receptors (toll-like receptors [TLR], nod-like and retinoic acid-inducible gene-I-like receptors) for signal transduction as well as genes encoding downstream effectors important for inflammation and apoptosis, including cytokines and chemokines, and genes encoding immune cell-expressed antimicrobial peptides). Per experiment, a set of five housekeeping genes was included to normalize the data. Controls were also included in each array for genomic DNA contamination, RNA quality, and general PCR performance.

cDNA template from each MEF sample was mixed with the appropriate ready-to-use PCR master mix, aliquoted in equal volumes to each well of the same PCR array and amplification performed (10 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 1 minute at 60°C), according to the manufacturer's guidelines by using a CFX96 real-time PCR instrument (Bio-Rad Laboratories, Hercules, CA). Only genes with threshold cycle (Ct) values of <35 were considered to be detectable, and the Ct values of the genes were normalized with the average Ct value of all five housekeeping genes on the array data analysis. The analysis was performed based on the $\Delta\Delta C_t$ method.²² The relative gene expression of different genes was calculated as ΔC_t sample = (C_t sample gene) – (C_t sample average of 5 housekeeping genes). The average ΔC_t , 2^{- ΔC_t} , fold change, and *p* value were calculated by using the software by SA Biosciences Valencia, CA.

Measurement of Chemokine Protein Levels in the MEF Supernatant

Based on RT-PCR data, we elected to measure interleukin (IL) 8, chemokine (C-C motif) ligand (CCL) 2 (monocyte chemoattractant protein-1), CCL5 (RANTES), and interferon (IFN) α protein levels in which we found higher expression for IL-8, no difference for CCL2, and low expression for CCL5 and IFN- α chemokines in the children who were sOP. Measurement of selected chemokines in the MEF supernatant was performed with a Bio-Plex assay kit (Bio-Rad) per the manufacturer's instructions. MEF supernatants stored at -80°C were thawed at room temperature. Undiluted samples were incubated with antibody conjugated magnetic beads for 1 hour at room temperature and then biotin-labeled cytokine-detection antibodies were added, followed by incubation at room temperature for 30 minutes. After incubation, phycoerythrin-conjugated streptavidin antibodies were added and incubated for 30 minutes. The samples were run per the manufacturer's instructions on a Bio-Plex 200 system with Luminex xMAP technology (Bio-Rad). IFN- α levels were detected in separate experiments with the MEF according to Bio-plex assay instructions. To correct for differential dilution effects that occurred during the MEF sample collection, total protein concentrations were determined by using the Pierce BCA Protein assay kit (Ther-

moFisher Scientific, Waltham, MA) according to the manufacturer's protocol, and data were normalized with total protein concentrations. Chemokine protein levels obtained from the Luminex readout were then corrected according to the total protein concentration in the sample. The results were expressed as a ratio of the chemokine protein level to the total protein level in the same sample as described previously.¹³

Statistical Analysis

Statistical analyses for RT² PCR array data was performed according to the manufacturer's instructions (SA Biosciences). Changes in gene expression were more than twofold and *p* < 0.05 were considered significant. Graph Pad Prism Software version 6.04 (GraphPad Software, Inc., La Jolla, CA) was used for statistical analysis of Luminex data. A two-tailed Student's *t*-test was used for comparisons between the groups, and *p* < 0.05 was considered significant. Demographic comparisons between the two groups were performed with Fisher's exact test.

RESULTS

Child Population

Twenty-four children (median age, ~12 months old) with AOM were included in this study. Detailed demographic information about the children, including culture results, is presented in Table 1. The sOP population included 72% boys compared with 40% boys in the NOP population, but this difference was not significant (*p* = 0.214).

Innate Immune Response Difference in Children Who Were sOP and Children Who Were NOP

