ORIGINAL ARTICLE

Reduced Nasal Viral Load and IFN Responses in Infants with Respiratory Syncytial Virus Bronchiolitis and Respiratory Failure

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Abstract

Rationale: Respiratory syncytial virus (RSV) bronchiolitis is a major cause of morbidity and mortality in infancy. Severe disease is believed to result from uncontrolled viral replication, an excessive immune response, or both.

Objectives: To determine RSV load and immune mediator levels in nasal mucosal lining fluid by serial sampling of nasal fluids from cases of moderate and severe bronchiolitis over the course of infection.

Methods: Infants with viral bronchiolitis necessitating admission ($n = 55$) were recruited from a pediatric center during 2016 and 2017. Of these, 30 were RSV infected (18 "moderate" and 12 mechanically ventilated "severe"). Nasal fluids were sampled frequently over time using nasosorption devices and nasopharyngeal aspiration. Hierarchical clustering of time-weighted averages was performed to investigate cytokine and chemokine levels, and gene expression profiling was conducted.

Measurements and Main Results: Unexpectedly, cases with severe RSV bronchiolitis had lower nasal viral loads and reduced IFN-γ and C-C chemokine ligand 5/RANTES (regulated upon activation, normal T cell expressed and secreted) levels than those with moderate disease, especially when allowance was made for disease duration (all $P < 0.05$). Reduced cytokine/chemokine levels in severe disease were also seen in children with other viral infections. Gene expression analysis of nasopharyngeal aspiration samples ($n = 43$) confirmed reduced type-I IFN gene expression in severe bronchiolitis accompanied by enhanced expression of *MUC5AC* and *IL17A*.

Conclusions: Infants with severe RSV bronchiolitis have lower nasal viral load, CXCL10 (C-X-C motif chemokine ligand 10)/IP-10, and type-I IFN levels than moderately ill children, but enhanced *MUC5AC* (mucin-5AC) and *IL17A* gene expression in nasal cells.

Keywords: viral bronchiolitis; respiratory syncytial virus; nasosorption; interferon; innate immunity

At a Glance Commentary

Scientific Knowledge on the Subject: Respiratory syncytial virus is the most common cause of bronchiolitis in children. However, the pathogenesis of severe disease is incompletely understood.

What This Study Adds to the Field: Through noninvasive serial sampling of hospitalized infants, we report that, surprisingly, both viral load and IFN responses in severely ill infants are lower than those of moderate cases.

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Acute viral lower respiratory tract infections (AVLRI) pose a major healthcare burden, especially in infancy (1, 2). Respiratory syncytial virus (RSV) is the single most common cause of moderate to severe acute respiratory infection during infancy, resulting in more than 3 million hospital admissions globally per annum (3). Most infants have been infected with RSV by 2 years of age, the majority of cases being mild (1). However, RSV infection sometimes causes respiratory failure, resulting in an estimated 118,200 deaths annually, mostly in the developing world (3). Hypoxia is a major indicator of severity (4), but there is an incomplete understanding of why some infants develop life-threatening disease, while most babies develop a mild illness that quickly resolves. Prematurity and congenital heart disease are risk factors for disease, but only a minority of RSV bronchiolitis cases have comorbidities (5, 6). Despite the clinical need, no vaccine for RSV is currently available, and there is no specific therapeutic for RSV bronchiolitis (7). Prophylactic treatment with palivizumab, an anti-RSV F-protein monoclonal antibody, reduces hospitalization rates, but the cost of treatment limits its use to high-risk cases (7, 8).

The mechanisms underlying severe RSV disease are unclear, but two main hypotheses have been advanced (9). First, heightened viral load (VL) has been associated with severe disease (10, 11), as shown by association between VL and length of hospital stay (a measure of disease severity); this has also been demonstrated in moderately ill, unventilated infants (12–14). In addition, viral challenge studies in adult humans show that higher doses are associated with greater disease severity (15). The second, potentially overlapping, hypothesis is that severity reflects a pathogenic overexuberant immune response to virus (9). This theory is supported by animal studies (16, 17) and by observations of high levels of inflammatory mediators and infiltration of neutrophils, monocytes, and T cells to the airway of severely ill human infants (18–20). An immune response biased toward generation of the type II cytokine IL-4 has also been associated with development of lower respiratory tract symptoms (21, 22), although this has not been replicated in other studies (23, 24).

Many respiratory sampling techniques are too invasive to permit repetitive sampling, therefore limiting our ability to obtain detailed time-series information over the course of disease. We recently reported the use of nasosorption as a minimally invasive technique to identify RSV infection and quantify immune mediators in the upper airway of infants with bronchiolitis (25). In this study, we sought to characterize in detail the course of the immune response during AVLRI in infants through serial nasosorption sampling. By comparing infants with moderate and severe disease, we sought to determine the contribution of VL and cytokine/chemokine responses to the pathogenesis of life-threatening bronchiolitis.

Methods

Patients and Ethical Approval

Cases of viral bronchiolitis were recruited at St. Mary's Hospital, Paddington, London between October 2016 and March 2017. This diagnosis was based on standard clinical criteria (cough, tachypnea, wheezes and crackles on auscultation, and hyperinflation). Infants were aged between 2 weeks to 2 years of age and weighed 2 kg or more. Infants were excluded from the study if any of the following criteria were met: *1*) any local or systemic factor that could influence the safety of nasal sampling (e.g., bleeding disorders), *2*) presence of nasal cannulae in both nostrils (e.g., high-flow nasal oxygen therapy or bilateral nasal feeding tubes), *3*) palliative care, or *4*) a potentially confounding respiratory comorbidity. Infants with nonrespiratory comorbidities, including prematurity (<36 weeks' gestational age), were not excluded from the study. Only infants with a PCR-confirmed viral infection were included in the analysis. Healthy control infants were recruited from ophthalmology or fracture clinics and had no symptoms of respiratory infection. Informed consent was sought from parents or guardians. The study was ethically approved by the Black Country Research Ethics Committee (reference 15/WM/0343).

