Differential Impact of Respiratory Syncytial Virus and Parainfluenza Virus on the Frequency of Acute Otitis Media Is Explained by Lower Adaptive and Innate Immune Responses in Otitis-Prone Children

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Background. Acute otitis media (AOM) is a leading cause of bacterial pediatric infections associated with viral upper respiratory infections (URIs). We examined the differential impact of respiratory syncytial virus (RSV) and parainfluenza virus URIs on the frequency of AOM caused by Streptococcus pneumoniae (Spn) and nontypeable Haemophilus influenzae (NTHi) in stringently defined otitis-prone (sOP) and non-otitis-prone (NOP) children as a potential mechanism to explain increased susceptibility to AOM.

Methods. Peripheral blood and nasal washes were obtained from sOP and NOP children (n = 309). Colonization events and antiviral responses consisting of total specific immunoglobulin G (IgG) responses, neutralizing antibody responses, and T-cell responses were determined. Isolated neutrophils were infected with varying multiplicities of infection of both viruses, and opsonophagocytosis potential was measured.

Results. A significant increase was found in frequency of AOM events caused by Spn and NTHi, with a concurrent RSV infection in sOP children. These results correlated with diminished total RSV-specific IgG, higher viral nasal burdens, and lower IgG neutralizing capacity. The sOP children had diminished T-cell responses to RSV that correlated with lower Toll-like receptor 3/7 transcript and decreased expression of HLA-DR on antigen-presenting cells. RSV interfered with the Spn phagocytic capacity of neutrophils in a dose-dependent manner. Parainfluenza virus infections did not differentially affect AOM events in sOP and NOP children.

Conclusions. Lower innate and adaptive immune responses to RSV in sOP children may slow the kinetics of viral clearance from the nasopharynx and allow for viral interference with antibacterial immune responses, thus contributing to increased frequency of AOMs.

Keywords. adaptive immune responses; acute otitis media; otitis-prone children; respiratory syncytial virus; parainfluenza virus.
Differences in sOP and NOP children in innate and adaptive immunological control of bacterial and viral infections could impact whether nasopharyngeal (NP) bacterial colonization during a concurrent viral upper respiratory infection (URI) proceeds to AOM, sinusitis, or pneumonia. Prior work has shown an association of AOM with concurrent respiratory syncytial virus (RSV)–induced URI [6-9]. RSV is also a significant pathogen in the elderly [10, 11], likely due to immunosenescence; our study may have implications for RSV infections in the elderly.

We have previously shown that sOP children have a poor adaptive immune response to Spn and NTHi [12]. Children generate antibody responses to RSV; however, memory to RSV is generally poor [13, 14]. Lower adaptive immune responses to viral replication in the nasal or lung mucosa.

This is the first study to examine the contribution of immune deficits in driving higher upper respiratory bacterial infection rates during RSV infection. We included human parainfluenza virus type 3 (hPIV3) as a comparator because both viruses commonly cause respiratory viral infections, albeit during different seasons of the year [15], there is no vaccine for either, and they share many similar characteristics with respect to host response modifications by viral proteins [16, 17].

MATERIALS AND METHODS

Patients

Children were enrolled at age 6 months in a prospective study and defined as sOP or NOP as previously described [1-3]. NP secretions were obtained from NP washes [18]. Whenever a child experienced an AOM, blood, NP secretions, and middle ear fluid were obtained by tympanocentesis. Bacterial identification was accomplished as previously reported [3]. The Rochester General Hospital research subjects review board approved the study.

Respiratory Viral Diagnosis

Viral species were identified in NP samples obtained with flocked brushes. Viral RNA was isolated using Viral RNA extraction kits (Qiagen), amplified by real-time reverse transcription polymerase chain reaction (qRT-PCR) using RSVa, RSVb, hPIV3, influenza A, influenza B, adenovirus, enterovirus, and rhinovirus kits specific for each virus (Primer Design, UK) and concentration determined per the manufacturer’s instructions.

