Impaired Proinflammatory Response in Stringently Defined Otitis-prone Children During Viral Upper Respiratory Infections

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Background. Viral upper respiratory infections (URIs) are common and often precipitate acute otitis media (AOM), caused by bacterial otopathogens, in young children. Acute inflammatory responses initiated in the early phase of viral URI contribute to preventing the development of AOM. Stringently-defined otitis-prone (sOP) children are susceptible to recurrent AOM.

Methods. We assessed proinflammatory cytokine and chemokine levels in the nasopharynxes during viral URIs, and examined the different nasopharyngeal responses between viral URI events and the following AOM episodes in both sOP and non-otitis-prone (NOP) children.

Results. The sOP children exhibited significantly more AOM episodes per child (8.86-fold higher), viral URIs ($P < .0001$), and viral URIs followed by AOMs ($P < .0001$) than the NOP children. The sOP children had lower nasal proinflammatory levels of interleukin (IL)-6 ($P = .05$), IL-10 ($P = .001$), tumor necrosis factor (TNF)- α ($P = .004$), and regulated on activation, normal T-cell-expressed and -secreted (RANTES; $P = .002$) than NOP children during viral URIs. NOP children had higher levels of IL-6 ($P = .02$), IL-10 ($P = .02$), interferon- γ ($P = .003$), TNF- α ($P = .006$), IL-1 β ($P = .022$), monocyte chemoattractant protein 1 ($P = .028$), RANTES ($P = .005$), IL-2 ($P = .002$), and IL-17 ($P = .007$) during viral URIs versus AOMs following the URIs, when compared to sOP children.

Conclusions. We conclude that sOP children have more frequent viral URIs than NOP children, due to deficient antiviral nasopharyngeal proinflammatory cytokine and chemokine responses.

Keywords. acute otitis media; cytokine; chemokine; innate immune response; nasopharyngeal colonization.

Viral upper respiratory tract infections (URIs) are caused by rhinoviruses, coronaviruses, enteroviruses, respiratory syncytial viruses (RSVs), influenza A and B viruses, parainfluenza viruses (PIVs), adenoviruses, and human metapneumoviruses [1, 2]. Innate and adaptive immune responses play a key role in combating viral URIs [3]. Viruses activate multiple target cells in the upper respiratory tract to produce an array of proinflammatory cytokines and chemokines [3, 4]. Proinflammatory responses are the early wave of innate immune defenses against viral URIs that limit the proliferation of viral pathogens and activate adaptive immune responses [3, 4].

Acute otitis media (AOM) is a common pediatric infection. *Streptococcus pneumoniae* (Spn), non-typeable *Haemophilus influenzae* (NTHi), and *Moraxella catarrhalis* (Mcat) are 3 species of bacteria that frequently cause AOMs [5]. When viral URIs occur in young children, these bacteria, residing in the

nasopharynx (NP), may cause AOMs as a complication of the viral URIs [6]. Viruses have been shown to increase the adherence of bacteria to epithelial cells [7], suppress neutrophil functions [8], and delay the clearance of bacteria from the middle ear to facilitate the development of AOMs [2, 7, 9].

Viruses induce a broad spectrum of acute, proinflammatory cytokines/chemokines during URIs, which may promote or inhibit bacterial colonization in the NP and progression to AOMs [3, 4, 7]. Inflammation is a double-edged sword [3, 10]. Controlled and modest inflammatory responses can enhance the host's immunity against viruses and decrease bacterial colonization and infection, whereas excessive, uncontrolled inflammatory responses may increase the susceptibility to bacterial colonization and secondary infection and facilitate AOM pathogenesis [10]. Therefore, the NP proinflammatory response induced by respiratory viruses may play a significant role in the initiation and development of secondary bacterial AOM.

