ORIGINAL ARTICLE

Respiratory Syncytial Virus Disease Severity Is Associated with Distinct CD8⁺ T-Cell Profiles

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Abstract

Rationale: Respiratory syncytial virus (RSV) causes significant morbidity and mortality in infants worldwide. Although T-helper type 2 (Th2) cell pathology is implicated in severe disease, the mechanisms underlying the development of immunopathology are incompletely understood.

Objectives: We aimed to identify local immune responses associated with severe RSV in infants. Our hypothesis was that disease severity would correlate with enhanced Th2 cellular responses.

Methods: Nasal aspirates were collected from infants hospitalized with severe (admitted to the pediatric ICU) or moderate (maintained in the general ward) RSV disease at 5 to 9 days after enrollment. The immune response was investigated by evaluating T-lymphocyte cellularity, cytokine concentration, and viral load.

Measurements and Main Results: Patients with severe disease had increased proportions of CD8 (cluster of differentiation 8)-positive T cells expressing IL-4 (Tc2) and reduced proportions of CD8⁺ T cells expressing IFN γ (Tc1). Nasal aspirates from patients with severe disease had reduced concentrations of IL-17. Patients with greater frequencies of Tc1, CD8⁺ T cells expressing IL-17 (Tc17), and CD4⁺ T cells expressing IL-17 (Th17) had shorter durations of hospitalization.

Conclusions: Severe RSV disease was associated with distinct T-cell profiles. Tc1, Tc17, and Th17 were associated with shorter hospital stay and may play a protective role, whereas Tc2 cells may play a previously underappreciated role in pathology.

Keywords: RSV; infant; adaptive; immunity

At a Glance Commentary

Scientific Knowledge on the Subject: Most infants with respiratory syncytial virus are infected by their second birthday. Despite known risk factors, such as prematurity and congenital heart disease, most hospitalized infants are previously healthy. The mechanisms underlying the immunopathology of severe disease are incompletely understood.

What This Study Adds to the Field: Severe respiratory syncytial virus disease was associated with increased IL-4-expressing CD8 (cluster of differentiation 8)-positive T cells and reduced IFN γ -expressing T cells in nasal aspirates. IL-17 concentration in nasal aspirates was reduced in patients with severe disease, and patients with lower frequencies of IL-17-producing T cells had longer durations of hospitalization.

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Table 1. Patient Demographics and Clinical Characteristics

Characteristic	Moderate <i>n</i> = 38	Severe <i>n</i> = 24	<i>P</i> Value
Age at admission, d, median (range)	69 (13–362)	87 (11–357)	0.749
Birthweight, g, median (range)	2,977 (794–4,139)	2,821 (1,219–4,309)	0.5355
Sex, <i>n</i> (%)			0.4092
F	14 (37)	6 (25)	
M	24 (63)	18 (75)	
Race, <i>n</i> (%)			0.0779
White	7 (18)	10 (42)	
African American and Hispanic	31 (82)	14 (58)	
Birthplace, <i>n</i> (%)			0.4097
Neonatal ICU	11 (29)	10 (42)	
Well-baby nursery	27 (71)	14 (58)	
Premature, <i>n</i> (%)			0.4286
No	24 (63)	12 (50)	
Yes	14 (37)	12 (50)	
Gestational age, wk, median (range)	38 (24–41)	36.5 (29–40)	0.3842
Breastfed, <i>n</i> (%)			0.1139
No	20 (53)	7 (29)	
Yes	18 (47)	17 (71)	
Exposure to tobacco*, <i>n</i> (%)			1
No	28 (74)	18 (75)	
Yes	10 (26)	6 (25)	
Daycare, <i>n</i> (%)			0.5111
No	32 (84)	18 (75)	
Yes	6 (16)	6 (25)	
Delivery, <i>n</i> (%)			1
Cesarean	14 (37)	9 (38)	
Vaginal	24 (63)	15 (62)	
Family history of asthma, <i>n</i> (%)			0.0683
No	14 (37)	15 (63)	
Yes	24 (63)	9 (37)	
Oxygen supplementation, <i>n</i> (%)			0.0043
No	11 (29)	0 (0)	
Yes	27 (71)	24 (100)	
Mechanically ventilated, <i>n</i> (%)			<0.0001
No	38 (100)	8 (33)	
Yes	0 (0)	16 (67)	
RSV strain†, <i>n</i> (%)			0.39
A	16 (47)	12 (63)	
B	18 (53)	7 (37)	
LOS, d, median (range)	5 (2–11)	15 (7–103)	<0.0001
Duration of fever, d, median (range)	1 (0–5)	1 (0–6)	0.8185
Duration of congestion, d, median (range)	4 (1–14)	3 (0–14)	0.4492
Duration of increased work of breathing, d, median (range)	2 (0–14)	2 (0–6)	0.4043
Duration of cough, d, median (range)	4 (1–10)	3 (0–14)	0.3343
RSV viral load†, log TCID ₅₀ /ml, median (range)	6.29 (3.73–7.63)	6.47 (5.05–7.71)	0.3404

