Airway CD8⁺ T Cells Are Associated with Lung Injury during Infant Viral Respiratory Tract Infection

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Abstract

Infants and young children are disproportionately susceptible to severe complications from respiratory viruses, although the underlying mechanisms remain unknown. Recent studies show that the T cell response in the lung is important for protective responses to respiratory infections, although details on the infant/pediatric respiratory immune response remain sparse. The objectives of the present study were to characterize the local versus systemic immune response in infants and young children with respiratory failure from viral respiratory tract infections and its association to disease severity. Daily airway secretions were sampled from infants and children 4 years of age and younger receiving mechanical ventilation owing to respiratory failure from viral infection or noninfectious causes. Samples were examined for immune cell composition and markers of T cell activation. These parameters were then correlated with clinical disease severity. Innate immune cells and total CD3⁺ T cells were present in similar proportions in airway aspirates derived from infected and uninfected groups; however, the CD8:CD4 T cell ratio was markedly increased in the airways of patients with viral infection compared with uninfected patients, and specifically in infected infants with acute lung injury. T cells in the airways were phenotypically and functionally distinct from those in blood with

activated/memory phenotypes and increased cytotoxic capacity. We identified a significant increase in airway cytotoxic CD8⁺ T cells in infants with lung injury from viral respiratory tract infection that was distinct from the T cell profile in circulation and associated with increasing disease severity. Airway sampling could therefore be diagnostically informative for assessing immune responses and lung damage.

Keywords: infant immunity; viral pneumonia; lung injury; immunopathology

Clinical Relevance

This study demonstrates that the immune response in the airway is associated with disease severity, whereas the systemic response is uninformative. Specifically, we detected an increased ratio of CD8⁺ to CD4⁺ T cells in the airways of virally infected children with acute lung injury compared to those infected without lung injury. This finding raises the possibility of adaptive immune response dysregulation contributing to severe consequences of infection in children.

Viral respiratory tract infections (VRTIs) are the most prevalent cause of disease worldwide, with infants and young children under 5 years of age being particularly

susceptible to VRTI-associated morbidity and mortality (for a review *see* Ref. 1). Although respiratory syncytial virus (RSV) is the most commonly implicated virus

causing severe disease among infants, influenza, rhino-/enterovirus, and human metapneumovirus (HMPV) also cause clinically significant disease (2, 3).

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Treatment is mainly supportive once infection occurs, and prevention via vaccination is limited in scope and efficacy. It remains unclear why some infants are able to clear infection with no significant sequela, whereas others require hospitalization, develop respiratory failure, or succumb to disease.

Developmental differences in the adaptive immune system likely play an important role in the increased susceptibility of infants to VRTI (4, 5). Differentiation of CD4⁺ and CD8⁺ T lymphocytes to effector and memory T cells is required for virus clearance and the establishment of protective immunity (for reviews *see* Refs. 6, 7); however, infant/neonatal T cells exhibit distinct functional capacities compared with adult T cells (8). Infant CD4⁺ T cells are impaired in production of Th1-associated proinflammatory cytokines, such as IFN- γ , and are skewed in production of Th2-like cytokines, such as IL-4 and IL-5 (9, 10), whereas neonatal CD8⁺ T cells exhibit reduced expression of cytotoxic and inflammatory mediators (11). Infants are largely devoid of circulating memory T cells, and early-life immune responses thus derive from newly generated naive T cells. It is unknown how this nascent T cell response determines disease outcome and severity in infant VRTI.

Mouse models have revealed additional complexity in the T cell response to respiratory infections. Importantly, viral protection is due to T cells recruited to and retained in lung tissue (12, 13), emphasizing a need to study lung-localized immune responses. Moreover, specific targeting of CD8⁺ T cells to the lung can also result in immune-mediated lung damage (14). The ability of infant T cells to generate lung-homing effector cells, and the impact on disease outcome, including protection and immunopathology, remains undefined.

We analyzed both the spatial and temporal immune response to VRTI in infants and young children to identify how local immune responses may impact disease outcome in early life. We sampled the local respiratory environment over the disease course by collecting daily airway aspirates from patients with respiratory failure requiring mechanical ventilation due to VRTI and noninfectious causes. Our results reveal a striking increase in effector/memory CD8⁺ T cells with cytotoxic capacity in infected infants with acute lung injury (ALI) that increases over the course of

severe disease, and is resolved with recovery. This local T cell response is highly distinct from that in circulation, suggesting that prolonged T cell imbalance may be associated with immunopathology in infants, and airway monitoring could be informative to shape clinical care.