Otitis-prone (OP) children experience frequent AOMs. We have adopted a standard definition of OP as ≥ 3 AOM episodes in 6 months or ≥ 4 AOM episodes in 12 months. Our group introduced the concept of the stringently-defined otitis-prone (sOP) child, for whom every AOM episode is confirmed by the culture of bacterial otopathogens from middle-ear fluid (MEF), collected by tympanocentesis [11, 12]. Children who have 0–2 AOM episodes in 6 months or 0–3 AOM episodes in 12 months

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are termed non-otitis-prone (NOP) [12–15]. We previously demonstrated multiple immune deficits in sOP children and proposed that these children experience a prolonged, neonatal-like immune profile [12]. In a prior study, we showed that RSVs inhibit the phagocytosis of *Spn* by neutrophils, which may contribute to the increased frequency of AOMs in sOP children [16]. We also showed that sOP children have lower epithelial cell repair responses and lower expression of proinflammatory cytokines in the nasopharynx during AOMs with concurrent viral URIs [17].

In this work, we examined the frequency of clinically-diagnosed viral URIs (hereafter, viral URIs), AOMs, and the otopathogens causing the AOMs in both sOP and NOP children. We sought to further explore the contribution of viral URIs to the pathogenesis of recurrent AOMs in sOP children by comparing the levels of proinflammatory cytokines/chemokines during viral URIs that did not precipitate AOMs versus viral URIs that did precipitate AOMs and studying the difference of the levels of proinflammatory cytokines/chemokines in sOP versus NOP children during these clinical events.

MATERIALS AND METHODS

Study Cohort

The samples were obtained during a prospective study, as previously described [18, 19]. For this study, we assessed 645 children, who were followed prospectively until 36 months of age from 2010–2015 (Table 1). Samples were obtained 7 times during periodic visits at 6, 9, 12, 15, 18, 24, and 30–36 months of age. Whenever children experienced an AOM, a confirmatory tympanocentesis was performed and MEF was microbiologically assessed (an essential component to the definition of “stringently-defined”). Samples were also collected at the time of the clinical diagnosis of an AOM and 3 weeks following an AOM (convalescent stage). A study physician clinically diagnosed children with viral URIs by the symptoms of nasal congestion, rhinorrhea, coughing, and/or sore throat with or without fever, as previously described [20–22]. The Rochester General Hospital Research Subjects Review Board approved the study (CIC 1141-B-09-1).

Sample Collection

Serum, NP, and MEF sampling were conducted as previously described [18, 23]. The nasal wash supernatant was stored at –80°C until used for quantification of cytokines, chemokines, and total protein.

Microbiology

NP swabs (FLOQSwabs, COPAN) and MEF samples were used to identify *Spn*, *NTHi*, and *Mcat* by culture-based methods, as described previously [18, 24]. Viral ribonucleic acid was isolated using extraction kits (Qiagen) and amplified by real-time reverse transcription polymerase chain reaction

Table 1. Demographics of the Subjects

Item	n (% of Total)
Sex	
Male	310 (48%)
Female	335 (52%)
Ethnicity	
White	512 (79%)
African American	42 (7%)
Other	91 (14%)
OM susceptibility	
NOP	571 (89%)
sOP	74 (11%)
Breastfeeding	
<6 months	413 (64%)
≥6 months	232 (36%)
Atopy	
Yes	181 (28%)
No	464 (72%)
Daycare	
None	458 (71%)
Home or center	187 (29%)
Smokers in the home	
Yes	65 (10%)
No	580 (90%)
Familial history of AOM	
Yes	284 (44%)
No	361 (56%)
Sibling status	
No siblings	265 (41%)
≤5 years old	296 (46%)
>5 years old	31 (5%)
<5 years and >5 years	53 (8%)

Abbreviations: AOM, acute otitis media; NOP, non-otitis-prone; OM, otitis media; sOP, stringently-defined otitis-prone.

using RSVa, RSVb, PIV3, influenza A, influenza B, adenovirus b, adenovirus c, enterovirus, and rhinovirus kits specific for each virus (Primer Design, United Kingdom), as previously described [16].

Cytokine/Chemokine Assay

Interleukin (IL)-8; IL-6; IL-10; macrophage inflammatory protein (MIP)-1α; interferon (IFN)-γ; tumor necrosis factor (TNF)-α; IL-1β; monocyte chemoattractant protein (MCP)-1; IFN-α2; regulated on activation, normal T-cell-expressed and –secreted (RANTES); IL-2; IL-4; and IL-17 levels in nasal washes were detected using the Milliplex MAP human cytokine/chemokine magnetic bead panel kit (EMD Millipore, Billerica, Massachusetts). The plates were read on a Luminex 200 and a High-Throughput Fluidics instrument (Bio-Rad, Hercules, California). The cytokine/chemokine concentration (pg/ml) was normalized by the concentration of the total protein (μg/ml) in the nasal wash, which was quantitated using a micro bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Rockford, Illinois).

