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Type III Interferons, Viral Loads, Age, and Disease Severity in Young Children With Respiratory Syncytial Virus Infection

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Background. The interplay among respiratory syncytial virus (RSV) loads, mucosal interferons (IFN), and disease severity in RSV-infected children is poorly understood.

Methods. Children <2 years of age with mild (outpatients) or severe (inpatients) RSV infection and healthy controls were enrolled, and nasopharyngeal samples obtained for RSV loads and innate cytokines quantification. Patients were stratified by age (0–6 and >6–24 months) and multivariable analyses performed to identify predictors of disease severity.

Results. In 2015–2019 we enrolled 219 RSV-infected children (78 outpatients; 141 inpatients) and 34 healthy controls. Type I, II, and III IFN concentrations were higher in children aged >6 versus 0–6 months and, like CXCL10, they were higher in outpatients than inpatients and correlated with RSV loads ($P < .05$). Higher IL6 concentrations increased the odds of hospitalization (odds ratio [OR], 2.30; 95% confidence interval [CI], 1.07–5.36) only in children >6 months, while higher IFN-λ2/3 concentrations had the opposite effect irrespective of age (OR, 0.38; 95% CI, .15–.86). Likewise, higher CXCL10 concentrations decreased the odds of hospitalization (OR, 0.21; 95% CI, .08–.48), oxygen administration (OR, 0.42; 95% CI, .21–.80),PICU admission (OR, 0.39; 95% CI, .20–.73), and prolonged hospitalization (OR, 0.57; 95% CI, .32–.98) irrespective of age.

Conclusions. Children with milder RSV infection and those aged >6 months had higher concentrations of mucosal IFNs, suggesting that maturation of mucosal IFN responses are associated with protection against severe RSV disease.

Keywords. RSV; cytokines; innate immune response; mucosal interferons; IFN-γ; CXCL10 (IP-10); disease severity; type III interferon.

Respiratory syncytial virus (RSV) accounts for 33 million cases of lower respiratory tract infection (LRTI), and more than 100 000 deaths annually in children <5 years of age [1]. Despite the high disease burden, therapy remains supportive and preventive strategies are exclusively approved for high-risk children [2]. The upper respiratory tract is the primary site for RSV infection. Upon infection, the respiratory mucosa maintains a balance between the interferon (IFN) antiviral response, that limits viral replication, and a proinflammatory response that can lead to enhance airway inflammation and worse clinical outcomes [3].

Studies of in vitro models and in children hospitalized with RSV LRTI have described associations between a number of innate immunity cytokines measured in the upper respiratory mucosa and disease severity with conflicting results [3–9]. Most of those studies did not comprehensively evaluate the role of mucosal type III IFNs, or the impact of age. Another important gap to help define the significance of mucosal cytokines in shaping RSV disease severity is to better define the mucosal cytokine profile of infants with mild RSV disease managed as outpatients, as most studies have been conducted in children hospitalized with severe disease. Understanding the interdependence between mucosal IFN responses, RSV loads, and the impact of age in children with mild RSV infection has important implications. On the one hand, the mucosal cytokine profile of mild disease could be used as a marker of ideal vaccine-elicited safe and protective responses, which is especially relevant to optimize the development of live attenuated vaccines designed to be administered intranasally [10]. On the other hand, from a practical perspective, this knowledge may aid with patient stratification

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in the clinical setting, and identification of the ideal therapeutic window for targeted therapeutic interventions.

The goal of this study was to define how the interplay between antiviral and proinflammatory mucosal cytokine responses, viral replication as defined by RSV loads, and age influences disease severity and clinical outcomes in young children with RSV infection.

METHODS

Study Design

This was a prospective, single-center, observational study that included a convenience sample of previously healthy children with RSV infection and healthy controls <2 years of age. Patients with RSV infection were enrolled at Nationwide Children's Hospital (NCH) within a median (25%–75% interquartile range (IQR) time of 13 (5–19) hours of hospitalization, or as outpatients at NCH Primary Care Pediatric Offices, Emergency Department, and Urgent Care Clinics over 5 respiratory seasons. RSV diagnosis was established by rapid antigen testing or as part of a polymerase chain reaction (PCR) respiratory panel obtained per standard of care [8]. We also enrolled as a reference a cohort of asymptomatic, healthy, age-matched controls during well child visits or minor elective surgeries not involving the respiratory tract [11]. Exclusion criteria for RSV patients included gestational age <36 weeks, underlying medical conditions (ie, congenital heart disease, chronic lung disease, congenital or acquired immunodeficiency), concomitant bacterial infections, and systemic use of immunomodulatory drugs including systemic steroids within 2 weeks of presentation. We also excluded children with symptoms for more than 7 days, previous history of wheezing or hospitalization for RSV bronchiolitis, and RSV hospitalization for apnea or social reasons. Healthy controls were excluded if they met any of those criteria, or if they had a history of fever or respiratory symptoms within 2 weeks of enrollment (Supplementary Material).

The study was approved by the Institutional Review Board at NCH, classified as a level 1 risk clinical study—no greater than minimal risk (pursuant under 45 CFR 46.404; and 21 CFR 50.51). Informed consent was obtained from legal guardians before study participation, in compliance with Nationwide Children's Research Responsible Conduct Guidelines.