Respiratory Sampling

When clinically permissible, participants were sampled up to twice daily throughout their admission. Nasosorption was performed using FXi-13 nasosorption devices (Hunt Developments UK, Ltd.); these were manipulated precisely up the nostril lumen, avoiding brushing, and the nostril lightly depressed for 30 seconds to make mucosal contact. After a 1-minute interval, nasosorption was repeated in the opposing naris. If one naris was unavailable (e.g., obstructed by a nasal catheter), then sampling was repeated in the same naris. Nasosorption devices were returned to their collection tube before processing. Nasopharyngeal aspiration was then performed by direct suction of secretions from the nasopharynx, followed by flushing of the suction catheter with 3.5 ml of saline. After collection, all samples were stored at 4°C and processed within 3 hours.

Results

Fifty-five infants with PCR-confirmed viral bronchiolitis were recruited to the study; 34 were cases of moderate severity admitted to general pediatric wards and were not mechanically ventilated (henceforth referred to as "moderate"), and 21 severely ill, mechanically ventilated infants were recruited from the pediatric ICU (PICU) ("severe"). Severe cases were significantly younger ($P = 0.028$), weighed less ($P = 0.0057$), were more likely to have nonrespiratory comorbidities ($P = 0.006$), and stayed in the hospital for longer ($P < 0.0001$) than moderate cases (Table 1). RSV was the mostly commonly identified pathogen (55% of cases), followed by rhinovirus (31%), human metapneumovirus (13%), adenovirus (11%), parainfluenza viruses (9%), and influenza-A (4%); 16% of cases were coinfected with two or more viruses. No significant difference were observed between groups in the infecting virus(es) (Table 1; *see* Table E1 in the online supplement). Demographic differences between bronchiolitic infants with and without RSV are detailed in Table 1; RSV cases were significantly younger (moderate, $P = 0.021$; severe, $P = 0.004$) and weighed less (moderate, $P = 0.020$; severe = 0.005), in line with previous reports (26). Samples were collected from infants up to twice daily during hospitalization; the mean number of sampling visits per participant was 3.2 (range, 1–13); the number of sampling visits per patient is outlined in Figure E1. A total of 175 study visits yielded

Table 1. Demographics of Infants with Viral Bronchiolitis

	Moderate				Severe				Moderate vs. Severe		
	All AVLRI (*n* = 34)	**Moderate RSV (*n* = 18)**	**Moderate Non-RSV (*n* = 16)**	***P* Value (RSV vs. Non-RSV)**	**All AVLRI (*n* = 21)**	**Severe RSV (*n* = 12)**	**Severe Non-RSV (*n* = 9)**	***P* Value (RSV vs. Non-RSV)**	***P* Value (All AVLRI, *n* = 55)**	***P* Value (RSV, *n* = 30)**	***P* Value (Non-RSV, *n* = 25)**
Age, d, median (range)*	180 (30–630)	76.5 (30–540)	255 (60–630)	**0.021**	56 (15–630)	32 (15–300)	395 (28–630)	**0.0042**	**0.028**	**0.002**	0.923
Sex, male:female†	21:13	13:5	8:8	0.291	13:8	9:3	4:5	0.203	>0.999	>0.999	>0.999
Weight, kg, median (range)*	7.3 (3.56–12.8)	5.52 (3.56–12.8)	8.85 (4.98–12.3)	**0.020**	3.9 (2.2–13.7)	3.6 (2.2–9.1)	9.9 (3.6–13.7)	**0.005**	**0.0057**	**0.0001**	0.967
Prematurity, *n* (%)†	11 (32)	7 (39)	4 (25)	0.477	7 (33)	5 (42)	2 (22)	0.642	>0.999	>0.999	>0.999
Nonrespiratory comorbidity, *n* (%)†	11 (32)	4 (22)	7 (44)	0.274	15 (71)	10 (83)	5 (55)	0.331	**0.006**	**0.002**	0.688
Length of hospital stay, d, median (range)*	2 (0.5–7)	2 (0.5–5)	2 (0.5–7)	0.710	14 (3–38)	14 (8–38)	12 (3–31)	0.254	**<0.0001**	**<0.0001**	**<0.0001**
Time between first symptoms and admission, d, median (range)*	3 (0–15)	4.5 (2–7)	2 (0–15)	**0.012**	3 (0–15)	1.5 (0–10)	5 (0–15)	0.057	0.330	**0.0038**	0.173
Viral coinfections, *n* (%)†‡	6 (18)	5	1	0.180	3 (14)	0	3 (33)	0.063	>0.999	0.066	0.116

Definition of abbreviations: AVLRI = acute viral lower respiratory tract infections; RSV = respiratory syncytial virus.

The study recruited infants with bronchiolitis of moderate severity from the pediatric wards (moderate, $n = 34$) and severe, mechanically ventilated cases from the pediatric ICU (severe, $n = 21$). Prematurity was defined as birth at <36 weeks' gestational age. Bold text highlights significant differences ($P < 0.05$).

*Two-tailed Mann-Whitney *U* tests were used for statistical evaluation of nonparametric continuous variables.

†Fisher exact tests were used for statistical evaluation of categorical variables.

‡Diagnosis of viral infection was based on clinical virology multiplex PCR respiratory viral screen of nasopharyngeal aspirates.

124 nasopharyngeal aspiration (NPA) samples and 175 nasosorption samples; NPA was refused by parents on 29% of study visits because of parental concern regarding tolerability.

Extracting RNA from nasosorption samples using RNA extraction buffer increased RSV RNA yields 5.8-fold, relative to nasosorption samples eluted with immunoassay buffer (data not shown). Nasosorption, eluted with RNA extraction buffer, was therefore compared with NPA samples for multiplex PCR-based respiratory viral screening. Using NPA multiplex results as a gold standard, nasosorption samples had a sensitivity of 78% and a specificity of 67% across all tested viruses. These results are similar to other reported noninvasive airway sampling techniques (27, 28) but indicated that nasosorption had poorer diagnostic utility than NPA when using a multiplex PCR assay. However, using a more sensitive single quantitative PCR (qPCR) assay, RSV was detected at 100% sensitivity and 100% specificity, relative to NPA, in line with our previous report (25), thereby validating nasosorption as a tool for respiratory virus detection when using high-sensitivity qPCR assays (data not shown).