Statistical Analysis

Either Fisher's exact test or the Chi-square test was used to assess the difference of percentages of URIs, URIs followed by AOMs, and NP colonization of otopathogens. A ratio-paired *t*-test was employed to analyze cytokine differences between URI and AOM visits. *P* values of <.05 were considered significant.

Logistic regression analysis was used to assess the relationship between sOP classification frequency and cytokines/chemokines. Details of the analysis are described in the Figure 5 legend and R statistical software environment was used for this analysis [25]. *P* values of <.05 were considered significant.

RESULTS

Frequency of Acute Otitis Media, Viral Upper Respiratory Tract Infection, and Acute Otitis Media Preceded by Upper Respiratory Tract Infection in Non-otitis-prone and Stringently-defined Otitis-prone Children

We compared the frequency of AOM episodes per child in the NOP and sOP cohorts in the study population. The averages were 0.50 and 4.43 AOM episodes/child in 571 NOP and 74 sOP children, respectively, an 8.86-fold difference.

We tested 225 samples by polymerase chain reaction for identification of a respiratory virus. We detected RSVa (21%), RSVb (8%), PIV3 (16%), influenza A (6%), influenza B (1%), adenovirus b (7%), adenovirus c (17%), enterovirus (8%), and rhinovirus (19%), with 3% of children being detected with more than one viral species per subject, consistent with a prior report from our group [16].

There were 586 and 136 viral URI episodes in 2129 and 348 periodic sampling visits of 571 NOP and 74 sOP children, respectively (Figure 1). The sOP children had a higher viral URI frequency (39.1%) than the NOP children (27.5%; Figure 1, $P < .0001$; relative risk of sOP:NOP = 1.42). There were 259 and 215 clinically-diagnosed viral URI episodes at 283 and 235 AOM visits in 196 NOP and 68 sOP children, respectively (Figure 1). Both sOP (91.5%) and NOP (91.5%) children had a similarly high frequency of concurrent viral URIs when bacterial AOMs occurred ($P = 1.000$, Figure 1).

The sOP children had higher rates of AOMs ≤ 4 days (61.5%, $P < .0001$), ≤ 1 week (60.7%, $P < .0001$), and ≤ 2 weeks (61.4%, $P < .0001$) after a viral URI than the NOP children (29.0%, 30.7%, and 30.8%, respectively; Figure 1). The relative risk of AOM for sOP:NOP children was 2.12, 1.98, and 1.99 for ≤ 4 days, ≤ 1 week, and ≤ 2 weeks after a viral URI, respectively, suggesting that sOP children are more likely to get an AOM than NOP children 4–14 days after a URI (Figure 1).

Frequency of Nasopharyngeal Colonization of Bacterial Otopathogens in Non-otitis-prone and Stringently-defined Otitis-prone Children at Healthy, Viral Upper Respiratory Tract Infection, and Acute Otitis Media Visits

We calculated the NP colonization frequencies of *Spn*, NTHi, and *Mcat* for different health statuses: healthy, URI, and AOM visits were examined for 1525 and 211 visits of 571 NOP and

Figure 1. Rates of upper respiratory tract infection (URI) and URI followed by acute otitis media (AOM) in non-otitis-prone (NOP) and stringently defined otitis-prone (sOP) children. We evaluated 571 NOP and 74 sOP children aged 6–36 months old. A, URI rates at periodic sampling visits and AOM visits. URI rates were assessed for 2129 and 348 periodic sampling visits of 571 NOP and 74 sOP children, respectively, and 283 and 235 AOM visits of 196 NOP and 68 sOP children, respectively. B, Rates of URI followed by AOM. Rates were evaluated for URIs ≤ 4 days, ≤ 1 week, and ≤ 2 weeks after the initiation of a URI, for 845 URI visits of 571 NOP children and 350 URI visits of 74 sOP children. All the differences of the percentage between sOP and NOP children were analyzed by using Fisher's exact test, except that the difference of URI rates at periodic sampling visits were assessed by using chi-square approximation. Data are represented as percentage $\pm$ 95% confidence interval. Abbreviations: AOM, acute otitis media; NOP, non-otitis-prone; sOP, stringently-defined otitis-prone; URI, upper respiratory tract infection.