At enrollment we collected patients' demographic and clinical data using a questionnaire purposely designed for this study [8]. In parallel, we also collected a midturbinate swab for RSV quantification and typing by real-time PCR and for cytokine analysis. Disease severity was assessed using standardized clinical disease severity score (Ohio-RSV CDSS; Supplementary Table 1) that ranged from 0 (normal) to 15 (most severe disease). This CDSS has been validated in other studies [8, 11–14]. Among inpatients, we evaluated additional parameters of severity including administration and duration of supplemental oxygen, need and duration of noninvasive and invasive ventilatory support, admission to the pediatric intensive care unit (PICU), and duration of hospitalization.

Sample Collection and Processing

Briefly, midturbinate nasal swab samples (FLOQswabs; Copan Diagnostics) were introduced into a nostril and rotated once to soak up secretions. The tip of the swab was then placed in 3 mL of viral transport media (M4-VTM; Thermo Fisher Scientific), transported on ice to the laboratory, vortexed for 30 seconds within 2–4 hours of sample collection, and aliquoted into 5 vials with approximately 500 μL in each. All vials were stored at −80°C until further processing in batches.

When ready for processing, samples were thawed in a laminar flow hood at room temperature for 20 minutes, and separate aliquots from the collected specimen were used for mucosal cytokine concentrations and RSV load quantitation. None of the samples included for cytokine analyses underwent more than 1 thawing cycle. For complete protocol, see Supplementary Material.

We decided to use midturbinate swabs instead of nasal wash samples to maximize enrollment and to improve retention. Before implementing midturbinate sampling, we performed validation studies as described below. In addition, nasopharyngeal or midturbinate nasal swab samples have been successfully used in studies assessing the host mucosal immune response of children and adults with RSV and other respiratory viral infections [15–17].

RSV Loads and Viral Coinfections

RSV loads were measured by quantitative real-time PCR targeting the N gene, as described [12]. In addition, to assess for viral coinfections all samples underwent testing using the FilmArray Respiratory Viral Panel (BioFire; BioMerieux) [8].

Cytokine Analysis

We measured the concentrations of 13 cytokines, in duplicates, using a bead-based multiplex assay (LEGENDplex Human Anti-Virus Response Panel; BioLegend) following manufacturer instructions and used a BD FACS flow cytometer and FACSDiva software for cytokine analyses. This assay allowed for the simultaneous quantification of type I ([IFN-α2], [IFNβ]), type II ([IFN-γ]), and type III ([IFN-λ1], [IFN-λ2/3]) interferons, interferon-γ–induced protein 10 (CXCL10 [IP-10]), interleukins ([IL-1β], IL-6, CXCL8 [IL-8], IL-10, IL-12), (TNF-α), and (GM-CSF). The lower limit of detection of the assay ranged from 0.7 to 12.8 pg/mL [18–21]. To assess the value of midturbinate samples for cytokine measurement, validation studies were performed with paired midturbinate swab and nasal wash samples in the first 55 children enrolled (RSV inpatients, n = 34; RSV outpatients, n = 16; healthy controls, n = 5). Overall, cytokine concentrations were higher in

Table 1. Demographic and Clinical Characteristics of RSV Patients and Healthy Controls

Characteristic	Healthy Controls (n = 34)	All RSV Patients (n = 219)	RSV Outpatients (n = 78)	RSV Inpatients (n = 141)	*P* Value[a]	*P* Value[b]
Demographic						
Age, mo	4.9 (3.0–7.2)	4.1 (1.8–8.6)	6.0 (3.4–10.4)	3.1 (1.5–7.9)	.38	<.01
Female, sex	6 (17.6)	107 (48.9)	37 (47.4)	70 (49.6)	<.01	.86
Race					.32	<.01
White	26 (76.5)	144 (65.7)	42 (53.8)	102 (72.3)		
Black	3 (8.8)	42 (19.2)	26 (33.3)	16 (11.3)		
Other	5 (14.7)	33 (15.1)	10 (12.8)	23 (16.3)		
Breastfed[c]	18 (52.9)	91 (41.5)	41 (52.6)	50 (35.5)	.26	.02
Delivery type, vaginal	22 (64.7)	157 (71.7)	59 (75.6)	98 (69.5)	.4	.35
Daycare attendance	6 (17.6)	76 (34.7)	28 (35.9)	48 (34.0)	.06	.88
Smoke exposure	6 (17.6)	60 (27.4)	19 (24.0)	42 (29.8)	.29	.34
Vaccines up to date	31 (91.2)	194 (88.6)	69 (88.5)	125 (88.6)	>.99	>.99
Family history of asthma	10 (29.4)	134 (61.2)	44 (56.4)	90 (63.8)	<.01	.31
Clinical parameters						
Days of symptoms	…	4 (3–5)	4 (3–5)	4 (3–5)	…	.15
Ohio RSV CDSS	…	5 (3–7)	3 (2–4)	8 (6–10)	…	<.001
Steroids use	…	35 (15.9)	11 (14.1)	24 (17.0)	…	.70
Virology data						
RSV type						.04
RSV-A	…	113 (51.6)	33 (42.3)	80 (56.7)	…	
RSV-B	…	106 (48.4)	45 (57.7)	61 (43.2)	…	
RSV load, $\log_{10}$ copies/mL	…	7.7 (6.7–8.3)	7.91 (±1.12)	7.24 (±1.24)	…	<.001
Viral coinfections[d]	13 (52.0)	66 (30.1)	20 (25.6)	46 (32.6)	.28	.35
Rhinovirus/enterovirus	10	28	9	19	…	
hCoVs, non-SARS-CoV-2	0	17	5	12	…	
Adenovirus	0	7	1	6	…	
Parainfluenza virus	0	2	1	1	…	
Influenza virus	0	2	0	2	…	
hMPV	0	1	1	0	…	
Bocavirus	0	1	1	0	…	
>1 respiratory virus[e]	3	8	2	6	…	