To confirm the utility of nasosorption for quantifying mucosal inflammatory mediators in the upper airway, relative to NPA, matched samples from 124 study visits were compared. Levels of all mediators were lower in nasosorption samples (all $P < 0.05$; Figure E2A), with a variable scale in this difference between mediators (Figure E2B). However, levels of all mediators significantly correlated between matched NPA and nasosorption samples (Figure E2C and Table E2). Furthermore, levels of cytokines and chemokines were more variable in NPA than in nasosorption, shown by the larger interquartile values in NPA. RSV load was also determined in matched nasosorption and NPA samples ($n = 47$), where VL was significantly higher in NPA ($P < 0.0001$, Figure E2D), but closely correlated ($R_S = 0.692$; $P < 0.0001$; Figure E2E). Bland-Altmann analysis of sampling techniques for viral quantification demonstrated consistent bias toward higher levels in NPA (Figure E3). Given that VL and mediator measurements from nasosorption samples were representative of NPA, and more nasosorption samples were available in a serial manner from infants, these samples were analyzed in further detail.

As RSV was the most commonly identified pathogen associated with bronchiolitis in this cohort, we first looked at RSV cases in isolation, to allow characterization of VL and the immune response to a specific pathogen. At the first sampling visit, RSV load was higher in moderate cases relative to severe cases in both nasosorption (Figure 1A) and NPA (Figure E4). In addition, at visit 1, levels of C-X-C motif chemokine ligand 10 (CXCL10)/IP-10, IFN-γ, and CCL5 (C-C chemokine ligand 5)/RANTES (regulated upon activation, normal T cell expressed and secreted) were lower in severe RSV, relative to moderate cases (Figures 1B–1D, respectively), although this did not reach statistical significance for CXCL10/IP-10. Associating VL with the duration of symptoms, regardless of study visit number, confirmed that moderate cases had higher RSV loads than severe cases, at equal symptom durations (Figure 1E). Similarly, levels of CXCL10/IP-10, IFN-γ, and CCL5/RANTES levels were elevated in moderate cases, relative to severe, over symptom duration (Figures 1F–1H, respectively). Most other measured cytokines were similarly elevated in moderate disease, relative to severe (Figure E5).

Given our observation of lower mediator levels in severe RSV disease, we next looked at RSV-negative cases. A similar trend toward elevated levels of CXCL10/IP-10 was observed in moderate cases, relative to severe, at visit 1 ($P = 0.09$; Figure 2A). Combining RSV-positive and RSV-negative cases resulted in significantly elevated CXCL10/IP-10 levels in moderate disease relative to severe ($P = 0.016$; Figure 2A). Trends toward elevated levels of IFN-γ ($P = 0.124$; Figure 2B) and CCL5/RANTES ($P = 0.142$; Figure 2C) were observed in moderate AVLRI. Conversely, IL-6 levels were equivalent between groups (Figure 2D), suggesting that lower levels in severe disease were not common to all mediators. Figure E6 characterizes additional inflammatory mediators from study visit 1. Because the greatest difference between RSV severity groups was observed in the first 7 days of symptoms, we focused on this period in all AVLRI cases. During the first week of illness, levels of CXCL10/IP-10 were higher in moderate cases, relative to severe (Figure 2E). IFN-γ levels were also higher in moderate cases (Figure 2F), where IFN-γ peaked around Day 4 of symptoms, which was not observed in severe cases. Similar kinetics were observed for CCL5/RANTES (Figure 2G). Elevated mediator levels in moderate cases were not universal, as levels of IL-6 were equivalent between groups at matched symptom durations (Figure 2H). The full time course for all mediators demonstrated that many were higher in moderate AVLRI cases, across symptom durations (Figure E7). These results indicated that severe AVLRI was associated with lower levels of some mediators, particularly IP-10, relative to moderate severity disease.

Importantly, although RSV load was associated with inflammatory mediator levels (e.g., CXCL10/IP-10; Figure E8A), differences in VL at the first study visit were still significant when only infants younger than 100 days old were analyzed ($P = 0.013$; Figure E8B). Premature and non-premature infants with severe disease had equivalent VL ($P = 0.662$; Figure E8C), indicating that this most frequent preexisting health condition did not influence the observed results. In addition, VL was not directly associated with age (Figure E8D). Neither were mediator levels associated with age (e.g., CXCL10/IP-10; Figure E8E). This suggested that the younger age of infants with severe viral AVLRI did not solely account for the observed results.

The lower RSV load and inflammatory mediator levels observed in infants with severe AVLRI was particularly evident during the first 7 days of symptoms. To determine significance between nasosorption inflammatory mediator levels of severe and moderate AVLRI cases, a time-weighted average (TWA) was determined for each case during the first 7 days of symptoms. These cases ($n = 44$; moderate, $n = 29$; severe, $n = 15$) were hierarchically clustered based on the TWA levels of all measured inflammatory mediators (Figure 3A). Severe cases (red) predominantly clustered at the lower level of expression for all mediators, in contrast to moderate cases (blue), which generally had higher mediator levels. There was no evident clustering of data between RSV-positive (purple, $n = 27$) and non-RSV (green, $n = 17$) bronchiolitis. To compare these mediator levels to healthy infants, a *Z*-score of the TWA was determined for each case, relative to the median level observed in a cohort of healthy control (HC) infants (for CXCL10/IP-10 and