Definition of abbreviations: LOS = length of stay; RSV = respiratory syncytial virus; TCID₅₀/ml = 50% tissue culture infectious dose per milliliter.

Patient demographics and clinical information by pediatric ICU status. Statistical significance was determined by Fisher's exact test for categorical data and Mann-Whitney test for continuous data. No adjustment was made for multiple testing. *P* values for continuous data were computed by Mann-Whitney test. *P* values for categorical data were computed by Fisher's exact test. Bold indicates *P* < 0.05.

*Any household member smokes.

†Unknown data not accounted for in analyses.

Respiratory syncytial virus (RSV) is the number one cause of lower respiratory infections in children (1) and hospitalization with severe RSV disease in infancy is linked to asthma development later in life (2). There are no vaccines or specific therapeutics available, and prophylactic treatment with palivizumab is approved for high-risk infants only (3). Although there are known risk factors for

severe RSV disease, such as prematurity, <6 months of age upon initial infection, and congenital heart disease, the majority of infants who are hospitalized are previously healthy without known risk factors (4). Studies have suggested a role for immune dysfunction in RSV disease severity. Nasal lavages of infants with RSV bronchiolitis exhibit a greater IL-4/IFN γ ratio compared with infants

without RSV bronchiolitis (5), suggesting a role for T-helper type 2 (Th2) (CD4 [cluster of differentiation 4]-positive IL-4⁺ T cells) in immunopathology. Disease severity in infants hospitalized with RSV has been shown to correlate with the level of immunoregulatory IL-10-expressing B cells, which dampen protective Th1 responses, in the nasopharyngeal aspirate (6). Nasopharyngeal

Figure 1. Patients with severe disease have a CD8 (cluster of differentiation 8)-skewed T-cell response. Nasal aspirates from infants collected 1 week after enrollment were analyzed by flow cytometry. (A and B) The percentages of CD4⁺ and CD8⁺ T cells (A) and the ratio of CD8⁺/CD4⁺ T cells (B) are compared between patients with moderate disease ($n = 31$) and patients with severe disease ($n = 16$). ** $P < 0.01$ by bivariate analysis.

aspirates have also been shown to contain enhanced proinflammatory cytokines such as IL-6 and IL-8 (7–9), which may contribute to the development of pathogenic CD4⁺ expressing IL-17 (Th17) responses. Furthermore, infants hospitalized with a severe episode of RSV disease maintain high levels of proinflammatory cytokines in their nasopharyngeal aspirates 1 year after hospitalization, with 85% of infants suffering at least one wheezing incident (10). Despite strong evidence that severe RSV disease is caused by a dysfunctional immune response and animal studies that suggest a role for Th2 cells in immunopathology, there have been few studies looking at the cellular immune response to RSV in the nasal mucosa of human infants.