Categorical data expressed as frequencies (%) and analyzed using Fisher or χ^2 test. All continuous variables except RSV loads are expressed as medians (interquartile range) and analyzed using Mann-Whitney rank test. RSV loads expressed in means ± SD.

Abbreviations: CDSS, clinical disease severity score; hCoV, human coronaviruses; hMPV, human metapneumovirus; RSV, respiratory syncytial virus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

[a]Healthy controls versus all RSV patients.

[b]RSV inpatients versus outpatients.

[c]Breastfed at any time since birth.

[d]Film array panel performed in all RSV patients, and 25 of 34 healthy controls (73%). No coinfections with *Bordetella pertussis, Chlamydophila pneumoniae,* and *Mycoplasma pneumoniae* were identified.

[e]For >1 respiratory virus combinations included rhinovirus/enterovirus and hCoV or adenovirus in 4 RSV patients and 2 controls, or hCoV and parainfluenza virus or adenovirus in 4 RSV patients and 1 control.

nasal wash than in midturbinate samples and were strongly correlated (Supplementary Table 2).

Statistical Analysis

Data are reported with frequencies and percentages for categorical variables and means (SD) or medians with 25%–75% IQR for continuous variables according to data distribution. Categorical data were analyzed by χ^2 or Fisher exact test, and continuous variables by Mann-Whitney or Kruskal-Wallis tests with Benjamini-Hochberg tests to adjust for multiple comparisons. Nonlinear associations between cytokine concentrations and viral loads were analyzed using polynomial and restricted cubic spline regression, and linear associations using Pearson correlation coefficient. To account for differential batch assignment in the samples included in the study, we applied the censored likelihood multiple imputation method, as described [22]. Briefly, cytokine concentrations that were below the level of quantitation were imputed according to the batch and the subject's cohort (healthy controls, RSV inpatients and outpatients) using the *lodi* package in R environment (version 4.1.2). This technique allowed to standardize the cytokines' lower limit of detection across different batches, generating uniform and reliable results. In addition, sensitivity analyses using nonimputed data were performed.

Table 2. Cytokine Concentrations in Children With RSV Infection and Healthy Controls

Cytokine	Healthy Controls (n = 34)	All RSV Patients (n = 219)	RSV Outpatients (n = 78)	RSV Inpatients (n = 141)	*P* Value[a]	*P* Value[b]
IFN-α2	0.1 (0.03–0.43)	0.5 (0.2–1.3)	0.9 (0.3–1.7)	0.4 (0.2–0.9)	<.001	<.001*
IFN-β	0.2 (0.03–1.1)	1.5 (0.4–5.5)	2.8 (0.6–6.9)	1.1 (0.4–5.1)	<.001	<.001
CXCL10	12.9 (2.9–77.4)	136.6 (36.4–328.0)	268.6 (118.5–825.6)	87.6 (15.4–221.2)	<.001	<.001*
IFN-γ	0.6 (0.1–1.5)	0.9 (0.4–2.4)	1.6 (0.6–3.5)	0.8 (0.4–2.1)	.154	.011*
IFN-λ1	2.2 (0.3–4.0)	4.0 (1.6–10.6)	5.2 (2.5–14.2)	3.6 (1.3–9.2)	.022	.004*
IFN-λ2/3	0.39 (0.08–2.3)	3.5 (1.3–12.3)	13.7 (2.3–24.9)	2.8 (1.1–6.4)	<.001	<.001*
IL-1β	2.1 (0.3–4.1)	11.6 (3.5–54.3)	13.4 (5.9–49.9)	10.4 (2.4–67.4)	<.001	<.001
TNF-α	1.4 (0.1–3.9)	4.4 (1.3–18.9)	5.6 (2.1–18.6)	3.5 (0.8–21.1)	<.001	<.001
IL-6	1.2 (0.2–2.3)	15.4 (4.2–55.3)	21.6 (5.3–53.7)	13.8 (3.6–59.5)	<.001	<.001
CXCL8	414.1 (208.9–777.9)	1471.0 (722.6–3011.0)	1431 (669.5–3114)	1484 (747.7–3101)	<.001	<.001
IL-10	0.4 (0.1–1.3)	1.0 (0.4–2.6)	1.5 (0.7–2.8)	0.7 (0.3–2.2)	.008	<.001*

Cytokine concentrations (pg/mL) are expressed as medians (25%–75% interquartile range). The asterisk indicates significant differences between inpatients and outpatients by post hoc analyses using Dunn multiple comparison test. The Bonferroni correction was applied to adjust for multiple test comparisons within the 11 cytokines analyzed.