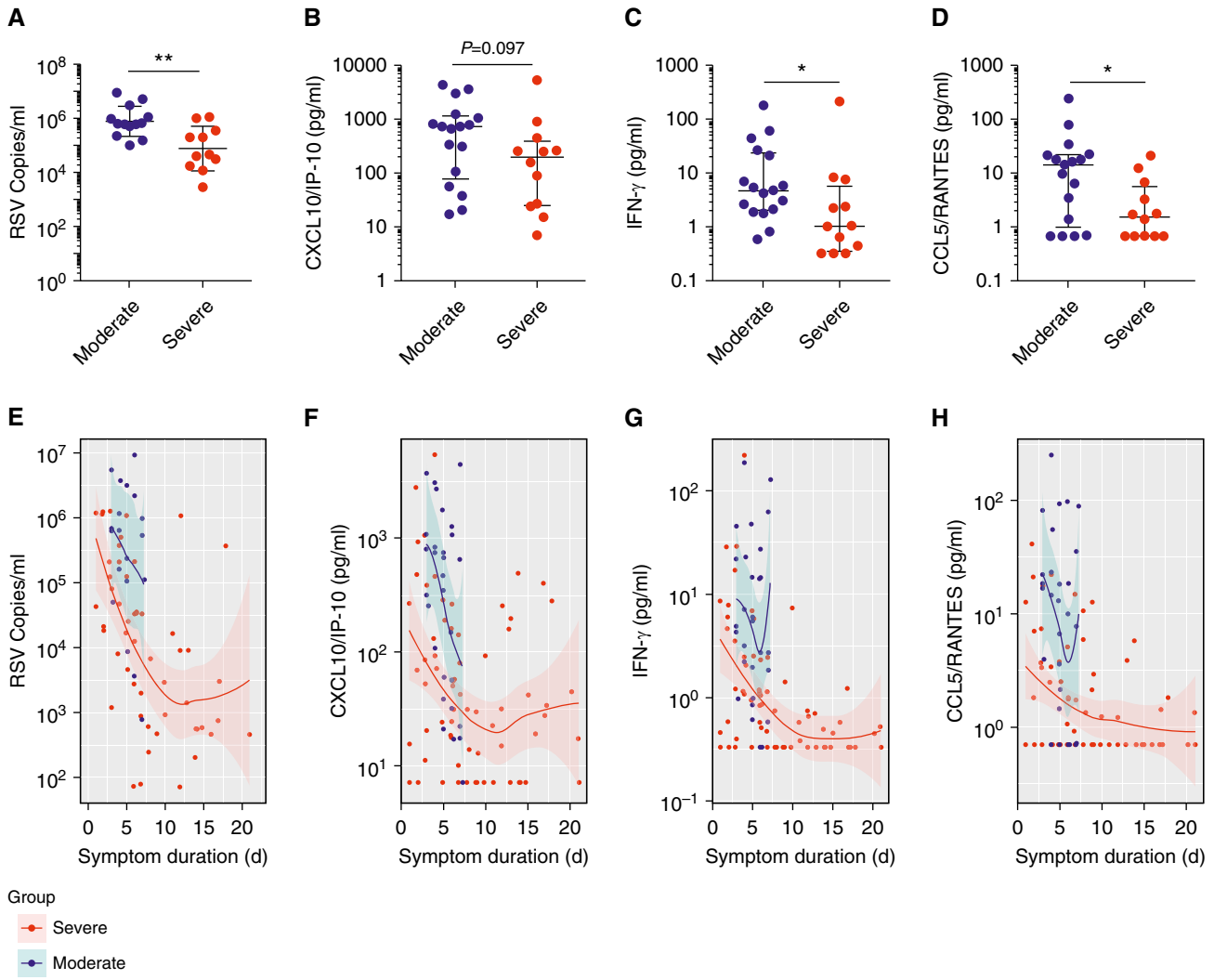

Figure 1. Lower respiratory syncytial virus (RSV) load and inflammatory mediators in severe RSV bronchiolitis. Where available, nasosorption fluid from the first sample collected from RSV-positive cases were tested for (*A*) RSV load, using quantitative PCR, in mechanically ventilated patients in the pediatric ICU (severe, $n = 11$, red) and the pediatric wards (moderate, $n = 13$, blue); and (*B*) CXCL10 (C-X-C motif chemokine ligand 10)/IP-10, (*C*) IFN-γ, and (*D*) CCL5 (C-C chemokine ligand 5)/RANTES (regulated upon activation, normal T cell expressed and secreted) (moderate, $n = 17$; severe, $n = 12$). Symptom duration at each study visit was plotted against (*E*) RSV load, (*F*) CXCL10/IP-10, (*G*) IFN-γ, and (*H*) CCL5/RANTES levels at that visit, shown as Loess fitted regressions for moderate (blue) and severe (red) groups. Fewer individuals are included in *A*, as nasosorption samples for virology were not collected in six individuals at baseline. *A–D* were analyzed for statistical significance using two-tailed Mann-Whitney *U* tests (*$P < 0.05$, **$P < 0.01$). In *E–H*, the dark line represents the Loess fitted regression, and the shaded areas represent the 95% confidence intervals.

CCL5/RANTES, $n = 6$; mean age, 183 d; range, 33–313 d; three boys; and for other mediators, $n = 13$; mean age, 177 d; range, 33–313 d; 7 boys), where a score of 0 denoted equivalence to HC. *Z*-score analysis demonstrated that several mediators were elevated in moderate cases relative to severe, including IFN-γ, CCL5/RANTES, and TNF-α (tumor necrosis factor-α); however, the principal increase observed was in CXCL10/IP-10 levels, which was observed in both RSV-positive and RSV-negative AVLRI (Figure 3B). By contrast, levels of IL-6 were higher than HC in all groups, independent of severity. TWAs for CXCL10/IP-10 were significantly higher in moderate RSV cases, relative to severe RSV ($P = 0.027$), whereas a similar trend ($P = 0.103$) was evident for RSV-negative AVLRI (Figure E9A). A large effect size was observed for IP-10 TWA levels between moderate and severe RSV groups (Cohen's d = 1.04). However, this effect size was more conservative than those of some individual days (Table E3), reflecting the greater distinction between groups in the earliest days of symptoms (Figure 1F). Interestingly, IFN-γ was also significantly higher in moderate RSV cases relative to severe RSV ($P = 0.011$); however, this difference was not evident in the non-RSV groups (Figure E9B). TNF-α, IL-1β, and CCL5/RANTES were also all significantly elevated in moderate RSV cases relative to severe RSV (all $P < 0.05$; Figures E9C–E9E, respectively), although levels were not typically above the HC range. TWAs from the first 7 days of symptoms also confirmed that nasosorption RSV loads were significantly higher in moderate cases, relative to severe ($P = 0.001$; Figure E9F). A similar trend