Viral Expansions

RSVa (A2), RSVb (B WV/14617/85), and hPIV3 (C 243) were obtained from ATCC (Manassas, Virginia) and grown in Hep2 cells (RSV) or LLC-MK2 cells (hPIV3) for 7 days prior to harvest. Supernatants were clarified by centrifugation at 14 000 RPM for 15 minutes, and viral aliquots were frozen at −80°C.

Antibody Assays

RSVa/b, or hPIV3 viral particles, 1 × 10⁶/mL in phosphate-buffered saline, were heat-inactivated (65°C for 30 minutes) and added to Immulon II enzyme-linked immunosorbent assay plates (ThermoFisher, Hampton, New Hampshire) overnight at 4°C. Plates were blocked with nonfat milk. Child and reference plasma (obtained from adults with known immunoglobulin G [IgG] concentrations) antibody levels were assessed.

Viral Neutralizations

RSVa or hPIV3 (multiplicity of infection [MOI] of 1) were incubated with serially diluted plasma (heat inactivated) for 1 hour at 37°C in Dulbecco modified Eagle’s medium without fetal bovine serum (FBS). RSV neutralization was determined using an MTT (Promega, Fitchburg, Wisconsin) assay [19]. hPIV3 neutralization was determined similar to other studies [20].

Hemagglutination Inhibition Titers

Hemagglutination inhibition (HAI) titers were determined as previously described [21].

Nasal Cytokine Assays

Frozen nasal washes were thawed and total protein was calibrated to 1 mg/mL using a bicinchoninic acid assay (Pierce Scientific, Rockford, Illinois). Two-hundred-microliter aliquots were assayed for cytokines by ElisaMax (BioLegend, San Diego, California) for interferon (IFN) γ, interleukin (IL) 2, IL-6, IL-1α, and IL-1β.

T-Cell Assays

Peripheral blood mononuclear cells (PBMCs) were thawed and rest at 37°C. RSVa, RSVb, or hPIV3 was added at an MOI of 0.1. After 18 hours of infection, brefeldin A (10 µg/mL) and anti-CD28 (1 µg/mL) were added and cells cultured for 6 hours prior to harvest. Additional cells were cultured in the presence of brefeldin A with or without Staphylococcal enterotoxin B (positive and negative controls, respectively). Cells were surface-stained using the LIVE/DEAD amine dye (Invitrogen, Carlsbad, California) and antibodies to CD4, CD3, and CD69 (BioLegend) followed by intracellular staining for IFN-γ, IL-2, IL-4, IL-5, IL-17a, and tumor necrosis factor α (BioLegend). Data were acquired on an LSRII (BD Biosciences) with live amine dye and doublet discrimination and analyzed with FlowJo software (TreeStar, Ashland, Oregon).

Antigen-Presenting Cell Analysis

PBMCs were thawed and rest overnight at 37°C and infected with either RSVa or hPIV3 at an MOI of 0.1. HLA expression was compared by flow cytometry prior to infection and

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24 hours postinfection on dendritic cells (DCs) (CD3, CD20, CD56, CD14 negative gating with HLA-DR and CD11c positive gating) with plasmacytoid dendritic cells/myeloid dendritic cells determination by antibodies for CD123 and B cells (CD3, CD56, CD14 negative gating, IgM positive staining).

**qRT-PCR for Toll-like Receptors**

PBMCs were thawed and rested overnight at 37°C followed by CD3 and CD20 magnetic depletion. Cellular RNA was extracted utilizing a RNA Plus extraction kit (Qiagen, Gaithersburg, Maryland). One hundred nanograms of RNA was reverse-transcribed using SuperscriptIII (Invitrogen). qRT-PCR was used with SYBR Green (Bio-Rad, Hercules, California) to amplify targets using G3PDH as an internal calibrator or Toll-like receptor (TLR) 2, 3, 4, 7, and 8 primers designed using Primer-Bank [22] (Harvard, Cambridge, Massachusetts). Data were calculated by the $\Delta\Delta$CT method and expressed as fold change between groups. Each sample was amplified twice and averaged.