Most of the 84 genes analyzed had similar expression in the two study groups. The genes that showed significant differences are listed in Table 2. Three genes differed significantly in upregulation. IL-8 plays a major role in neutrophil recruitment in otitis media,²³ and expression was 4.4-fold higher in the middle ears of children who were sOP. CCL3 protein is involved in the acute inflammatory state in the recruitment and activation of neutrophils, and expression was 2.1-fold higher in children who were sOP. Secretory leukocyte protease inhibitor (SLPI) is considered to be the predominant neutrophil elastase inhibitor in secretions. It was 3.2-fold higher in children who were sOP. Six genes were significantly different in downregulation. IFN regulatory factor 7 (IRF7), which is a key transcriptional regulator of type 1 INF-dependent immune responses, showed 2.72-fold downregulation. The corresponding genes, IFN- α and Z-DNA binding protein 1 (ZBP1) (Z-DNA-dependent activator of IFN-regulatory factors), were 3.79- and 2.43-fold downregulated, respectively, in children who were sOP. The CCL5 gene, which is chemotactic for T cells, eosinophils, and basophils, and plays an active role in recruiting leukocytes into inflammatory sites, was 2.3-fold downregulated in children who were sOP. Similarly, mitogen-activated protein kinase 8 (MAPK8) and Toll-like receptor adaptor molecule 2 (TICAM2) genes were 2.20- and 2.31-fold downregulated in children who were sOP compared with children who were NOP.

Innate gene expression might vary according to the causative otitis pathogen; therefore, we compared gene expression in relation to the presence of either *S. pneumoniae* (Fig. 1 A) or nontypeable *H. influenzae* (Fig. 1 B) in children who were sOP and in children who were NOP. Some genes, e.g., IL-8, TICAM2, and ZBP1 (zinc binding proteins) showed a similar trend of differential expression in relation to both *S. pneumoniae* and nontypeable *H. influenzae* (Fig. 1) However, pathogen-specific innate immune response differences in children who were sOP versus children who were NOP were identified as shown in Fig. 1, including -3.6-fold downregulation of the Caspase Recruitment Domain Family (CARD) 6 gene and 1.8-fold upregulation of heat shock proteins (HSP90AA1) in children who were sOP with AOM

Table 1 Demographic information of children

	sOP	NOP
Average age, \pm SD, mo	12.24 \pm 3.2	13.3 \pm 2.7
Girls/boys, %	28/72	60/40
Race, %		
White	93	90
Black	7	
Multiracial		10
Pathogens in MEF	<i>Streptococcus pneumoniae</i> or nontypeable <i>Haemophilus influenzae</i>	<i>S. pneumoniae</i> or nontypeable <i>H. influenzae</i>
Daycare attendance, %	66	40
Smoking exposure, %	8	20
Average upper respiratory infections when the sample was taken, days	6	6
Atopy presence, %	30	20
Breast-feeding at the time of sampling, %	30	10

sOP = Stringently defined otitis prone; NOP = not otitis prone; SD = standard deviation; MEF = middle ear fluid.

Table 2 Fold regulation of genes that shows significant difference in children who were sOP ($n = 12$) compared with age-matched children who were NOP ($n = 12$)

Gene Description	Gene Acronym	Fold Up- or Downregulation	p Value
Upregulated genes in sOP compared with NOP			
Interleukin 8	<i>IL8</i>	4.41	0.021
Secretory leukocyte peptidase inhibitor	<i>SLPI</i>	3.22	0.047
Chemokine (C-C motif) ligand 3	<i>CCL3</i>	2.09	0.039
Downregulated genes in sOP compared with NOP			
Chemokine (C-C motif) ligand 5	<i>CCL5</i>	-2.27	0.004
Interferon α 1	<i>IFNα1</i>	-3.79	0.08
Interferon regulatory factor 7	<i>IRF7</i>	-2.72	0.02
Mitogen-activated protein kinase 8	<i>MAPK8</i>	-2.20	0.03
Toll-like receptor adaptor molecule 2	<i>TICAM2</i>	-2.31	0.010
Z-DNA binding protein 1	<i>ZBP1</i>	-2.43	0.014

sOP = Stringently defined otitis prone; NOP = not otitis prone.

caused by *S. pneumoniae*. CARD6 plays a role in immune defense,²⁴ the HSP90AA1 protein is an inducible molecular chaperone and aids in the proper folding of specific target proteins by use of an adenylpyrophosphatase activity that is modulated by co-chaperones.

Protein Analysis

We analyzed chemokine protein levels in the MEF of four proteins (IL8, CCL5, IFN- α , and CCL2) to determine whether messenger RNA differences observed between the two groups correspond to protein levels. IL-8 protein levels were significantly higher in children who were sOP, which corresponded to the higher levels of gene expression (Fig. 2 A). In contrast, protein levels of CCL5 were not reflective of gene expression differences (Fig. 2 B). IFN- α protein levels were significantly lower in children who were sOP (Fig. 2 C), which corresponded to lower levels of gene expression. CCL2 protein levels corresponded to gene expression levels in that they were not significantly different in children who were sOP versus children who were NOP.