Here, for the first time, we present data comparing the mucosal T-cell immune responses of infants hospitalized with RSV disease of varying severity. We tested the hypothesis that patients admitted to the pediatric ICU (PICU; severe) would have a greater proportion of Th2 cells in their nasal aspirates compared with patients admitted to the general ward (moderate). This study reveals that RSV disease severity is associated with a specific T-cell profile and that CD8⁺ T cells expressing IL-4 (Tc2) cells may play an important, underappreciated role in the development of RSV immunopathology.

Methods

Study Design

Infants hospitalized with a positive RSV quantitative PCR result in Le Bonheur

Children's Hospital, Memphis, Tennessee, during the respiratory seasons of 2014 to 2017 were enrolled in this study. Patients who were admitted to or transferred to the PICU within 10 days of hospital admission were defined as having severe disease, while those admitted to the general floor were defined as having moderate disease. Nasal aspirates were collected from 62 patients at day of enrollment for viral load determination and between 5 and 9 days after enrollment for flow cytometry and cytokine analysis. At this later time point, some patients had been discharged and returned to the hospital for sample collection. Of the 62 patients who provided samples up to this time point, 24 patients had disease severe enough for admission to the PICU and 38 patients with moderate disease were maintained on the general ward (see Figure E1 in the online supplement).

Sample Collection

Nasal aspirates were collected by trained study personnel using a standardized method described previously (11). Cell and supernatant isolation are described in the online supplement.

Flow Cytometry

Cell staining is described in detail in the online supplement. Signature cytokine expression was used to classify nasal aspirate T cells, and gating on cytokine positive populations was set based on fluorescence minus one controls (see Figures E2 and E3). Within the live CD3⁺CD4⁺ population, T cells were

defined as Th1 (IFN γ ⁺IL-4[−]IL-17[−]), Th2 (IFN γ [−]IL-4⁺IL-17[−]), and Th17 (IFN γ [−]IL-4[−]IL-17⁺). Within the live CD3⁺CD8⁺ population, T cells were defined as T cells expressing IFN γ (Tc1) (IFN γ ⁺IL-4[−]IL-17[−]), Tc2 (IFN γ [−]IL-4⁺IL-17[−]), and CD8⁺ T cells expressing IL-17 (Tc17) (IFN γ [−]IL-4[−]IL-17⁺). Samples with less than 100 CD4⁺ or CD8⁺ T cells were excluded from further analyses.

Real-Time RT-PCR

Viral RNA was extracted from cell-free fractions of nasal aspirates collected on the day of enrollment using QIAamp Viral RNA kit (Qiagen). Viral load was interpolated by qRT-PCR from a standard curve produced from an RSV stock at known concentration in 50% tissue culture infectious dose per milliliter. Details are provided in the online supplement.

Multiplex Cytokine Analysis

Cell-free supernatants from nasal aspirates were evaluated for IFN γ , IL-4, IL-13, and IL-17A concentrations using the Luminex technology multiplex cytokine kit, Milliplex Human High Sensitivity T-cell Panel (EMD Millipore) with a BioPlex 200 instrument (Bio-Rad). Samples with cytokine concentrations below the limit of detection of the assay were replaced by a value equal to the limit of detection divided by the square root of 2 (12).

Statistical Analysis

Frequencies and percentages were used to summarize categorical data, whereas median and interquartile ranges were used to describe continuous data. Comparison of patient demographics, clinical characteristics, and cellular and cytokine responses in nasal aspirates were tested using Fisher's exact test for categorical data and the Mann-Whitney test for continuous data. Luminex coefficients were computed to examine the association between clinical data. All data were considered statistically significant at $P < 0.05$. Bivariate analysis was used to determine statistical differences in T-cell subsets between patients with moderate disease and patients with severe disease. Statistical analyses were performed using GraphPad Prism version 6.07 for Windows and SAS v9.3.