Abbreviations: IFN, interferon; IL, interleukin; RSV, respiratory syncytial virus; TNF, tumor necrosis factor.

[a] *P* value for comparisons between healthy controls and all RSV patients by Mann-Whitney *U* test.

[b] *P* value for comparison between healthy controls, RSV inpatients, and RSV outpatients by Kruskal-Wallis rank test.

To determine which factors were independently associated with worse clinical outcomes, defined as need for hospitalization, oxygen administration, PICU admission, and duration of hospitalization, we used multivariable logistic regression with Firth penalized likelihood, when warranted, to avoid small sample size bias. Covariates were included in the models if they were clinically relevant or had univariate *P* values of <.15, and were retained in the models if they had an adjusted *P* value of <.1, or if their inclusion had a substantial impact on models' goodness of fit based on Akaike information criterion [23]. These covariates included age (0–6 and >6–24 months), viral loads, viral coinfections, and cytokine concentrations that underwent $\log_{10}$ transformation. Analysis plan, including definitions of disease severity and age categories, were defined a priori. Analyses were conducted using R for statistical computing, and GraphPad Prism version 9 with a 2-sided *P* value <.05 considered statistically significant.

RESULTS

Characteristics of Study Population

From February 2015 to April 2019, we enrolled a convenience sample of 219 previously healthy children <2 years of age with RSV infection and 34 healthy controls. Among children with RSV infection, 78 (36%) were diagnosed with mild disease in the outpatient setting, and 141 (64%) required hospitalization for RSV LRTI (inpatients). Median age was 6.0 (IQR, 3.4–10.4) months for outpatients, 3.1 (IQR, 1.5–7.9) months for inpatients, and 4.9 (IQR, 3.0–7.2) months for healthy controls. The demographic and clinical characteristics of the study cohorts are summarized in Table 1.

Median duration of symptoms at enrollment was 4 (IQR, 3–5) days for RSV outpatients and inpatients, and as expected the CDSS was higher in inpatients. Overall, rates of RSV A and RSV B infections were similar (51.6% vs 48.4%), with a slightly higher proportion of RSV B infections in outpatients than inpatients (58% vs 43%; *P* = .04). RSV loads were higher in outpatients (7.91 [SD 1.12] $\log_{10}$ copies/mL) than in inpatients (7.24 [SD 1.24] $\log_{10}$ copies/mL; *P* < .001).

Other respiratory viruses were identified in 30% of RSV patients and at similar rates in outpatients (26%) and inpatients (33%). Rhinoviruses followed by seasonal coronaviruses were the most commonly codetected viruses.

Mucosal Cytokine Concentrations in Children With RSV Infection and Healthy Controls

First, we analyzed whether cytokine concentrations differed between children with RSV infection and healthy controls. Of all cytokines evaluated, GM-CSF and IL-12 concentrations were below the limit of detection in most RSV patients and controls and were not included in further analyses.

Concentrations of FN-α2, IFN-β, CXCL10, IFN-λ1, IFN-λ2/3, IL-1-β, TNF-α, IL-6, CXCL8, and IL-10 were significantly higher in RSV patients compared with controls after adjusting for multiple comparisons (Table 2). Further analyses according to disease severity showed that concentrations of type I (IFN-α2), type II (IFN-γ), type III (IFNλ1; IIFN-λ2/3) IFNs, and CXCL10 were significantly higher in outpatients with mild RSV infection than among inpatients with severe RSV disease (Figure 1).

Cytokine Concentrations in Children With RSV Infection According to Age and Viral Loads

Next, we compared cytokine concentrations according to age. Within the RSV cohort, 140 (64%) infants were 0–6 months and 79 (36%) children >6–24 months of age. Of all cytokines