DISCUSSION

Here, to our knowledge for the first time, we assessed the innate response in the middle ear in children who were sOP with recurrent AOM infections compared with children who were NOP. We used a real-time PCR array that focused on inflammatory cytokines whose role in AOM has been studied extensively^{21,25-27} but not previously in an sOP population. We found differential expression in the two

populations of children for important genes that relate to host defense. IL-8 showed higher expression in children who were sOP. A previous study showed that overstimulation and dysfunction of recruited neutrophils within the lung result in release of a number of proinflammatory molecules and proteases, which results in damage of lung tissue.²⁸ This is likely the case in children who were sOP and who experience AOM as well.

IL-8 levels are increased by oxidant stress, which thereby causes recruitment of inflammatory cells and induces a further increase in oxidant stress mediators, which makes it a key cytokine involved in localized inflammation.²⁹ Observing higher levels of IL-8 messenger RNA as well as higher levels of IL-8 protein in children who were sOP indicated that these children with excessive neutrophil recruitment and localized middle ear mucosa damage due to greater inflammation than occurs in children who were NOP. Elevated IL-8 gene expression was similar whether *S. pneumoniae* or nontypeable *H. influenzae* caused AOM. In a previous study, we found lower expression levels of IL-8 in the nasopharynx in children who were sOP.¹² We speculated that the difference is attributable to the time of measurement. The measurement of expression of IL-8 in the nasopharynx was after 5-7 days of upper respiratory infection (URI), whereas measurement of IL-8 in MEF was on day 1 of clinical illness. The role and requirement for IL-8 in chemotaxis for neutrophil recruitment during recurrent infections have been described in other studies that involved other disease conditions.^{30,31}

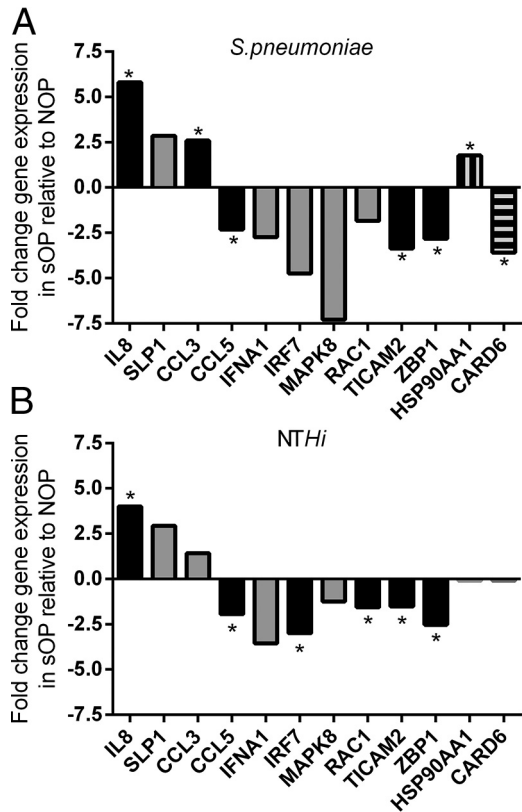


Figure 1. Comparison of innate immune response in the middle ear fluid (MEF) in relation to the presence of *Streptococcus pneumoniae* (*Spn*) or nontypeable *Haemophilus influenzae* (*NTHi*) of children who with stringently defined otitis prone (sOP) versus children who were not otitis prone (NOP) in which more than twofold differences were observed. Black bar shows similar trend in gene expression in relation to *S. pneumoniae* and *NTHi*. Gray bar represent no statistical significance was found and solid box bar represents genes that showed expression in *S. pneumoniae* only. *Significant difference ($p < 0.05$) was found in children who were sOP compared with children who were NOP.