74 sOP children, respectively. The sOP children had a higher frequency of NP colonization by *Spn* (47.6%, $P < .0001$), NTHi (32.2%, $P < .0001$), *Mcat* (48.0%, $P < .0001$), and ≥ 1 of the any of the 3 otopathogens (*Spn*/NTHi/*Mcat*, 81.8%, $P < .0001$) than the NOP children (31.1%, 11.3%, 36.0%, and 56.8%, respectively) in all 3 health states examined (Figure 2). The

Figure 2. Rates of nasopharyngeal (NP) colonization of otopathogens in non-otitis-prone (NOP) and stringently-defined otitis-prone (sOP) children at healthy, upper respiratory tract infection (URI) only, and acute otitis media (AOM) visits. We examined 571 NOP and 74 sOP children aged 6–36 months old. **A.** Rates of NP colonization of otopathogens. The rates of NP colonization with *Spn*, *NTHi*, *Mcat*, and ≥ 1 of any of the 3 otopathogens (*Spn/NTHi/Mcat*) were examined for 2406 visits of 571 NOP children and 571 visits of 74 sOP children. **B.** Rates of NP colonization of the 3 otopathogens at healthy, URI, and AOM visits. The rates of NP colonization of ≥ 1 of any of the 3 otopathogens (*Spn*, *NTHi*, and *Mcat*) were examined for 1525 healthy, 582 URI-only, and 275 AOM visits of 571 NOP children, and 211 healthy, 135 URI-only, and 224 AOM visits of 74 sOP children. All the differences of the percentages between sOP and NOP children were analyzed by using Fisher's exact test, except that the difference of rates for NP colonization of *Spn/NTHi/Mcat* were assessed by using chi-square approximation. Data are represented as a percentage $\pm$ 95% confidence intervals. Abbreviations: AOM, acute otitis media; *Mcat*, *Moraxella catarrhalis*; NOP, non-otitis-prone; NP, nasopharyngeal; *NTHi*, non-typeable *Haemophilus influenzae*; sOP, stringently-defined otitis-prone; *Spn*, *Streptococcus pneumoniae*; URI, upper respiratory tract infection.

sOP children showed a higher frequency of NP colonization of ≥ 1 of any of the 3 bacterial otopathogens at healthy visits (66.8%, $P < .0001$), URI-only visits (84.4%, $P = .018$), and

AOM visits (94.2%, $P = .039$) than the NOP children (44.5%, 74.6%, and 88.7%, respectively; Figure 2). The sOP children also exhibited a higher frequency of NP colonization with each otopathogen of *Spn*, *NTHi*, and *Mcat* in each health condition of healthy, URI, and AOM visits, respectively, than the NOP children (Supplementary Figure S1).

Comparison of Nasopharyngeal Cytokine and Chemokine Levels Between Viral Upper Respiratory Tract Infection and Acute Otitis Media for Stringently-defined Otitis-prone and Non-otitis-prone Children