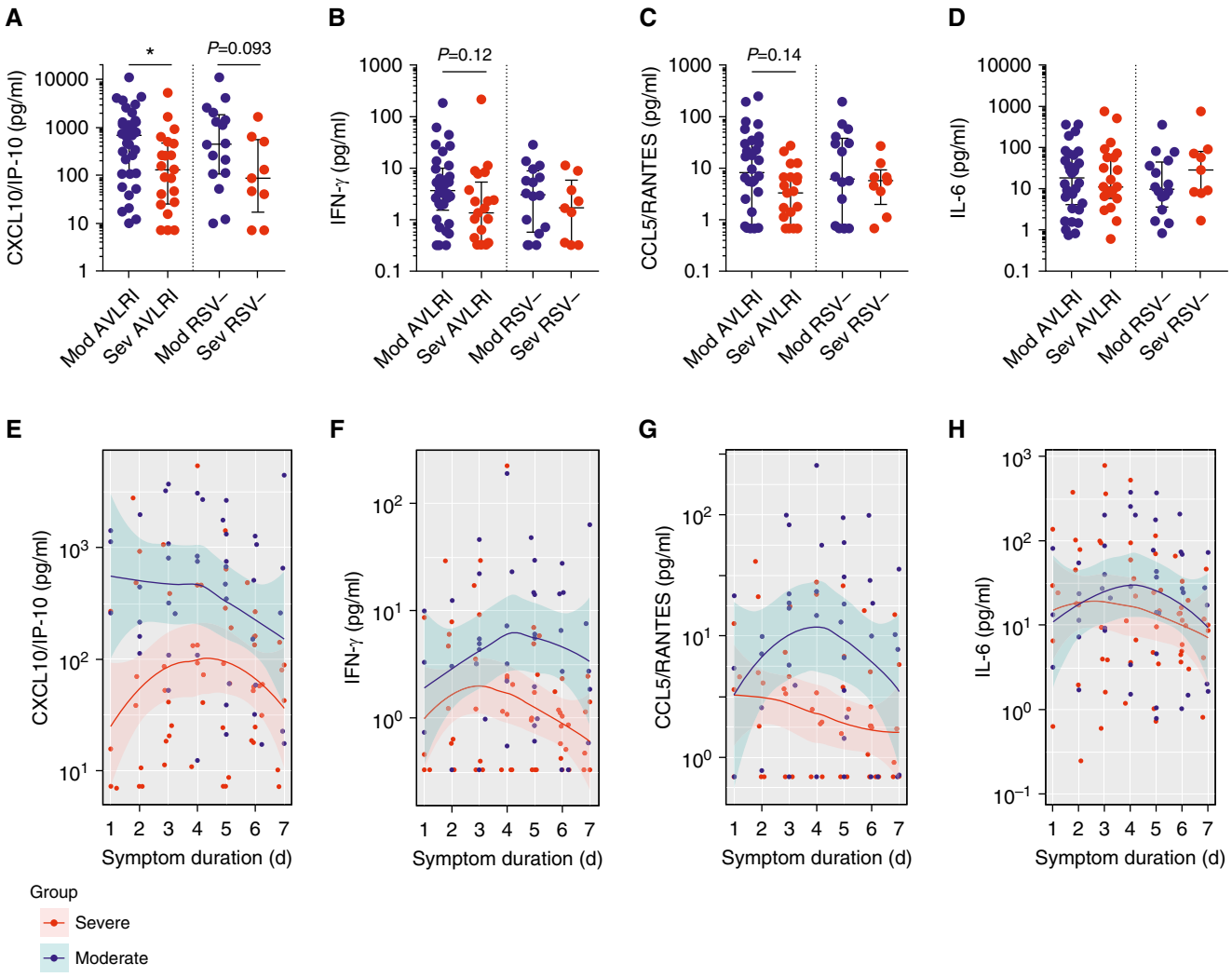

Figure 2. Lower mediator levels in both respiratory syncytial virus (RSV) and non-RSV severe viral bronchiolitis. Nasosorption samples were used for measurement of inflammatory mediators from all infants with viral bronchiolitis (acute viral lower respiratory tract infection [AVLRI]; moderate [Mod], $n = 34$; severe [Sev], $n = 21$) and RSV-negative subgroup (RSV−; Mod, $n = 16$; Sev, $n = 9$). At the first study visit, levels of (*A*) CXCL10 (C-X-C motif chemokine ligand 10)/IP-10, (*B*) IFN-γ, (*C*) CCL5 (C-C chemokine ligand 5)/RANTES (regulated upon activation, normal T cell expressed and secreted), and (*D*) IL-6 were determined in these populations. Levels of inflammatory mediators over the first 7 days of symptoms were also related to the symptom duration at the time of sampling for (*E*) CXCL10/IP-10, (*F*) IFN-γ, (*G*) CCL5/RANTES, and (*H*) IL-6. Data in *A–D* were analyzed for statistical significance using two-tailed Mann-Whitney *U* tests (*$P < 0.05$). In *E–H*, the dark line represents the Loess fitted regression and the shaded areas represent the 95% confidence intervals.

was observed in this analysis of NPA samples ($P = 0.10$; data not shown). Power calculations confirmed decreased CXCL10 levels in the severe RSV group, relative to moderate RSV ($P = 0.027$; Figure E9A), at 80% power, given $\alpha = 0.05$. Similarly, decreased VL in severe RSV, relative to moderate ($P = 0.001$; Figure E9F), was determined at 97% power, given $\alpha = 0.05$. Therefore, once duration of illness was accounted for, moderate disease was associated with significantly heightened RSV load and levels of inflammatory mediators in the first 7 days of symptoms.

The pronounced increase in CXCL10/IP-10 levels early in disease, in both RSV-positive and RSV-negative bronchiolitis, led us to further investigate IFN levels in the early response to infection.