We found fourfold higher levels of SLP1 gene expression in children who were sOP. SLP1 is generally expressed at mucosal surfaces and plays a role in the defense against the destruction of epithelial tissues by neutrophil elastase.³² Its higher expression is consistent with the need to control neutrophil elastase damage as well as to indicate that this antineutrophil elastase defense mechanism is not impaired in children who were sOP. In addition, SLP1 exerts an impact on mucin gene expression, which is known to be an important regulator in otitis media.³³ Previous studies on lung pathology showed that increased levels of SLP1 in nasal secretions and bronchoalveolar fluids may indicate inflammatory lung conditions.^{32,34}

Almost always AOM is universally preceded by viral upper respiratory infections (URI).^{35,36} Immune responses to control viral URI are critical to prevent the progression to AOM. We observed a significantly lower expression of IRF7 in children who were sOP. We also observed lower IFN- α expression in children who were sOP. Lower IRF7 signaling is consistent with our observations that children who were sOP were more prone to viral URIs and consequent bacterial AOM. Howie *et al.*³⁷ compared the association of viral URIs and IFN production and found the presence of IFN in most MEF specimens from which virus was absent and bacteria were isolated. Pitkaranta *et al.*^{38,39} indicated that leukocyte cultures of children with recurrent respiratory tract infections and otitis media with effusion produce less IFN than those of healthy children. Similarly, lower levels of ZBP1,

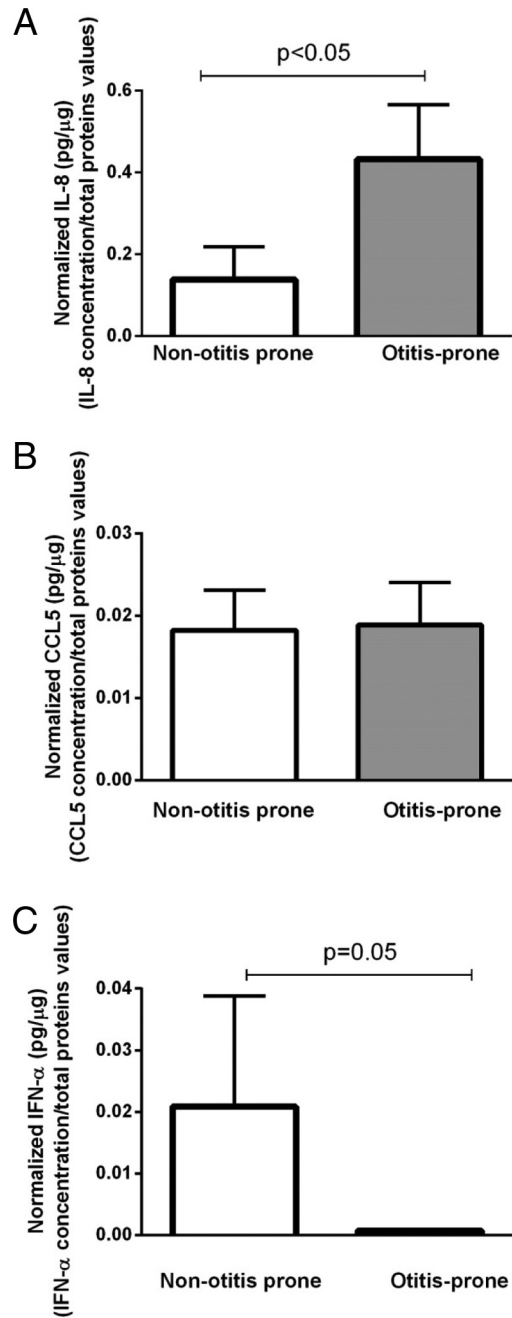


Figure 2. Comparison of (A) interleukin (IL) 8, (B) chemokine (C-C motif) ligand 5 (CCL5), and (C) interferon (IFN- α) at the proteomic levels in the middle ear fluid (MEF) of children with stringently defined otitis prone (sOP) versus children who were not otitis prone (NOP). Data were normalized with total proteins concentrations. Data shown in the figure represent the mean \pm SE.