To investigate the contribution of acute inflammatory responses to the OP condition, we evaluated differences of NP levels of proinflammatory cytokines/chemokines between viral URIs and their following AOMs in young children. Each nasal wash sample at a viral URI visit was paired within 1–2 weeks with a nasal wash sample obtained at the subsequent AOM visit, for both sOP and NOP children. To correct for differential dilution effects that occurred during the nasal wash sample collection, the cytokine/chemokine concentration was normalized by the concentration of the total protein and expressed as pg/ μ g. No difference was found between the 2 study groups at healthy visits ($P = .9$) or AOM visits ($P = .3$) for total protein concentrations (data not shown). We found higher nasal levels of IL-6 ($P = .024$), IL-10 ($P = .022$), IFN- γ ($P = .003$), TNF- α ($P = .006$), IL-1 β ($P = .022$), MCP-1 ($P = .028$), RANTES ($P = .005$), IL-2 ($P = .002$), and IL-17 ($P = .007$) at periodic sampling visits when a viral URI was present than at the subsequent AOM visits in NOP children (Figure 3). There were no differences for nasal chemokines IL-8 ($P = .11$) and MIP-1 α ($P = .39$) between URI and AOM visits in NOP children (Figure 3). In sOP children, no differences were found for nasal cytokines and chemokines IL-8 ($P = .17$), IL-6 ($P = .19$), IL-10 ($P = .85$), MIP-1 α ($P = .13$), IFN- γ ($P = .91$), TNF- α ($P = .39$), IL-1 β ($P = .31$), MCP-1 ($P = .87$), RANTES ($P = .15$), IL-2 ($P = .94$), and IL-17 ($P = .66$) at periodic sampling visits when a viral URI was present as compared to the levels when an AOM was present following the viral URI (Figure 4).

Relationship of Cytokine and Chemokine Levels With Frequency of Stringently-defined Otitis-prone Classification at Upper Respiratory Tract Infection Visits

To study the role of proinflammatory mediators in the development of the OP condition, we measured nasal secretion levels of cytokines/chemokines for 29 periodic sampling visits of 18 sOP children and 58 periodic sampling visits of 49 NOP children. We analyzed the relationship between the NP cytokine/chemokine level and sOP rate using a logistic regression analysis, generalized additive model (GAM). The analysis was confined to samples collected when the children were aged 6 to 15 months old, because the sparsity and variability of samples in the older age cohorts precluded a meaningful interpretation. Higher NP cytokine and chemokine levels of IL-6 ($P = .05$), IL-10 ($P = .001$), TNF- α ($P = .004$), and RANTES ($P = .002$) were associated with lower sOP classification frequencies (Figure 5). Of

Cytokine and chemokine levels in nasal wash of NOP children

Figure 3. Comparison of cytokine and chemokine levels between upper respiratory tract infection (URI) and acute otitis media (AOM) visits for non-otitis-prone (NOP) children. IL-8, IL-6, IL-10, MIP-1 α , IFN- γ , TNF- α , IL-1 β , MCP-1, RANTES, IL-2, and IL-17 levels in nasal washes were measured for URI visits and the AOM visits following the URIs in 18 NOP children using a Milliplex MAP human cytokine/chemokine magnetic bead panel kit. The cytokine/chemokine concentration (pg/ml) was then normalized by the concentration of the total protein (μ g/ml) in the nasal wash, which was quantitated using a micro bicinchoninic acid protein assay kit. A ratio-paired *t*-test was used to analyze the differences of the normalized cytokine/chemokine levels (pg per μ g of total protein). Data are represented as geometric means $\pm$ 95% confidence intervals. Abbreviations: AOM, acute otitis media; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; NOP, non-otitis-prone; RANTES, regulated on activation, normal T-cell-expressed and -secreted; TNF, tumor necrosis factor; URI, upper respiratory tract infection.

these proinflammatory mediators, IL-10, TNF- α , and RANTES were significant, with a false discovery rate <0.02 (Benjamini-Hochberg procedure). IL-8 ($P = .05$) and IL-2 ($P = .07$) showed positive relationships with the sOP classification frequency, but the relationships were not statistically significant (Figure 5).

DISCUSSION

Viral URIs and AOMs are common respiratory infections in young children. We found that about 92% of AOM events in both sOP and NOP children had a preceding, clinically-diagnosed viral URI. Mechanistically, for the first time we have shown that sOP children have deficient innate immune responses, as reflected in their NP proinflammatory responses during viral URIs. We further determined that NOP children have higher NP proinflammatory responses when viral URIs occur as compared

to the AOMs following the viral URIs, reflecting better innate immune responses associated with a reduced likelihood of secondary bacterial AOM infections. This study also showed that lower NP cytokines IL-10, TNF- α , and RANTES are associated with higher sOP classification frequencies, suggesting an association of sOP propensity and lower proinflammatory cytokine responses in the NP during viral URIs.