**Phagocytosis Assay**

Neutrophils were isolated from blood of sOP or NOP children by density gradient centrifugation after Ficoll separation of PBMCs. Cells were infected with increasing doses of hPIV3 or RSVa at an MOI of 0.001, 0.01, 0.1, 1, and 10, and incubated overnight at 37°C. Cells were washed and resuspended in RPMI 10% FBS with no antibiotics. Heat-treated human plasma containing type 6A capsular antibodies and rabbit complement (Thermo Fisher) at 0.1 μg/mL were added to 0.04% vol/vol Spn serotype 6A strain BG7322 [23] grown in Todd Hewitt broth (Difco) with 1% yeast extract media to an OD600 of 0.6 and prelabeled with lipophilic PKH67 dye [24]. The bacteria, antibody, and complement mix was incubated for 20 minutes at 37°C, then added to the neutrophils at a concentration of 10 bacteria per neutrophil. After 24 hours’ incubation at 37°C, cells were washed, stained with CD15, CD1c, and CD14, and analyzed by flow cytometry with double discrimination and live amine dye. Neutrophils were identified based on CD1c, CD14 negative gating with CD15 positive gating. Bacteria were identified by fluorescence of PKH67 dye in the fluorescein isothiocyanate channel.

**Statistical Analysis**

Data were analyzed by 2-tailed student t tests, 2-tailed Mann–Whitney U tests (data with nonnormal distribution), or Fisher exact tests, and $P < .05$ was considered significant.

**RESULTS**

The sOP and NOP cohorts were similar with respect to sex, ethnicity, frequency of breastfeeding, daycare attendance, and exposure to smokers in the home and family history of otitis media (Table 1), although sOP children were more frequently atopic ($P = .05$). Infected infants in the first 6 months of life may not generate innate or adaptive responses to RSV infection similar to those >6 months old, and reinfection rates in a second RSV season are high. Those not infected in a first season of exposure are likely to be infected in a second season given the winter prevalence of RSV and lack of a licensed vaccine. In the present study, children were enrolled at 6 months of age and followed until 24 months of age. To assess the role of first season of exposure as a possible confounder, we examined baseline immune status in blood obtained at 6 months of age and found that only 1 child from each group might have been infected with RSV in a prior season. We also compared the age of children in the sOP and NOP groups and found they were well matched, with a median age of 11.9 months for both cohorts.

**Association of RSV and PIV With Bacterial NP Colonization and AOM**

Increased NP colonization by bacterial otopathogens during months that have higher rates of circulating respiratory viruses could drive more AOM events irrespective of the type of

<table>
<thead>
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<th>Table 1. Patient Characteristics</th>
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Abbreviations: NOP, non–otitis prone; OM, otitis media; sOP, stringently defined otitis prone.

$^a$ Significantly different ($P < .05$) determined by Fisher exact tests.
We next examined whether a correlation existed between frequency of Spn or NTHi NP colonization during known peak viral infection months for RSV (December–February) and hPIV3 (March–May) and concurrent development of AOM. We found that Spn/NTHi bacterial otopathogen NP colonization during RSV season was associated more often with concurrent AOM in sOP compared with NOP children ($P = .02$; Figure 1A and 1B). During March–May, NOP children showed a trend for more frequent NP colonization without development of AOM compared with sOP children ($P = .15$; Figure 1A and 1B). We examined the frequency of RSV and hPIV3 infection in sOP and NOP children with regard to infecting bacterial otopathogen and found a significant association between AOMs caused by Spn and NTHi during RSV infections in sOP children ($P < .001$; Figure 1C). In contrast, NOP children developed AOM with similar frequency during RSV and hPIV3 infections (Figure 1C). These data indicate that RSV infection causes AOM more frequently in sOP than NOP children, suggesting that sOP children may not respond to RSV infection as well as NOP children do.