Protein levels of the type-I IFN, IFN-α2a, and IL-29/IFN-λ1 in nasosorption and NPA samples were below the limits of detection (data not shown). RNA was therefore extracted from NPA cell pellets collected at the infant's first study visit ($n = 41$) and analyzed for expression of a panel of type-I IFNs, IFN-stimulated genes (ISGs), and cellular markers. Elevated expression of type-I IFNs and neutrophil markers was evident in moderate cases, relative to severe (Figure 4A). Indeed, the type-I IFN *IFNA1* was significantly elevated in moderate AVLRI ($P = 0.04$) and moderate RSV-positive AVLRI ($P = 0.04$), relative to severe (Figure 4B and Figure E10A). Similar trends toward elevated expression of *IFNB1* and *IFNE1* were also evident in moderate AVLRI (Figures E10B and E10C, respectively). In addition, *SOCS1* expression was significantly elevated in moderate AVLRI relative to severe ($P = 0.004$; Figure 4B and Figure E10D), a

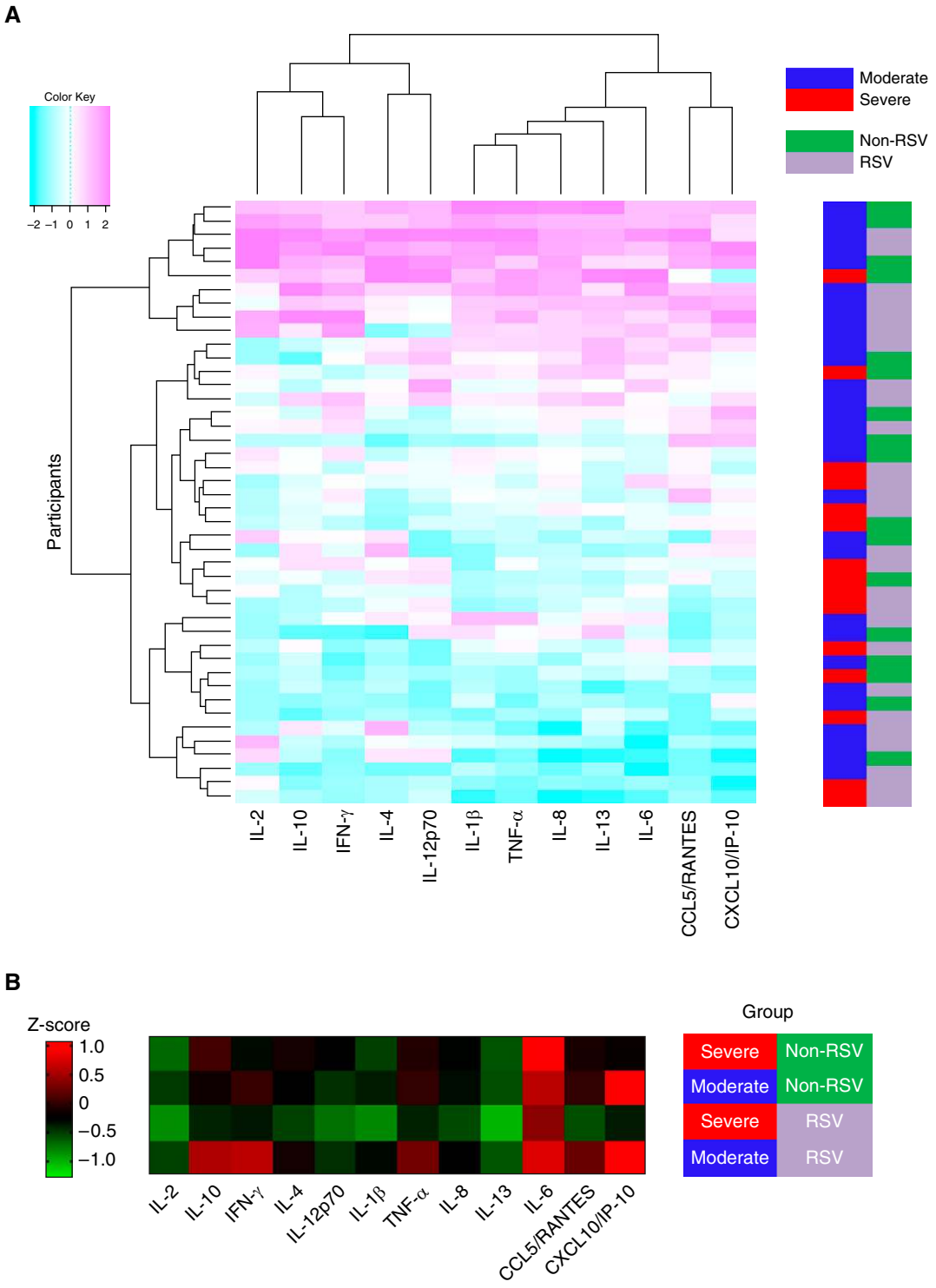

Figure 3. Mediator levels during the first week of symptoms distinguish moderate and severe viral bronchiolitis. Time-weighted averages (TWAs) compared cases of bronchiolitis ($n = 44$: moderate respiratory syncytial virus [RSV]-positive, $n = 17$; severe RSV-positive, $n = 10$; moderate RSV-negative, $n = 12$; severe RSV-negative, $n = 5$) over the first 7 days of symptoms. (*A*) Levels of all tested inflammatory mediators were used to hierarchically cluster cases. Blue, moderate; red, severe; green, RSV-negative viral infection; purple, RSV infection. (*B*) *Z*-scores of the median TWA in each group, compared with the healthy control (HC) median level, whereby 0 (black) indicates equivalence with HC, red is higher than HC, and green is lower. CCL5 = C-C chemokine ligand 5; CXCL10/IP-10 = C-X-C motif chemokine ligand 10; RANTES = regulated upon activation, normal T cell expressed and secreted; TNF = tumor necrosis factor.