Figure 2. Patients with severe disease have a greater frequency of CD8 (cluster of differentiation 8)-positive T cells expressing IL-4 (Tc2) and reduced frequency of CD8⁺ T cells expressing IFN γ (Tc1) than patients with moderate disease. A Boolean analysis was done of cytokine expression in T cells in nasal aspirates of patients with moderate (n = 31) and severe respiratory syncytial virus disease (n = 16). (A) CD3⁺ CD8⁺ T cells (*P < 0.05 by bivariate analysis) and (B) CD3⁺ CD4⁺ T cells. (C) Spearman's correlation coefficients were determined between clinical characteristics and T-cell subsets (n = 47 patients). (D) Spearman's correlation coefficients were determined between viral load and T-cell subsets. At day of enrollment (n = 42 patients) the viral load could not be determined owing to lack of samples for 5 patients for whom T-cell frequency is known. At 5 to 9 days after enrollment (n = 36 patients) viral load could not be determined owing to lack of samples for 8 patients and was undetectable in samples for 3 patients. Dotted lines represent the 95% confidence interval. Only significant correlations are shown. P < 0.05 by Spearman's correlation coefficient. Tc17 = CD8⁺ T cells expressing IL-17; TCID₅₀/ml = 50% tissue culture infectious dose per milliliter; Th17 = CD4⁺ T cells expressing IL-17.

Results

Characteristics of the Study Population

As expected, infants with severe disease were more likely to require supplemental oxygen ($P=0.0043$) or mechanical ventilation ($P<0.0001$) compared with patients with moderate disease (Table 1). Furthermore, patients with severe disease required a significantly longer length of hospital stay (median, 15 d) compared with patients with moderate disease (median, 5 d; $P<0.0001$). Importantly, there was no

difference in symptom duration (fever, congestion, increased work of breather, or cough) before enrollment between patients with severe disease and patients with moderate disease.

Severe Disease Is Associated with Type 2-Skewed CD8 T-cell Response

This study aimed to characterize the mucosal T-cell response in infants hospitalized with RSV. Our cohort of infants was enrolled at a median of 2 to 3 days after symptom onset (see Table 1); therefore, we chose to investigate T-cell profiles in

patients at 5 to 9 days after enrollment. We first compared the frequencies of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells in nasal aspirates between patients with moderate disease and patients with severe disease. Fifteen patients (seven moderate and eight severe) were excluded from flow cytometry analysis due to low recovery of nasal aspirate cells. Patients with severe disease had a significantly higher proportion of CD8⁺ T cells (median, 78.05%) compared with patients with moderate disease (median, 56.4%; $P=0.0017$) (Figure 1A). Additionally, patients with severe

Figure 3. Patients with severe disease have a type 2-skewed T-cell response. (A) Ratio of IL-4⁺/IFN γ ⁺ T cells, (B) ratio of CD8⁺ (cluster of differentiation 8-positive) T cells expressing IL-4 (Tc2)/CD8⁺ T cells expressing IFN γ (Tc1), and (C) ratio of Th2/Th1 in nasal aspirates from infants with moderate and severe respiratory syncytial virus disease ($n = 31$) and patients with severe disease ($n = 16$). * $P < 0.05$ by Mann-Whitney test. Th = T-helper cell.

disease had a significantly lower frequency of CD4⁺ T cells (median, 14.3%) compared with patients with moderate disease (median, 27%; $P = 0.0062$). Next, we evaluated the ratio of CD8⁺/CD4⁺ T cells within each patient. We found that patients with severe disease had a greater CD8⁺/CD4⁺ T-cell ratio (median, 5.63) compared with patients with moderate disease (median, 1.9; $P = 0.0049$) (see Figure 1B).

Our initial hypothesis was that severe disease would be associated with a Th2-biased cellular response in nasal aspirates. However, patients with severe disease had significantly increased frequencies of Tc2 cells (median, 40.1%) compared with patients with moderate disease (median, 14.4%; $P = 0.033$). Patients with severe disease also had significantly reduced frequencies of Tc1 cells (median, 12.51%) compared with patients with moderate disease (median, 26.9%; $P = 0.0261$) (Figure 2A and Table E1). An analysis of the CD3⁺CD4⁺ subsets revealed an increase in frequency of Th2 cells in patients with severe disease (median, 14.8%) compared with patients with moderate disease (median, 7.7%), although this difference did not reach significance. Patients with moderate disease and patients with severe disease had similar frequencies of all other CD4⁺ subsets (see Figure 2B). Gestational age was positively correlated with the frequency of Tc17 cells, whereas birthweight was positively correlated with the frequency of Th2 cells. Additionally, the age at hospital admission was positively correlated with the frequency of Tc1 and Th17 cells (see Figure 2C). There were no