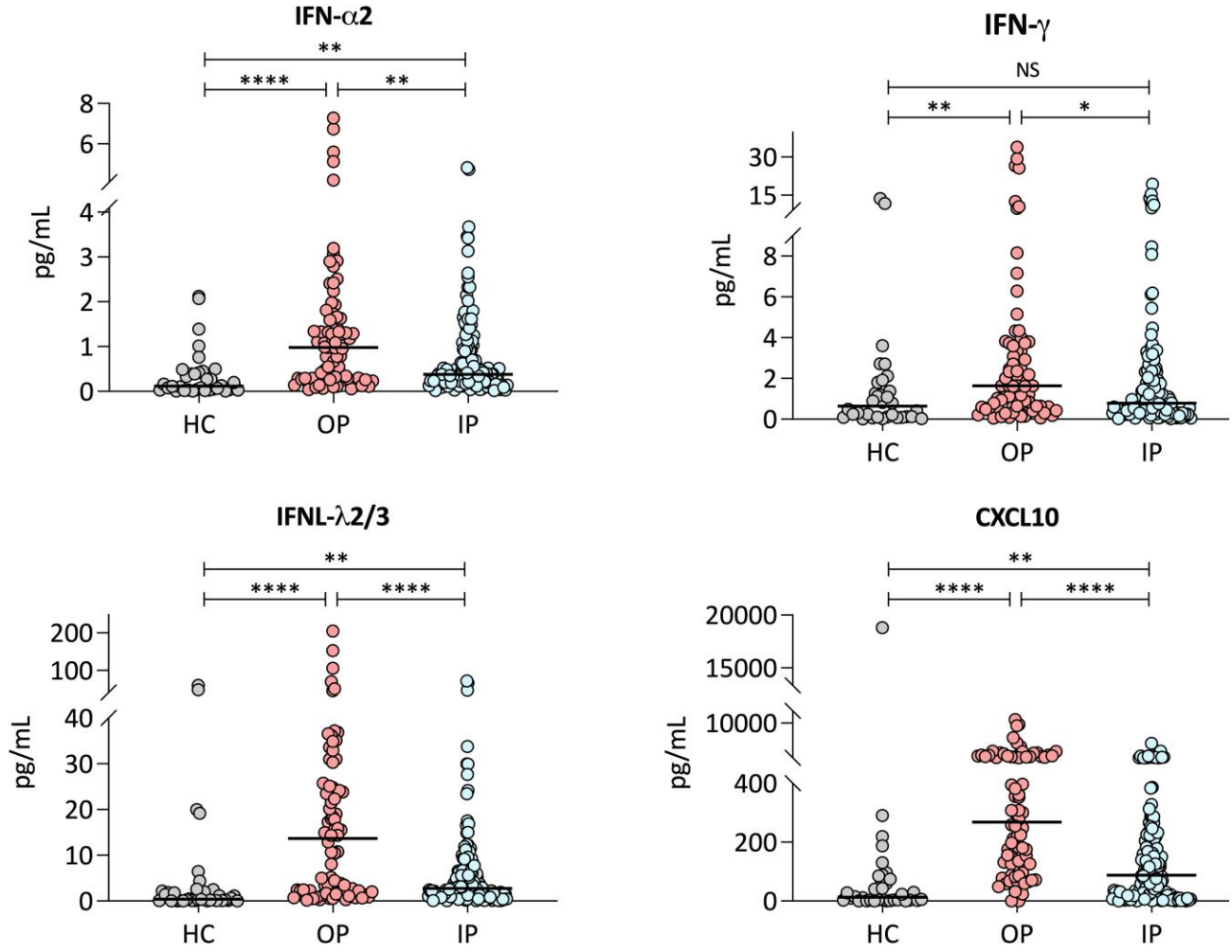

Figure 1. Mucosal cytokine concentrations in RSV patients and healthy controls. Concentrations of IFN-α2, IFN-γ, CXCL10, and IFN-λ2/3 in healthy controls (n = 34), RSV outpatients (n = 78), and RSV inpatients (n = 141). Y-axis represents cytokine concentrations in pg/mL and the X-axis the 3 study groups (healthy controls (HC), RSV outpatients (OP), and RSV inpatients (IP)). Analyses by Kruskal-Wallis and Dunn test for multiple test corrections. Number of asterisks indicate the strength of the *P* value: * < .01; ** < .001; **** < .0001. Abbreviations: NS, not significant; RSV, respiratory syncytial virus.

analyzed, concentrations of IFN-α, IFN-γ, mucosal interferons (IFN-λ2/3), as well as TNF-α, IL-1β, CXCL8, and IL-10 were significantly higher in RSV infants >6 months of age compared with the 0–6 months age group (Supplementary Table 3).

Differences in concentrations of these cytokines in children younger than and older than 6 months also differed according to disease severity (Table 3). CXCL10 concentrations were consistently higher in RSV outpatients (mild disease) versus inpatients (severe disease) in both age groups. Similarly, IFN-λ2/3 concentrations were higher in RSV outpatients, both younger and older than 6 months, with differences that reached statistical significance only in the youngest age group. IL-10 concentrations were significantly higher in outpatients 0–6 months of age.

We also assessed the associations between RSV loads and cytokine concentrations. Using polynomial regression and smoothed scattered plot curves (LOESS curves) we found significant direct linear associations between RSV loads and CXCL10, IFN-λ1, IL-6, CXCL8, and IL-10 in RSV outpatients and inpatients (Figure 2 and Supplementary Table 4).

Adjusted Risk Factors for Severe RSV Disease

Lastly, we constructed different multivariable models to identify which cytokines were predictive of disease severity after adjusting for age, viral loads, and viral coinfections. Disease severity was defined by the need for hospitalization in all children with RSV infection, and among hospitalized children by the administration of supplemental oxygen, need for PICU, and prolonged hospital stay, which was defined as >62 hours (2.6 days) based on the median duration of hospitalization of the complete inpatient cohort.