In the current study, we compared the inflammatory levels when clinically-diagnosed viral URIs were present versus when AOMs was present following viral URIs. Our study suggests that NOP children produce robust antiviral proinflammatory responses with higher levels of cytokines/chemokines during URIs, whereas sOP children do not, which reflects deficient innate immune responses to control acute respiratory tract viral infections in sOP children. This is consistent with our prior observation that sOP children have diminished antiviral

Cytokine and chemokine levels in nasal wash of sOP children

Figure 4. Comparison of cytokine and chemokine levels between upper respiratory tract infection (URI) and acute otitis media (AOM) visits for stringently defined otitis-prone (sOP) children. IL-8, IL-6, IL-10, MIP-1 α , IFN- γ , TNF- α , IL-1 β , MCP-1, RANTES, IL-2, and IL-17 levels in nasal washes were measured for URI visits and the AOM visits following the URIs in 21 sOP children using a Milliplex MAP Human cytokine/chemokine magnetic bead panel kit. The cytokine/chemokine concentration (pg/ml) was then normalized by the concentration of the total protein (μ g/ml) in the nasal wash, which was quantitated with a micro bicinchoninic acid protein assay kit. A ratio-paired *t*-test was used to analyze the differences of the normalized cytokine/chemokine levels (pg per μ g of total protein). Data are represented as geometric means $\pm$ 95% confidence intervals. Abbreviations: AOM, acute otitis media; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T-cell-expressed and -secreted; sOP, stringently-defined otitis-prone; TNF, tumor necrosis factor; URI, upper respiratory tract infection.

adaptive immune responses with lower neutralizing antibodies against RSV and a higher burden of nasal RSV infections than NOP children [16]. Our results are consistent with a previous observation that children with recurrent AOMs (otitis-prone) exhibited significantly lower levels of IL-1 β , IL-6, and TNF- α in NP secretions than healthy (NOP) children [21]. In contrast, Patel et al found that IL-1 β concentrations were higher during URI episodes with AOMs than those without AOMs, suggesting higher IL-1 β production is associated with the development of AOMs [22]. In their study, they did not differentiate OP from NOP children and did not do paired comparisons for each subject [22]. In addition, the ages of the children were within the range of 6–36 months old in their study [22]. Therefore, our current work elaborated the relationship between URIs and AOMs further by using paired comparisons for each subject and stratified analyses on each separate child population of sOPs and NOPs.

Bacterial colonization is an important event for the progression of a viral URI to a bacterial AOM. Influenza A virus, adenoviruses, and RSVs all are able to enhance the binding of otopathogens *Spn* and *NTHi* to human respiratory epithelial cell lines [7]. Although the detailed mechanisms are still unknown, a viral infection activates respiratory epithelial cells and induces the increased adherence of bacteria to the cells. The colonized bacteria then ascend to the middle ear through the dysfunctional Eustachian tubes induced by viral infections, to cause an AOM [7]. A controlled level of acute inflammation in the NP of children during viral URIs may help to inhibit bacterial adherence to respiratory epithelial cells. We found that NOP children experienced an ailment consistent with controlled viral URIs, since their illnesses frequently resolved without progressing to AOMs, whereas sOP children frequently experienced uncontrolled viral URIs that did progress to AOMs. The mechanism