significant correlations with the other T-cell subsets (data not shown). Patients with lower viral load at the day of enrollment had greater frequencies of Tc17 and Th17 cells. Additionally, patients with lower viral load at 5 to 9 days after enrollment had greater frequencies of Tc1 cells and a lower ratio of IL-4⁺ T cells/IFN γ ⁺ T cells (see Figure 2D). There were no significant correlations between viral load and the other T-cell subsets (data not shown).

There was a greater ratio of type 2/type 1 T cells in patients with severe disease (median, 1.29) compared with patients with moderate disease (median, 0.72; $P = 0.0368$ by Mann-Whitney test); however, this did not reach significance after adjusting for confounders ($P = 0.5758$) (Figure 3A). Within the CD8⁺ fraction, patients with severe disease had greater Tc2/Tc1 ratios (median, 1.54) compared with patients with moderate disease (median, 0.70; $P = 0.0202$ by Mann-Whitney test); however, this did not reach significance after adjusting for confounders ($P = 0.1672$) (see Figure 3B). There was no difference in the Th2/Th1 ratio between patients with moderate disease and patients with severe disease (see Figure 3C). Further investigation of the CD3⁺CD8⁺ subset revealed an association between the level of CD8 expression and the cytokine produced. CD3⁺CD8⁺ T cells diverged into two separate populations based on CD8 expression (Figure 4). A gate around the total CD8⁺ population includes cells expressing IL-4 (Tc2), IFN γ (Tc1), and IL-17 (Tc17) (see Figure 4A). However,

two populations appeared to exist within the total CD8⁺ gate based on CD8 expression. We found that cells expressing high levels of CD8 predominantly produced IFN γ , whereas those expressing low levels of CD8 produced predominantly IL-4 or IL-17 (see Figure 4B).

Tc1, Tc17, and Th17 Cell Subsets Are Associated with Shorter Hospital Length of Stay

As previously described, hospital length of stay was significantly greater in patients with severe disease compared with patients with moderate disease (see Table 1). To further investigate whether frequencies of T-cell subsets are associated with disease severity, we examined whether the frequency of T-cell subsets correlated with length of stay. Within the CD8⁺ compartment, Tc1 and Tc17 subsets were negatively correlated with length of stay ($r = -0.4333$; $P = 0.0023$ and $r = -0.3415$; $P = 0.0188$, respectively). In contrast, Tc2 cells were positively correlated with length of stay; however, this relationship did not reach statistical significance ($r = 0.2549$; $P = 0.0838$) (Figure 5A). Within the CD4⁺ compartment, Th17 cells were also negatively correlated with length of stay ($r = -0.2955$; $P = 0.0437$) (see Figure 5B).

Patients with Severe Disease Have Reduced IL-17 Concentrations in Nasal Aspirates

We tested the hypothesis that type 2 cytokine concentrations would be greater in the nasal aspirate supernatants of patients with severe disease compared with patients with moderate disease. Surprisingly, despite greater frequency of type 2 T cells in nasal aspirates of patients with severe disease, the concentration of the type 2 cytokines IL-4 and IL-13 were similar among patients with moderate disease and patients with severe disease. There was also no difference in the type 1 cytokine IFN γ between patients with moderate disease and patients with severe disease. Interestingly, however, patients with severe disease had significantly reduced IL-17 concentrations (median, 0.592 pg/ml) compared with patients with moderate disease (median, 1.262 pg/ml; $P = 0.0086$) (Figure 6). We also asked whether the frequency of T-cell subsets correlated with their signature cytokine