**Differences in Viral Load in sOP and NOP Children**

Viral burden could have a significant impact on mucosal epithelial damage and inflammation that in turn may contribute to increased otopathogen bacterial load and subsequent AOM [9, 25]. We found that RSV viral burdens were higher in nasal washes of sOP vs NOP children during an AOM ($P = .04$; Supplementary Figure 1A). Overall, viral burden of RSVa or RSVb was higher in sOP children than in NOP children (Supplementary Figure 1B), suggesting either differential permissiveness to infection or divergent immune responses between the populations.

**Table 2. Colonization Rates of Cohort Patients**

<table>
<thead>
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<th>No. Colonized</th>
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<tr>
<td>sOP</td>
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<td>52</td>
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<tr>
<td>NOP</td>
<td>205</td>
<td>136</td>
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<table>
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<tr>
<th>No. Colonized</th>
<th>$P$ Value</th>
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<tr>
<td>Viral seasons, 2009–2011</td>
<td></td>
</tr>
<tr>
<td>sOP</td>
<td>32</td>
</tr>
<tr>
<td>NOP</td>
<td>87</td>
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$P$ values determined by Fisher exact test.

Abbreviations: NOP, non–otitis prone; NTHi, nontypeable *Haemophilus influenzae*; sOP, stringently defined otitis prone; Spn, *Streptococcus pneumoniae*.

**Figure 1.** A strong association between respiratory syncytial virus (RSV) and acute otitis media (AOM) in stringently defined otitis-prone (sOP) children. Frequency of nasopharyngeal colonization or AOM by bacterial otopathogens across seasons in sOP (A) and non-otitis-prone (NOP) (B) children. *$P = .021$, comparing the frequency of AOM of sOP vs NOP children for similar months ($n = 42$ sOP, $n = 267$ NOP, mean). C. Frequency of RSV or human parainfluenza virus type 3 (hPIV3) in sOP and NOP children with respect to either AOM or no AOM. *$P < .001$, comparing frequency of AOM between RSV and hPIV3 for sOP vs NOP children ($n = 17$ sOP and $n = 65$ NOP, mean). *Fisher exact tests were used for statistical analysis. Abbreviations: AOM, acute otitis media; NOP, non–otitis prone; NTHi, nontypeable *Haemophilus influenzae*; PIV, parainfluenza virus; RSV, respiratory syncytial virus; sOP, stringently defined otitis prone; Spn, *Streptococcus pneumoniae*.
Children Who Are sOP Have Lower Total Specific IgG Responses to RSV but Not hPIV3

Antibody-mediated viral neutralization is a strong correlate of viral control. We found lower plasma RSV-specific IgG concentrations in sOP than NOP children ($P = .02$; Figure 2A). In contrast, there was no difference in titers to hPIV3 (Figure 2A).

We also compared neutralizing functionality of the plasma antibodies and found that plasma Ig from sOP children had lower RSV neutralizing titers compared with NOP children ($P = .04$; Figure 2B). In contrast, sOP and NOP children had comparable

Figure 2. Lower respiratory syncytial virus (RSV) specific antibodies during infection are associated with lower viral control in stringently defined otitis-prone (sOP) children. A. Concentration of plasma-derived immunoglobulin G (IgG)–specific antibody to RSV and human parainfluenza virus type 3 (hPIV3) in sOP and non-otitis-prone (NOP) children during an active infection (n = 10 sOP or n = 10 NOP for each viral species, geometric mean ± SD). B. Titer of neutralizing plasma IgG for RSVa in both groups of children. Reciprocal mean ± SD log$_2$ values shown (n = 10 sOP or n = 10 NOP, mean ± SD). C. Hemagglutination inhibition titers for hPIV3 in sOP and NOP children. Reciprocal mean ± SD log$_2$ values shown (n = 8 sOP or n = 10 NOP for each virus, mean ± SD). Two-tailed Student t tests were used. Abbreviations: HAI, hemagglutination inhibition; hPIV3, human parainfluenza virus type 3; IgG, immunoglobulin G; NOP, non–otitis prone; RSV, respiratory syncytial virus; sOP, stringently defined otitis prone.