Table 3. Cytokine Concentrations in Children With RSV Infection Stratified by Age and Severity

	Age 0–6 mo			Age >6 mo		
Cytokine	OP n = 41	IP n = 99	*P* Value	OP n = 37	IP n = 42	*P* Value
IFN-α2	0.6 (0.3–1.6)	0.3 (0.2–0.6)	.07	1.1 (0.3–1.7)	0.9 (0.2–1.7)	>.99
IFN-β	3.4 (0.3–7.7)	0.9 (0.3–3.8)	.76	2.4 (0.9–5.6)	2.9 (0.7–6.4)	>.99
CXCL0	278.1 (131.0–690.6)	83.7 (11.4–223.1)	<.01	259 (91.1–1324.0)	96.3 (20.9–175.3)	<.01
IFN-γ	1.2 (0.6–3.0)	0.7 (0.3–1.4)	.15	1.8 (0.6–3.8)	1.6 (0.6–3.5)	>.99
IFN-λ1	4.0 (1.8–12.9)	3.7 (1.4–8.9)	>.99	7.2 (2.9–21.6)	3.8 (1.2–9.7)	.45
IFN-λ2/3	10.4 (1.7–22.7)	2.6 (1.0–5.9)	<.01	15.8 (3.1–31.9)	3.2 (1.3–12.2)	.06
IL-1β	13.2 (4.8–32.9)	6.1 (1.4–53.4)	>.99	13.6 (6.2–62.7)	16.5 (7.2–105.7)	>.99
TNF-α	4.8 (1.6–12.4)	2.1 (0.6–15.1)	.43	8.7 (2.7–35.2)	9.0 (2.5–77.7)	>.99
IL-6	27.7 (3.7–51.4)	12.1 (3.1–50.8)	>.99	16.7 (5.3–55.7)	22.6 (6.6–66.5)	>.99
CXCL8	1212 (662.4–1964)	1226 (613.3–2249)	>.99	2301 (671.6–3946)	2060 (1233–4879)	>.99
IL-10	1.7 (0.8–3.6)	0.6 (0.3–1.6)	.02	1.5 (0.7–2.5)	1.3 (0.4–4.6)	>.99

Continuous data are expressed as median (interquartile range) and analyzed using Mann-Whitney rank test. Values in bold indicate significant 2-sided *P* values. Bonferroni correction was applied to adjust for multiple test comparisons.

Abbreviations: IFN, interferon; IL, interleukin; IP, inpatient (severe RSV infection); OP, outpatient (mild RSV infection); RSV, respiratory syncytial virus; TNF, tumor necrosis factor.

To assess for the odds for hospitalization, children were stratified according to age as 0–6 months and >6–24 months (Table 4). Adjusted for the covariates mentioned above, higher concentrations of CXCL10 and IFN-λ2/3 were independently associated with reduced odds of hospitalization in children 0–6 months and >6 months of age. On the other hand, higher IL-6 concentrations were significantly associated with increased odds of hospitalization only in children >6 months of age. Among inpatients, models were also adjusted for age, viral loads, and viral coinfections, and constructed separately for each cytokine to avoid overfitting. Of all cytokines analyzed, only higher concentrations of CXCL10 were significantly associated with reduced odds of supplemental oxygen administration, reduced odds of PICU admission, and reduced odds of prolonged hospitalization (Table 5). Sensitivity analyses using nonimputed data showed similar results (Supplementary Tables 5 and 6).

DISCUSSION

The role of mucosal type III IFNs in shaping disease severity in infants and young children with RSV infection has not been comprehensively evaluated. Nor has a careful assessment been made of the role of mucosal antiviral and inflammatory responses in children with mild RSV infection managed as outpatients, and whether those responses vary according to age. In this prospective study we showed that young children with mild RSV infection had significantly higher mucosal concentrations of type I, II, and more importantly of type III IFNs compared with children hospitalized with severe disease. In addition, we found that the concentrations of these cytokines were significantly higher in children >6 months of age and correlated with RSV loads. Of all cytokines measured, multivariable analyses showed that CXCL10 was consistently associated with improved clinical outcomes across age groups. Mucosal interferons (IFN-λ2/3) also played a protective role, being associated with decreased odds of hospitalization in younger and older infants.

The identification of biomarkers predictive of RSV disease severity has remained a topic of interest due to the high burden of disease and limited preventive and therapeutic strategies available. Because the primary site of RSV infection is the respiratory mucosa, there has been great interest in improving our understanding of the antiviral and proinflammatory mucosal immune response elicited by RSV [5–7, 24–29]. While studies have evaluated the role of various cytokines, including IFN-γ, IL1-β, IL-6, CXCL8, CXCL10, IL-10, or TNF-α, on RSV disease severity [30], the role of type III interferons, comprising IFN-λ1–4 [31, 32], is not completely defined.