Figure 4. Level of CD8 (cluster of differentiation 8) expression correlates with cytokine production. (A) T cells expressing IFN γ (Tc1), T cells expressing IL-4 (Tc2), and CD8 $^{+}$ T cells expressing IL-17 (Tc17) cells distinguished based on cytokine expression within the total CD8 $^{+}$ gate. (B) The majority of Tc1 cells exhibit high levels of CD8 expression, whereas the majority of Tc2 and Tc17 cells exhibit low CD8 expression. (C) Comparison of CD8 median fluorescence intensity in Tc1, Tc2, and Tc17 cells as defined in A. A representative sample is shown. MFI = median fluorescence intensity.

concentrations in nasal aspirates. Within the CD8 $^{+}$ subset, the frequency of Tc17 cells had a positive correlation with the concentration of IL-17 ($r = 0.3092$;

$P = 0.0463$). In contrast, Tc1, Tc2, and all Th subsets were not correlated with their signature cytokine concentrations (see Figure E4).

Discussion

Many studies describe the immune response to RSV as type 2-biased (5, 13–16). It is controversial, however, whether the level of type 2 cytokines (IL-4, IL-5, and IL-13) or proportions of type 2 cytokine-producing cells (Th2 and Tc2) are associated with varying severity of disease. Here, we tested the hypothesis that patients admitted to the PICU would have a greater proportion of Th2 cells in nasal aspirates compared with patients admitted to the general ward. Indeed, our results showed a greater proportion of IL-4 $^{+}$ T cells in patients with severe disease; however, statistical significance was reached only in the CD8 $^{+}$ fraction. In the CD4 $^{+}$ fraction, IL-4 $^{+}$ T cells were increased in patients with severe disease compared with patients with moderate disease; however, this difference did not reach significance. Two studies have shown enhanced IL-4/IFN γ ratios in nasal aspirates in patients with more severe RSV disease (5, 13). In our study there was no difference in the IL-4/IFN γ ratio, which might be a result of the late time point after the decline of these cytokines. Of course, we only evaluated one time point in this study and it is possible that the peak CD4 response has already occurred. It has been shown that CD4 responses occur before CD8 (17). Because patients often arrive in the hospital days after the onset of symptoms it is impossible to know when the patients were infected. This ambiguity in the time of infection could result in analysis at a later time point dominated by CD8 responses. Nevertheless, a pathogenic role for CD8 $^{+}$ T cells is also supported by mouse models of RSV infection (18). Depletion of CD8 $^{+}$ cells reduces illness in mice after primary RSV infection (19), and adoptive transfer of an RSV-specific cytotoxic T lymphocyte clone both exacerbated lung pathology in mice and mediated viral clearance (20). On the other hand, fatal cases of RSV infection in human infants show few lymphocytes (neither CD8 $^{+}$ nor CD4 $^{+}$) by immunohistostaining of postmortem lung tissue (21, 22). Importantly, cytokine staining was not performed, and it is possible that these patients succumbed to infection before lymphocyte recruitment. Indeed, IL-4-expressing CD8 $^{+}$ T cells have been found in the blood after RSV infection, but their proportions are negatively correlated with disease severity (23). It is possible that a

Figure 5. CD8⁺ T cells expressing IFN γ (Tc1), CD8⁺ T cells expressing IL-17 (Tc17), and CD4⁺ T cells expressing IL-17 (Th17) cell subsets are associated with shorter hospital length of stay. (A–B) Spearman's correlation coefficients (r) were determined between hospital length of stay and frequency of nasal aspirate Th1 (CD3⁺CD4⁺IFN γ ⁺), T-helper cell type 2 (Th2) (CD3⁺CD4⁺IL-4⁺), and Th17 (CD3⁺CD4⁺IL-17⁺) (A); and Tc1 (CD3⁺CD8⁺IFN γ ⁺), T cells expressing IL-4 (Tc2) (CD3⁺CD8⁺IL-4⁺), and Tc17 (CD3⁺CD8⁺IL-17⁺) (B). $n = 47$ patients. Dotted lines represent the 95% confidence interval. $P < 0.05$ by Spearman's correlation coefficient.

reduction in systemic Tc2 cells in severe RSV disease is a result of relocation to the respiratory tract. It has been shown that Tc2 cells induced by HIV infection have reduced cytotoxic activity (24). Future directions will include characterization of the Tc2 cells found in patients with severe RSV disease, such as activation status and cytotoxic activity. Altogether, these results suggest an important role for Tc2 cells in the severity of RSV disease.