Figure 3. Respiratory viral infections interfere with neutrophil phagocytic capacities. Blood-derived neutrophils isolated and infected with either respiratory syncytial virus (RSV) a or human parainfluenza virus type 3 (hPIV3) in escalating multiplicities of infection (MOIs) for 24 hours. A. Phagocytic capacities of neutrophils from children were tested with PKH67-labeled Streptococcus pneumoniae (Spn). Neutrophils were identified by CD15 gating (n = 6 each group, representative flow shown for each group with an MOI of 1 for both hPIV3 and RSVa). * $P < .05$ between sample and no viral controls. B. Percentages of neutrophils staining positive for Spn over the range of MOIs tested for hPIV3 and RSV. * $P$ values are between groups. Mann–Whitney $U$ test was used for statistical analysis. Abbreviations: hPIV3, human parainfluenza virus type 3; MOI, multiplicity of infection; RSV, respiratory syncytial virus; Spn, Streptococcus pneumoniae.
RSV compared with NOP children (Supplementary Figure 2). Responses with fewer singlet and polyfunctional responses to levels of neutralizing antibodies in sOP children to RSV may during RSV and PIV viral control. Data indicate that sOP children have lower adaptive T-cell responses in sOP or NOP hPIV3-infected children (data not shown). These children also had fewer polyfunctional CD8 T cells (Supplementary Figure 2A). In contrast, no significant differences were identified in hPIV3-stimulated CD4 or CD8 T cells from sOP or NOP hPIV3-infected children (data not shown). These data indicate that sOP children have lower adaptive T-cell responses to RSV compared with NOP children that could affect viral control.

**Decreased T-Cell Responses to RSV in sOP Children**

Diminished adaptive control could allow for continued innate inflammatory dominance during heightened and prolonged viral infection. To explore this possibility, we studied the magnitude of cytokine responses from CD4 and CD8 T cells after recall exposure to RSV and found the responses were divergent (Supplementary Figure 2A). SOP children had lower CD4 T-cell responses with fewer singlet and polyfunctional responses to RSV compared with NOP children (Supplementary Figure 2B). All cytokines were lower in sOP children; some showed a trend, while others were significant (Supplementary Figure 2B). SOP children also had fewer polyfunctional CD8 T cells (Supplementary Figure 2C). In contrast, no significant differences were identified in hPIV3-stimulated CD4 or CD8 T cells from sOP or NOP hPIV3-infected children (data not shown). These data indicate that sOP children have lower adaptive T-cell responses to RSV compared with NOP children that could affect viral control.

**Lower Expression of TLR7/8 on Antigen-Presenting Cells From sOP Children**

To explore a mechanism accounting for diminished responses to RSV in sOP children, we measured RNA expression (relative) of TLRs from isolated blood major histocompatibility complex class II–positive (MHCI+) antigen-presenting cells (APCs) (B cells, pDCs, and mDCs) from sOP and NOP children with no current URI. We found that expression of TLR3 was lower from MHCI+ APCs of sOP compared with NOP children (P = .05; Supplementary Figure 3A). We also examined TLR RNA expression during RSV and hPIV3 infection, concentrating on TLR7/8 expression because of its importance during single-stranded RNA activation of APCs. We found that sOP children had lower expression of TLR7 during RSV compared with hPIV3 infections (P = .04; Supplementary Figure 3B). No difference was observed in TLR8 expression from either population during RSV or PIV infections. These data indicate that lower T-cell responses in sOP children may derive in part from lower APC activation by RSV.