MAPK8, and TICAM2 in MEF samples of children with sOP was consistent with the observed downregulation of the entire IRF7-dependent signaling pathway. TICAM2 was shown to be required for IRF7 activation.⁴⁰

We found a 2.5-fold lower production of CCL5 (RANTES) expression in children who were sOP. CCL5 is a chemoattractant for T cells, eosinophils, and basophils. We did not detect differences in protein levels of CCL5 in sOP compared with children who were NOP. This may be due to issues of assay sensitivity or sample size and will

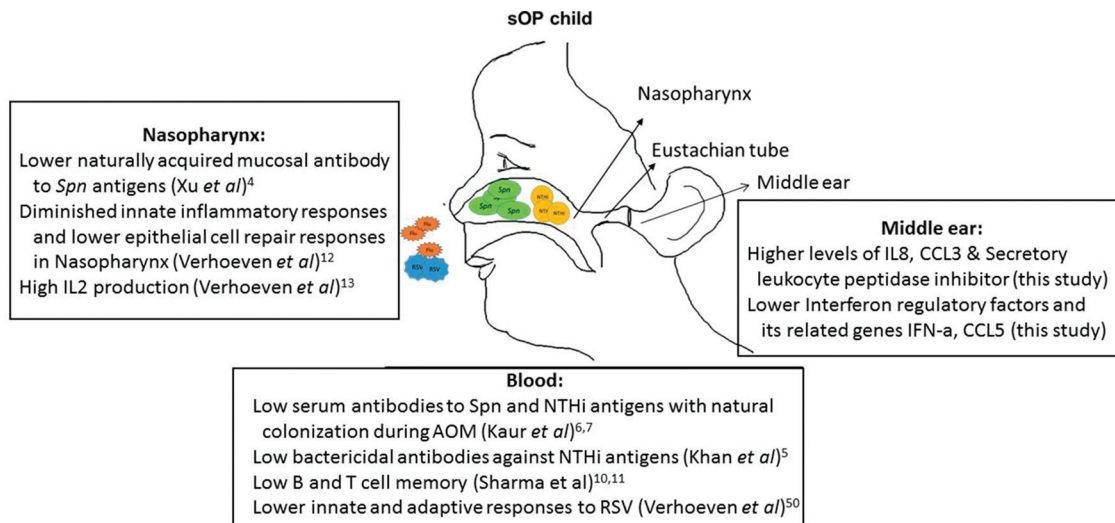


Figure 3. Overall immune responses found in the different compartments (blood, nasopharynx, and middle ear) of children with stringently defined otitis prone (sOP).

require further study. Because we previously showed differences in the transcriptome from peripheral blood mononuclear cells when young children experience AOM caused by *S. pneumoniae* versus nontypeable *H. influenzae*.^{41,42} we sought to determine if differences in the innate immune response gene expression occurred in MEF of children who were sOP versus children who were NOP. We found that most of the genes showed a similar pattern, consistent with our previous study²¹ However, a few genes showed pathogen-specific differences, including CARD downregulation in children who were sOP caused by *S. pneumoniae*.

Our study had some limitations. Differences in fluid volumes of MEF and dilutions of the samples could affect the levels of detection of messenger RNA and proteins. We sought to mitigate this by normalizing the data with total protein concentrations in MEF. To minimize variability attributable to other aspects of using human samples, we selected samples from children of similar age, race, and URI time points during an AOM event, and restricted to two major otopathogens, nontypeable *H. influenzae* and *S. pneumoniae*. Our results could be attributed to concurrent viral URI, bacterial colonization, or a combination of the two as well as AOM when the samples were collected. Previous studies that used a conventional culture method showed that 15% of the cases of AOM have bacteria and virus together in MEF and that 5% of MEFs contain viruses alone^{36,43}; by using molecular diagnostics assays, bacteria and virus together have been detected in up to 66% of AOM cases,^{43–47} therefore, the gene expression difference shown in this study could be attributed to viral co-infection in some of the children. Because IL-8 and IRF7 gene pathways are also affected by viral infection,^{48,49} it is not possible to determine if the noted gene expression differences are an epiphenomenon secondary to viral infection or represent a primary host immune response defect.

CONCLUSION

Our group previously identified adaptive and innate immunologic deficiencies in blood associated with the propensity to recurrent AOM infections in children who were sOP.^{6,7,8–11,14,50} Our previous work at the mucosal level in the nasopharynx^{12,13,50,51} identified dysfunction in multiple immune cells that play a key role in immunologic control of bacterial and viral infections in children who were sOP. The overall findings by our group to date are depicted in Fig. 3. As our group continues to study children who are sOP, we anticipate defining additional clinically significant aberrations in their immune sys-

tem. Here, we showed the innate immune response of children who were sOP differed from that of children who were NOP when measurements were made from MEF. The defect in innate response occurring in the middle ear likely contributes to otitis proneness.

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