Many studies attribute IL-4 protein detected in BAL fluid, nasal aspirates, or

peripheral blood to a Th2 response (25–27). This study reveals CD8⁺ T cells as an important source of IL-4 production in RSV-infected infants. It has been shown in mice that Th2 responses to lymphocytic choriomeningitis virus infection cause virus-specific CD8⁺ T cells to switch from IFN γ production to IL-5 production (Tc1 to Tc2 switch) (28). Furthermore, these Tc2 cells mediated eosinophil recruitment to the lungs upon lymphocytic choriomeningitis virus peptide challenge. It is possible that in human infants an initial Th2 response

induces a robust Tc2 response, and both T-cell compartments synergize to establish severe disease.

It was also observed that Tc2 cells in nasal aspirates of RSV patients expressed lower levels of CD8 than Tc1 cells (see Figure 4). Reduced CD8 expression in T cells has been associated with activation, reduced antigen sensitivity (29), and reduced degranulation (30). In a macaque model of acute simian immunodeficiency virus infection, antigen-specific CD8⁺ could also be separated into CD8^{high} and CD8^{low} populations (31). Importantly, the CD8^{low} population produced less IFN γ compared with the CD8^{high} population, similar to what we observe in human infants with RSV. Unfortunately, production of IL-4 was not tested in this model. However, it has been shown in mice that IL-4 reduces RSV-specific cytolytic activity and delays viral clearance (32). In fact, we found reduced proportions of Tc1 cells in patients with severe disease compared with patients with moderate disease (see Figure 2A). Additionally, CD8 T cells activated under type 2 conditions produce IL-4 while downregulating CD8 expression, granzyme B, and IFN γ *in vitro* (33). Further studies will determine differences in the phenotypes of Tc1 versus Tc2 cells in nasal aspirates from RSV-infected infants.

Higher frequencies of Tc2 cells and lower frequencies of Tc1 cells were associated with admission to the PICU in patients hospitalized with RSV. This prompted us to ask whether the frequencies of T-cell subsets are also associated with the total length of hospital stay. Indeed, Tc1, Tc17, and Th17 frequencies were negatively correlated with length of stay, suggesting that these cell types and/or their effector cytokines IFN γ and IL-17 confer protection to RSV disease (see Figure 5). Likewise, IL-17 concentration was significantly lower in patients with severe disease compared with moderate disease (see Figure 6). These results suggest a protective effect for IL-17 and are supported by a previous study that observed decreased IL-17 concentrations in nasal aspirates of ventilated infants compared with nonventilated infants at the time of hospital discharge (34).

Although the frequency of Tc17 cells was significantly correlated with IL-17 concentrations in the nasal aspirates, overall, the frequencies of T-cell subsets were not correlated with their signature

Figure 6. Lower IL-17 levels are observed in the nasal aspirates of patients with severe disease. Cytokine concentrations in the cell-free nasal aspirates were compared between patients with moderate and severe respiratory syncytial virus disease. Undetectable samples were set to the limit of detection divided by the square root of 2 (12). Cell-free nasal aspirates were not available for analysis from 3 patients with moderate disease ($n = 35$) and 5 patients with severe disease ($n = 19$). $**P < 0.01$ by Mann-Whitney test.

cytokine concentrations (see Figure E4). The lack of correlation may be due to the time point analyzed. As previously noted, it has been shown that IL-17 concentrations are higher later in the course of infection in patients with moderate disease. Indeed, IL-17 and IFN γ were detectable in the majority of patients (88%) at 5 to 9 days after enrollment, regardless of disease severity. In contrast, only 47% of patients had detectable IL-4, and 32% had detectable IL-13 levels at this time point. Therefore, our time point may be appropriate to compare frequencies of T-cell subsets that express their signature cytokines after stimulation *ex vivo* but too late to detect these cytokines in the nasal aspirate supernatant. Another possibility that may explain the discrepancy between the frequency of T-cell subsets and their signature cytokine concentration in the nasal aspirate is that the multiple cell types may be responsible for the overall cytokine concentration.