To determine the level of activation of APCs in sOP and NOP children during RSV and PIV infection, we measured the change in surface expression of HLA-DR (MFI) on different APC cell types prior to and after in vitro viral infection. B cells of sOP children infected with RSVa showed a lower level of induced HLA-DR expression than NOP children (P = .05; Supplementary Figure 3C). Other APCs infected with RSVa showed a trend of lower induced HLA-DR expression among sOP children (Supplementary Figure 3C). The same pattern of expression was found for RSVb (data not shown). APCs infected with hPIV3 showed a trend of lower induced HLA-DR expression in B cells among sOP children (P = .06; Supplementary Figure 3D). Again, this indicates that sOP children have lower APC responses to RSV that in turn affect their T-cell responses.

**Viral Infection Interferes With Neutrophil Function**

We also sought to better understand the interface of a diminished Ig neutralizing response to virus and correlative heightened viral replication in relation to neutrophil phagocytic function during AOM. We did not concentrate here on potential differences between neutrophils derived from sOP and NOP children but rather determined the effects of viral infection directly on neutrophil phagocytic function. High viral burdens not neutralized by Ig in the NP, as exhibited by NOP children, could allow RSV to move into the middle ear and infect infiltrating neutrophils or infect them prior to reﬂux up the Eustachian tube to the middle ear. We found that infection with hPIV3 and RSV interfered with the phagocytic capacity of neutrophils to ingest Spn in a dose-dependent manner in both groups of children with the more significant interference caused by RSV (Figure 3A and 3B). These data suggest that a failure to neutralize RSV could disrupt the capacity of neutrophils to engulf respiratory bacteria.

**DISCUSSION**

In this study, we examined the association between RSV and hPIV3 respiratory viral infections and AOM in an immunologically vulnerable population (sOP) compared with an immunologically age-appropriate population (NOP) of children. To our knowledge, this is the ﬁrst study to demonstrate a mechanistic association between RSV infection and extension of a bacterial secondary infection from the NP to a local infection in the form of AOM. The ﬁndings point to diminished innate and adaptive immune responses to RSV among sOP children.

We have previously shown that sOP children have an immature immune proﬁle resembling a prolonged neonatal-like proﬁle in responding to NTHi and Spn antigens [12, 18, 26, 27]. Here, for the ﬁrst time, we show that RSV viral burdens are higher in the nasal washes of SOP vs NOP children during an AOM, and that result correlated with diminished antibody and
adapting cellular immune responses. A failure to neutralize replicating RSV in the NP could serve as a catalyst for Eustachian tube inflammation and trapping of refluxed bacteria and virus within nasal mucus. In support of this, RSV Ig treatment leads to a significant reduction of AOMs in otitis-prone children [28]. We previously found that sOP children have lower antibody responses to Spn and NTHi antigens [12, 26, 27, 29] and in this study we add lower antibody and cellular responses to RSV, suggesting a global deficiency in the adaptive immune response in sOP children.

We found that sOP children had lower T-cell responses to RSV compared with NOP children with fewer singlet/polyfunctional responses. IL-2 and IFN-γ production are both associated with protection from RSV whereas polyfunctional cytokines are associated with better control in multiple infection models [29–31]. The lower observed T-cell responses in sOP children could be due to differences in antibody enhancement of T-cell activation [33], differences in stimulatory capacity of dendritic cells [34], or relate to intrinsic defects in T cells of sOP children.

Activation of APCs as part of the innate immunity response plays a significant role in the quantity and quality of the adaptive immune response. RSV can infect APCs [35–37]. We found that APCs of sOP children have lower expression of TLR7 and lower induced HLA-DR expression during RSV infections. TLR7 is directly involved with clearance of viral infections [38], or relate to intrinsic defects in T cells of sOP children.

Importantly, we showed that RSV could interfere with phagocytic capacity of neutrophils to phagocytize Spn in a dose-dependent manner. In agreement, other studies have shown that RSV can infect neutrophils in infants and undergo transcription [38]; these infections could disrupt neutrophil myeloperoxidase function [39]. Therefore, RSV infection of neutrophils may initiate or further exacerbate the diminished capacity of sOP children to control otopathogen replication, a notion that we are currently investigating.

Supplementary Data

Supplementary materials are available at Clinical Infectious Diseases online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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