Type III IFNs are relatively newer cytokines that play a fundamental role in mucosal immunity, promoting an antiviral state in mucosal barriers with less collateral damage than type I IFNs [33]. Studies in animal models described increased viral loads of RSV, influenza, human metapneumovirus (hMPV), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-1) in mice lacking the interferon-λ receptor IL28Raα [34], while exogenous treatment with IFN-λ1 in primary human nasal epithelial cell cultures resulted in reduced RSV replication [31, 35]. Data in children with RSV infection is limited. A study conducted in infants hospitalized with RSV infection showed increased IFN-λ1 and IFN-λ3 mRNA expression in nasal wash samples compared with those with rhinovirus infection. In addition, higher IFN-λ1 mRNA expression was associated with tachypnea and a higher disease severity index only in infants with RSV infection [24]. In contrast, we found significantly higher concentrations of IFN-λ1 and IFN-λ2/3 in outpatients with mild RSV infection compared with inpatients with severe RSV disease. These differences

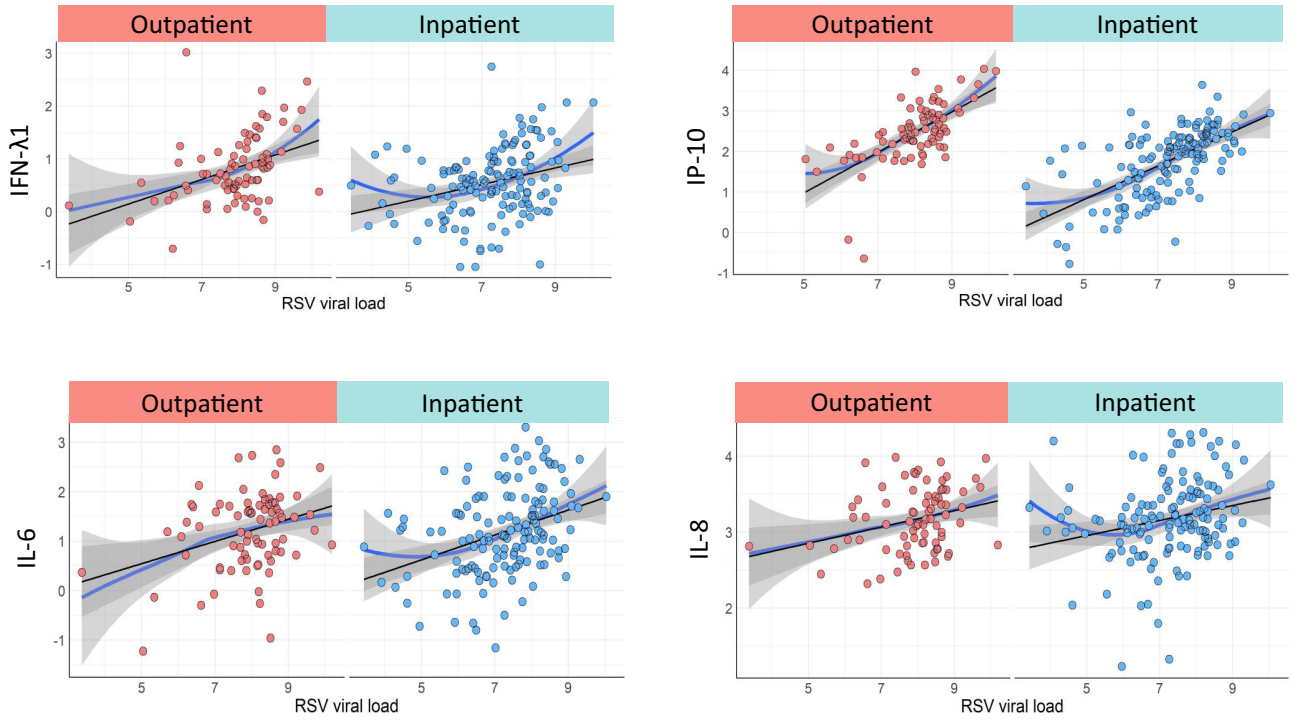

Figure 2. Association between RSV loads and cytokine concentrations in RSV patients. LOESS curves for CXCL10(IP-10), IFN-λ1, IL-6, and CXCL8 in RSV outpatients (n = 78), and RSV inpatients (n = 141). The Y-axis represents cytokine concentrations in pg/mL and the X-axis RSV load in $\log_{10}$ copies/mL in the 2 study groups (RSV outpatients in red and RSV inpatients in blue). Analyses by polynomial and restricted cubic spline regression with the linear regression line included as a reference. Abbreviations: IFN, interferon; IL, interleukin; RSV, respiratory syncytial virus.

may have been related to differences in study design, as the aforementioned study included exclusively children hospitalized with severe RSV infection, and lambda IFNs were measured by gene expression rather than at the protein level.

Two studies conducted in children <24 months of age with RSV bronchiolitis found that increased concentrations of IFN-γ and CXCL10 in nasal wash samples were associated with decreased risk of hospitalization [26, 27]. In agreement with those studies, we found that higher CXCL10 concentrations were protective in terms of hospitalization, but also associated with decreased odds of oxygen administration, need for PICU, and shorter duration of hospitalization. Secretion of CXCL10 is stimulated by type I, II, and III IFNs, and appears to promote dendritic cell maturation, T-cell stimulation, and IL-12 secretion that overall favors viral clearance of infected respiratory epithelial cells [36, 37]. The dynamic and functions of CXCL10 are nevertheless complex, as this cytokine can also promote a proinflammatory and profibrotic state [38]. Different studies have shown that high IL1-β, CXCL8, and TNF-α concentrations in nasal wash and bronchoalveolar lavage samples in children with RSV infection were associated with worse clinical outcomes [3, 25, 28, 29, 39–42]. In the present study, concentrations of IL1-β, TNF-α, CXCL8, and IL-6 were significantly higher in children with RSV infection compared with healthy controls; however, only in older children higher IL-6 concentrations were independently associated with increased risk of hospitalization.