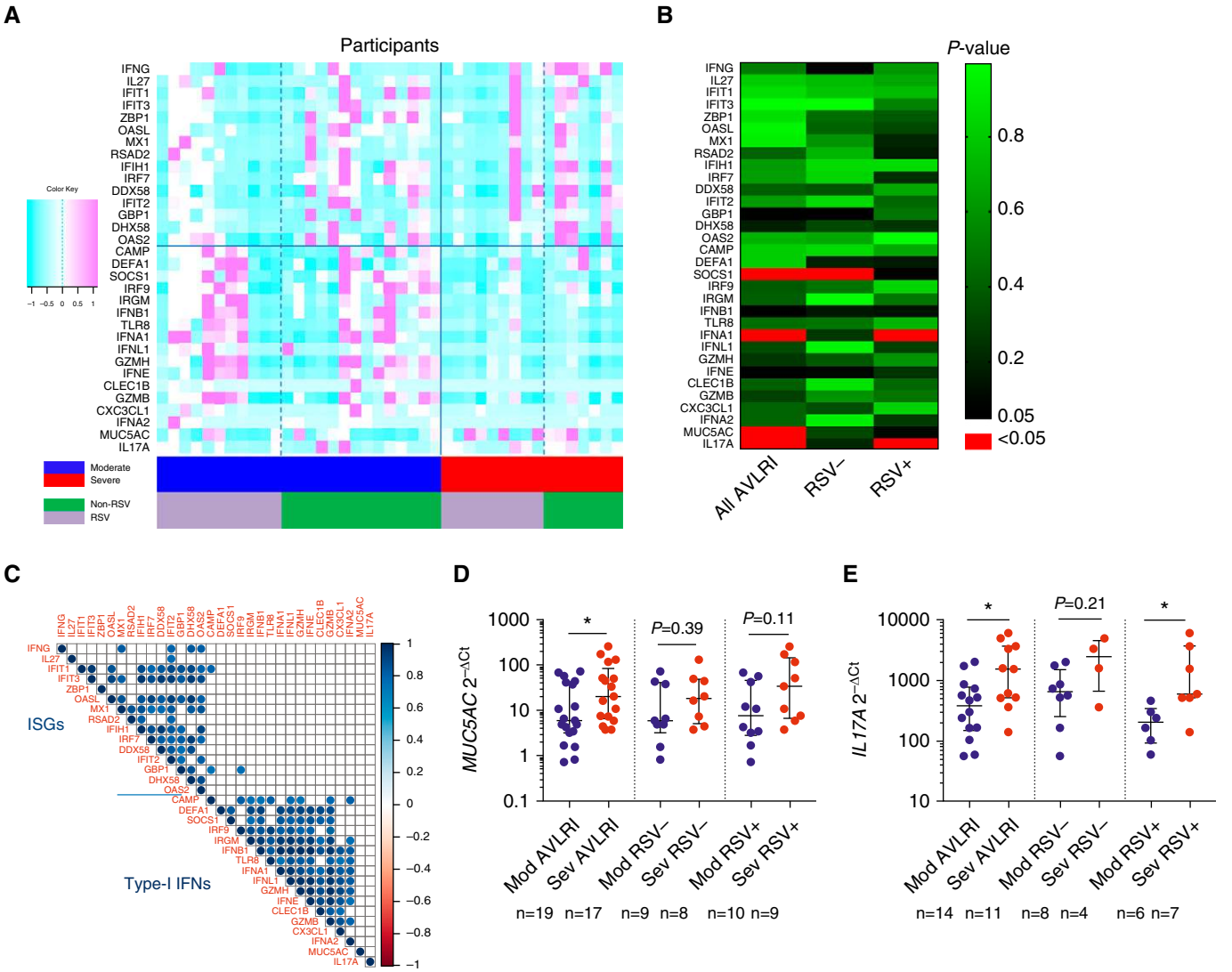

Figure 4. Lower type-I IFN gene expression and higher *MUC5AC* and *IL17A* in severe bronchiolitis. Gene expression of a panel of IFNs, IFN-stimulated genes (ISGs), and cell markers were analyzed in nasopharyngeal cells from bronchiolitic infants ($n = 43$), collected at their first study visit. (*A*) Hierarchically clustered gene expression of all analyzed genes. (*B*) *P* value heat map for expression of each gene between moderate and severe cases in all cases (all acute viral lower respiratory tract infection [AVLRI]) and respiratory syncytial virus (RSV)-negative (RSV−) and RSV-positive (RSV+) cases. (*C*) Hierarchically clustered correlation plot of all tested genes in RSV+ cases, where significant correlations are denoted by a circle and the *R* value by the color of the circle. (*D* and *E*) Expression of *MUC5AC* (*D*) and *IL17A* (*E*) in these samples. Statistical analysis used two-tailed Mann-Whitney *U* tests (*$P < 0.05$). Correlation was assessed by Spearman's tests. Mod = moderate; Sev = severe.

difference also observed in both RSV-negative and RSV-positive subgroups. Gene expression formed two distinct clusters, the first cluster composed mostly of ISGs and the second cluster containing IFNs and neutrophil markers (Figure 4C; RSV-positive cases). In contrast to greater type-I IFN levels in moderate cases, severe cases had elevated expression of *MUC5AC* (mucin-5AC) ($P = 0.04$; Figure 4D) and *IL17A* ($P = 0.03$; Figure 4E) relative to moderate AVLRI cases. These data associated lower VL, type-I IFNs, and CXCL10/IP-10 levels, along with an elevated mucogenic response, with severe viral bronchiolitis in infants.

Discussion

The relative role of viral damage to the epithelium and the host immune response to the severity of viral disease is a topic of considerable debate. In this study, we measured the VL and the levels of many immune mediators in nasal fluids from virally infected infants admitted to hospital wards and were surprised to find that moderate bronchiolitis was associated with elevated levels of IFN-related proteins when compared with severe (mechanically ventilated) cases. The only indicator of enhanced responses in severe disease was greater *MUC5AC* and *IL17A* expression. RSV was the most common pathogen associated with AVLRI in this study, where RSV load was surprisingly also significantly lower in severe cases. These results indicate that severe disease is not accompanied by

elevated VL or enhanced host responses but is instead associated with diminished IFN levels and indications of IL-17 production and local mucogenesis.

A crucial strength of this study is the combination of a longitudinal design with minimally invasive sampling techniques to associate the immune response during bronchiolitis to the duration of symptoms. This allowed us to sample 55 infants repeatedly, on up to 13 occasions, generating detailed kinetic data. However, further large multicenter studies are required to confirm and extend our findings. Respiratory failure, and requirement for supportive mechanical ventilation, is a feature of the most severe cases of bronchiolitis. Such cases reflect those most likely to be life threatening in low- and middle-income countries and represent a major health care economic burden in developed nations (3, 29). Despite the particular importance of this group, such infants have been relatively understudied in attempts to establish correlates of disease severity.