There are conflicting reports in the literature regarding whether viral load is associated with disease severity. Many reports show a positive correlation between viral load and disease severity (11, 35–37). However, some studies report that disease severity is not associated with viral load (13, 38, 39). Recently, it was reported that viral load is lower in patients with severe compared with moderate disease when adjusted for symptom duration (40). Discrepancies within the literature are likely the result of differences in characterization of disease severity, patient demographics, variability in the time after symptom onset that sampling occurs, sampling method, and even method of viral load determination. In this study, admission to

the PICU versus general ward was not associated with viral load at the day of enrollment or the day of flow cytometry analysis (see Figure E5). However, patients with lower viral load at the day of enrollment had increased frequencies of Tc17 and Th17 cells, and patients with lower viral load at 5 to 9 days after enrollment had higher frequencies of Tc1 and a reduced ratio of total IL-4 $^{+}$ /IFN γ^{+} T-cell cells (see Figure 2D). Likewise, it has been shown in experimental RSV infection of adults that RSV-specific CD8 $^{+}$ T cells are inversely correlated with viral load (41).

We acknowledge that, in contrast to our results, there are a number of reports indicating a correlation between age and disease severity. The majority of those reports use hospitalization as the definition of severe disease and some include children over the age of 12 months (42–46). In contrast, our study involved only hospitalized infants (<1 yr of age). Additionally, 53 out of 62 (85.5%) of our patient population were less than 6 months of age. Of the studies using the same definition of severity (i.e., PICU versus ward) and comparing children of similar age, one failed to demonstrate a correlation with age and severity (11, 47–50). We do not understand the reasons for these discrepancies, but we reiterate that differences in characterization of disease severity, differences in patient demographics, variability in the time after symptom onset that sampling occurs, sampling method, and even method of viral load determination exist across these studies and could be responsible for the different outcomes.

This study has some limitations. This study was performed in a predominantly African American population. In our study,

African Americans had significantly reduced Tc2 cells compared with white individuals (see Table E2). These results are in line with a previous study showing that African Americans in Tennessee are less likely to be hospitalized for bronchiolitis within the first year of life compared with white individuals (51). A multicenter study that includes areas of multiple racial backgrounds would provide a more comprehensive picture of RSV disease among genetically diverse populations. Additionally, disease severity is a subjective classification. Here, we chose to define severe disease as admission to the PICU. However, the majority of patients with moderate disease required supplemental oxygen (71.1%). Characterization of disease severity by PICU status could be refined further by incorporating objective clinical parameters to determine severity. Several severity scoring methods have been used to categorize infants with varying disease severity (52–59). The adoption of a single, standardized RSV severity scoring system would allow better comparisons between studies performed by different groups. Interestingly, Tc2 and Th2 cells were significantly higher in patients on oxygen supplementation. Another limitation is our small sample size. Out of 243 infants enrolled between 2014 and 2017 over three respiratory seasons, only 62 patients provided samples out to the 5 to 9 days after the enrollment time point. Furthermore, only 24 of these patients were admitted to the PICU. Additionally, some nasal aspirates could not be analyzed by flow cytometry due to low numbers of live lymphocytes. Statistically significant differences in immune profiles between

patients were found despite our small sample size.

Conclusions

This study suggests that a specific mucosal T-cell subset may contribute to severe RSV disease. To our knowledge, this is the first

study to directly evaluate cytokine-producing T cells in nasal aspirates. These results show that RSV disease severity is associated with a specific immune profile and suggest a role for CD8⁺ T cells in the development of RSV immunopathology. ■

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