Figure 5. Logistic regression analysis of the relationship between cytokine and chemokine levels and the rate of stringently-defined otitis-prone (sOP) children at upper respiratory tract infection (URI) only visits. IL-8, IL-6, IL-10, MIP-1 α , IFN- γ , TNF- α , IL-1 β , MCP-1, IFN- α 2, RANTES, IL-2, IL-4, and IL-17 levels in nasal washes were measured for 29 URI visits of 18 sOP children and 58 URI visits of 49 non-otitis-prone children using a Milliplex MAP human cytokine/chemokine magnetic bead panel kit. The cytokine/chemokine concentration (pg/ml) was then normalized by the concentration of the total protein (μ g/ml) in the nasal wash, which was quantitated with a micro bicinchoninic acid protein assay kit. The cytokine level (pg per μ g of total protein) was log-transformed and, where possible, the log mean of the 2 duplicates was used as a single outcome. Cytokine/chemokine levels below the detection limits were treated as missing, because the protein concentrations were generally very low or not detectable, suggesting poor success in obtaining an adequate nasal wash sample. For 10 of 13 cytokine/chemokines, repeated measures were available. Kernel smoothers were used to assess the relationship between cytokine/chemokine levels and sOP classification frequencies. In general, little change in the sOP classification frequencies was evident at lower cytokine/chemokine levels. In some cases, rapid changes in sOP classification frequencies were observed at higher cytokine/chemokine levels. In addition, even after log transformation, outlier cytokine/chemokine levels were evident, with potentially disproportionate influences on any regression models. Therefore, cytokine/chemokine levels were transformed into ranks and then normalized into the unit interval. To model the rapid change in sOP classification frequencies at higher cytokine/chemokine levels, a power transformation was then applied to the rescaled levels. This predictor was then used in a logistic regression model: $P(OP) = \phi(\eta)$, where, for transformed cytokine/chemokine level X , $\eta = \beta_0 + \beta_1 X^k$ and $\phi(\eta) = e\eta / (1 + e\eta)$. Application of the Bayesian information criterion yielded an optimal choice of k near 8 for those cytokines/chemokines with a significant association with the sOP classification frequency, so this parameter was used for the remaining analysis. The data contain subject-level repeated measures, but since sOP is fixed for each subject, logistic regression models that incorporate subject-level correlations, such as generalized estimating equation models, are unable to produce stable model estimates. Therefore, in order to assess significance, a bootstrap procedure with subject-level resampling was used. To model the rapid change in sOP rates at higher cytokine levels, a power transformation was then applied to the rescaled cytokine levels. In order to assess significance, a bootstrap procedure with subject-level resampling was used. Abbreviations: AOM, acute otitis media; IFN, interferon; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; RANTES, regulated on activation, normal T-cell-expressed and -secreted; sOP, stringently-defined otitis-prone; TNF, tumor necrosis factor; URI, upper respiratory tract infection.

appears to be relatively higher levels of acute proinflammatory cytokines/chemokines in the NP of NOP children during viral URIs, resulting in better protection as compared to sOP children.

Although 74 sOP children were identified in this study, there were only 22 paired samples that were collected from children during URI visits and subsequent AOM visits within a time interval of 1–2 weeks. An AOM bacterial infection is generally

regarded as a secondary infection following a primary viral infection. Our previous study has shown that >90% AOM bacterial infections have a preceding viral infection [26]. The paired samples are most likely representative of viral and bacterial infection states in the same episodes. Therefore, we only used the paired samples to further evaluate differences in innate immunity. The same sample size was randomly selected from all paired samples that were collected from age-matched NOP children and that met the criterion. This experimental design resulted in a reduced sample size, but allowed the results to be optimally comparable.

This study has limitations. We did not differentiate the viral pathogen causing each URI, due to limited samples available to analyze each virus-specific illness. Different viruses may elicit different innate and adaptive immune responses. In a prior study, we showed that RSVs have a higher interference effect on the phagocytosis activity of neutrophils than PIVs [16]. However, there are common innate immune pathways in target cells that are activated by various viruses to produce an array of proinflammatory mediators during a URI, such as the cytokines/chemokines secreted by the proinflammatory cells in the NP that were analyzed in this work [4]. The URI episodes reported here were recorded and sampled only from the periodic sampling visits and AOM visits. Therefore, we missed some URI episodes when the children did not come to our clinical sites to seek medical care, which may have affected the rates associated with URIs. But this would not significantly influence the outcome of comparisons between sOP and NOP children, because all information and samples were collected under the same conditions. A recent study has shown that viral infections are associated with the nasopharyngeal density of common otopathogens and the subsequent risk of otitis media [27], which is consistent with our current findings. However, we did not quantify the bacterial loads in our study.

In conclusion, sOP children have more frequent viral URIs, lower acute inflammatory responses during viral URIs, and a high frequency of NP colonization by bacterial otopathogens and viral URIs progressing to bacterial AOMs. Modulating the acute inflammation induced by viral infections during URIs may be beneficial to protect from recurrent AOMs in sOP children.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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