A major limitation of our study is our sampling of the upper airway (and especially the anterior compartment of the nose) to reach our conclusions, whereas the disease driving bronchiolitis is in the lower airway and lung. We were unable to compare upper and lower airway samples in the current study, and the relationship between responses at these sites was not assessed. One important demographic difference in our study was the younger age of severe cases. The decreased response to infection in severe cases could therefore be influenced by age; however, no correlation was evident between age and inflammatory mediator levels or RSV load. The greater frequency of nonrespiratory comorbidities in severe cases was another important difference between groups, which is intrinsically associated with the most severe RSV cases (9, 30). Large multicenter studies of patients with severe disease are required to confirm the present observations, using multivariate analyses to compensate for confounding demographic and clinical differences. Symptom duration at the time of sampling was based on a subjective assessment by the infants' caregivers. However, as the time from infection cannot be known, basing time courses on the duration of symptomatic illness was the closest possible approximation. Host genetics may contribute to bronchiolitis severity, although a recent multicenter genome-wide association study did not observe any significant associations with SNPs (31). Finally, mechanical ventilation has been demonstrated to increase mucosal proinflammatory mediator levels (32); however, this confounding difference between groups is unavoidable and would be expected to have the reverse effect to our observation of lower mediator levels in ventilated infants.

Levels of most mediators on nasosorption samples decreased over time during illness, and the greatest differences between severe and moderate cases of bronchiolitis were observed in the first 7 days of symptoms. In addition, the relative scarcity of moderately ill cases with more than 7 days of symptoms made comparisons between groups difficult beyond this point. Use of TWAs confirmed that elevated mediator levels in moderate cases, as observed in scatterplots over time, were significant. This approach also confirmed that RSV load was significantly lower in PICU-admitted RSV cases, after allowance for symptom duration. Other reports have observed insignificant differences in RSV load between PICU and non-PICU RSV cases using NPA (12, 13) or elevated VL in severe disease (10, 11). Two key differences between these reports and our study are our close accounting for symptom duration and our focused comparison of ventilated cases with moderately ill hospitalized infants. Within a moderately ill hospitalized cohort, RSV load correlates with length of hospitalization (12, 13); however, our data demonstrate that this association cannot be extended to severely ill cases with respiratory failure.

Recently, Nicholson and colleagues highlighted that a heightened nasal inflammatory response during bronchiolitis of infancy was associated with decreased risk of hospitalization (24). This study took nasal wash specimens from children attending the emergency department to measure VL and cytokines/chemokines and compared hospitalized and nonhospitalized children. Our current study only includes hospitalized patients with RSV and compares those on the wards (moderate) and children given mechanical ventilation (severe). Hence, our results confirm and extend the observations of Nicholson and colleagues (24), suggesting that a continuous decrease in the scale of the immune response to infection is observed from mild (nonhospitalized) cases to moderate and then severe disease.

Infants with severe RSV may have lower cytokine responses because of cell exhaustion, a feature of many autoimmune and infectious diseases (33). Exhaustion and cell death involve the immune checkpoint molecule programmed death ligand 1 in T cells, B-cells, and dendritic cells (34), and various mechanisms of programmed cell death have been delineated (35). During chronic viral infections, $CD8^+$ T-cell exhaustion occurs (33), viral nucleotide analogs cause dendritic cell necroptosis (36), and influenza A (H1N1) induces programmed death ligand 1 on human T cells and dendritic cell (37); natural killer cell exhaustion is also described in viral infections (38).

Neutrophils are the predominant infiltrating cell in the lungs of babies with RSV bronchiolitis, and these cells can limit viral spread but also cause damage (39). In severe RSV bronchiolitis, it has been shown that the virus undergoes transcription in blood neutrophils (40) and that neutrophil TLR4 (Toll-like receptor 4) expression and function are reduced in the blood and airways (41), with distinct blood neutrophil subsets in severe viral infection (42). Interestingly, in our study, gene expression of several granzymes and antimicrobial peptides, including *CAMP* (cathelicidin) and *DEFA1* (defensin-α1), associated with neutrophils were clustered with type-I IFNs, but were not significantly differentially expressed between severity groups. Alveolar macrophages have been reported to generate type-I IFNs in the early response to RSV infection, thereby limiting disease (43). In addition, M1-monocytes are a major determinant of severity in life-threatening influenza (44). Given our observation of lower type-I IFN expression in severe disease, we speculate that alveolar macrophages and/or neutrophils may mediate these differences between moderate and severe infants.

We did not observe any elevation of the type-II mediators IL-4 or IL-13 during bronchiolitis. Indeed, levels of these mediators were equivalent to HC infants, in contrast to some previous observations of a type-II biased immune response (21, 22). Furthermore, we demonstrate that severe disease was associated with elevated expression of the mucin *MUC5AC* in the nasopharynx, which may contribute to mucus plugging of the airway and

respiratory failure (45). IL-17 has been associated with mucus production, and *IL17A* expression was similarly elevated in severe cases (46).

This study also confirmed the utility of nasosorption for quantifying RSV load (25). Relative to NPA samples, RSV load on nasosorption samples were lower but still detectable using a sensitive qPCR assay. This difference in viral yield between methodologies was reflected in the lower sensitivity of nasosorption samples in a multiplex clinical PCR assay for 10 viral pathogens. Identification of viral infection using nasosorption may therefore be of limited clinical utility but provides a less-invasive alternative to NPA that is well suited to clinical studies, where some yield of virus and immune mediators can be sacrificed for improved standardization, repeatability, and tolerability.

Common respiratory viruses, including RSV, disrupt the host antiviral IFN response, and different strains of virus achieve differing scales of immunosuppression (47, 48), which may be differentially achieved between moderate and severe cases, accounting for our observations. However, despite lower type-I IFN levels, RSV load was lower in severe cases. Interestingly, gene expression of NPA cells in this study suggested that IFN expression was similar between RSV-positive and RSV-negative moderate cases, but ISG expression may be lower in RSV. This difference might reflect enhanced inhibition of type-I IFN receptors by RSV, relative to other respiratory viruses. Deep genome sequencing of each viral isolate may shed light on whether our observation of differing scales of type-I IFN production between severe and moderate disease are the result of subtly different infecting RSV strains.

Our observations cast doubt on the two major theories advanced to explain the severity of disease in children with viral infections of the respiratory tract. Our conclusion that neither VL nor a "storm" of immune responses is associated with respiratory failure requires further studies of the responses of the lower airway (in situations where access can be gained) and direct measurements of IL-17 and mucin production (indicated by gene expression to be enhanced in severe cases). In future studies, we aim to assess the contribution of age and host genetic/epigenetic factors and viral diversity to severity using enhanced, standardized respiratory sampling techniques. ■

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