We observed that RSV loads were significantly higher among outpatients with mild disease than in infants hospitalized with severe RSV infection. In addition, we found that RSV loads were directly correlated with CXCL10, IFN-λ1, IL-6, CXCL8, and IL-10 concentrations. These observations conflict with earlier studies that reported higher viral loads and an exaggerated

Table 4. Multivariable Analyses of Factors Associated With the Need for Hospitalization in Children With RSV Infection

	Age 0–6 mo		Age >6 mo	
Factor	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
RSV load, $\log_{10}$ copies/mL	1.01 (.63–1.58)	.98	0.67 (.33–1.24)	.22
IFN-α2	0.43 (.16–1.13)	.09	0.77 (.26–2.20)	.63
CXCL10	0.21 (.08–.48)	**<.01**	0.30 (.10–.82)	**.02**
IFN-λ2/3	0.44 (.19–.95)	**.04**	0.38 (.15–.86)	**.03**
IL-6	1.59 (.91–2.87)	.11	2.30 (1.07–5.36)	**.04**

Adjusted odds of hospitalization among patients in the study stratified by age. Bolded values indicate significant 2-sided *P* values ($P < .05$).

Abbreviations: CI, confidence interval; IFN, interferon; IL, interleukin; OR, odds ratio; RSV, respiratory syncytial virus.

Table 5. Multivariable Analyses of Factors Associated With Clinical Outcomes in Hospitalized Children With RSV Infection

	Supplemental O_2		PICU		Prolonged Hospitalization	
Factor	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value	OR (95% CI)	*P* Value
Age >6 mo	1.06 (.99–1.16)	.121	0.57 (.22–1.38)	.228	0.83 (.39–1.75)	.625
RSV load, $\log_{10}$ copies/mL	1.08 (.71–1.62)	.702	1.3 (.87–2.04)	.220	1.34 (.94–1.96)	.113
Viral coinfections	1.57 (.67–3.86)	.311	1.92 (.84–4.35)	.117	1.46 (.70–3.10)	.315
CXCL10	0.42 (.21–.80)	**.011**	0.39 (.20–.73)	**.004**	0.57 (.32–.98)	**.047**

Prolonged hospitalization was defined as length of stay >62 hours based on median duration of hospitalization. Bolded values indicate significant 2-sided *P* values ($P < .05$).

Abbreviations: CI, confidence interval; OR, odds ratio; O_2, oxygen; PICU, pediatric intensive care unit; RSV, respiratory syncytial virus.

immune response associated with severe RSV disease [9, 43, 44]. There has been, however, recent cumulative evidence suggesting that higher viral loads early in RSV infection coupled with a robust antiviral interferon response may lead to more favorable clinical outcomes in children with RSV infection [8, 12, 27, 29, 45]. Overall, these results suggest that clinical outcomes in RSV infection depend on the interplay between RSV replication and an adequate balance between the interferon and proinflammatory responses and that those responses are influenced by age.

Our study has limitations. We analyzed innate immunity cytokines in the nasal mucosa, which may not reflect the innate immune response occurring in the lower respiratory tract. However, we and others have shown in previous studies the value of upper respiratory tract samples as a surrogate of lower respiratory tract disease [3, 5, 26, 39, 40, 46]. In addition, the primary objective of our study was to understand the mucosal innate immune response at the initial site of infection and to potentially identify factors that may confer protection against severe RSV disease. Additionally, nasal mucosa sampling is a convenient, noninvasive procedure that will facilitate its implementation in large multicenter clinical trials. We did not perform sequential measurements of cytokine concentrations to assess the dynamics of mucosal innate immune responses. Nonetheless duration of symptoms at enrollment was comparable between children with mild and severe disease, and we limited study participation to children with up to 7 days of illness. In addition, a recent study that analyzed sequential samples in the first 7 days of symptoms of children hospitalized with RSV infection showed that the greatest differences in the concentrations of most mediators, particularly CXCL10 and IFN-γ, were observed in the first 7 days of illness [29]. As previously mentioned, children in the RSV inpatient cohort were younger than outpatients, and this may be an intrinsic limitation of studying RSV disease, given that younger age is a known risk factor for more severe illness. We performed age-matched analyses between inpatients and outpatients to confirm the consistency of our observations, and age was included as a covariate in all multivariable models.

In summary, we were able to consistently measure and define the protective role of mucosal type III interferons and other antiviral cytokines in a large cohort of infants with RSV infection. We found that those responses were influenced by age, correlated with viral loads, and demonstrated significant associations with clinical outcomes. This novel information will help with the development and evaluation of RSV live attenuated vaccines designed to be administered via the intranasal route [10]. In addition, these findings may aid patient stratification in the clinical setting and could facilitate the clinical evaluation of therapeutic interventions for RSV infections in children.

Supplementary Data

Supplementary materials are available at *The Journal of 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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