Materials and Methods

Study Design

Patients 4 years of age or younger admitted to the Pediatric Intensive Care Unit at Morgan Stanley Children's Hospital/New York Presbyterian Hospital (New York, NY) requiring mechanical ventilation via an endotracheal tube or tracheostomy due to VRTI, elective surgery, aspiration pneumonia, or other respiratory failure were enrolled in the study from April 2012 to April 2015 (Table 1). Patients with VRTI had viral pathogen confirmed by multiplex PCR by FilmArray (BioFire Diagnostics, Salt Lake City, UT). Excluded were patients with primary immunodeficiency, trisomy 21, those receiving immunosuppressants, or those infected with human immunodeficiency or herpes viruses. Airway aspirates were collected daily for 14 days or until cessation of mechanical ventilation. Blood draws (1 ml for patients up to 3 kg; then 1 ml/kg up to maximum of 10 ml), when permitted, were obtained on the first and last day of mechanical

ventilation (or Day 14). The Institutional Review Board of Columbia University Medical Center (CUMC) (New York, NY) approved the conduct of this study, and informed consent from parents was obtained before study enrollment.

Sample Preparation

Blood samples were processed by centrifugation through ficoll and washing with saline. Airway aspirates were obtained as part of routine suctioning of the artificial airway by infusing 1 ml of 0.9% saline into the airway, followed by three to five manual breaths, and passage of a catheter for suctioning into a sterile sputum trap. Samples were centrifuged, and supernatants collected and stored at -80°C for cytokine analysis. Cell pellets were resuspended in PBS and analyzed by flow cytometry.

Flow Cytometry

Cells were stained with innate cell- and T cell-specific antibody panels, as described previously (15, 16), and analyzed using the LSRII flow cytometer (BD Biosciences, San Diego CA) and Flowjo software (Treestar, Ashland, OR).

Cytokine Assays

Supernatants from airway aspirates were analyzed for cytokine content using Luminex BioPlex 200 multiplex array system (Bio-Rad, Hercules, CA) performed by the Irving Institute for Clinical and

Table 1. Patient Demographics

Patient Demographics*	Uninfected	Infected	P Value
<i>n</i>	20	34	
Male sex, <i>n</i> (%)	13 (65)	18 (53)	0.41
Median age, mo	6.5	3.5	0.29
<3 mo, <i>n</i>	3	12	
3–12 mo, <i>n</i>	10	12	
12 mo to 4 yr, <i>n</i>	7	10	
Uninfected patients (with acute lung injury)			
Postoperative, general, <i>n</i>	9 (2)		
Postoperative, cardiac, <i>n</i>	8 (1)		
Aspiration, <i>n</i>	2 (2)		
Neuromuscular, <i>n</i>	1		
Clinical characteristics, median			
Hospital length of stay, d	8.5	20.5	0.03
PICU length of stay, d	6	14	0.002
Duration of mechanical ventilation, d	3	10	<0.0001
Minimum PaO ₂ :FIO ₂ ratio	218	138	0.1

Definition of Abbreviations: FIO₂, fraction of inspired oxygen; PaO₂, partial pressure of oxygen, arterial; PICU, pediatric intensive care unit.

*For individual patient data, see Table E1.

Translational Research Biomarkers Core Laboratory at CUMC and the Human Immunology Core at the University of Pennsylvania (Philadelphia, PA). The samples were read using a BioPlex200 multiplex array system (Bio-Rad, Hercules, CA).

Clinical Data

Demographic and clinical data were extracted from electronic medical records, and included hospital length of stay, Pediatric Intensive Care Unit length of stay, duration of mechanical ventilation, and minimum partial pressure of oxygen (PaO_2)/fraction of inspired oxygen ratio (PF ratio). ALI was defined as respiratory distress with a PF ratio less than 300 and bilateral infiltrates on chest X-ray without left-sided heart failure, based on the consensus definitions (17, 18). Lymphocyte, monocyte, and neutrophil counts in peripheral blood were obtained from complete blood counts.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 5.00 (GraphPad, San Diego, CA) and R multiple regression software (<http://www.r-project.org/>). Mann-Whitney and Fisher's exact test were used to determine significance between groups. The primary immune response variable studied was the per diem area under the curve (AUC) of the CD8:CD4 ratio for each patient divided by the number of sample days for that patient. The natural logarithm of the per diem AUC was entered as the dependent variable in multiple regression analyses to improve goodness of fit and satisfy the normality of errors assumption. All hypothesis tests were conducted at the 0.05 level of statistical significance.

Results

A total of 54 pediatric patients requiring mechanical ventilation were enrolled. A total of 34 patients had VRTI and 20 patients required mechanical ventilation for noninfectious causes (Table 1). The majority of patients enrolled were infants under 12 months of age (37/54), with the remaining patients aged 1–4 years (17/54; Table 1). We collected a total of 178 airway aspirates—132 from infected patients and 46 from uninfected patients, and 22 blood

samples were collected—12 from infected patients and 10 from uninfected patients.

Cellular Composition of Airway and Blood in Infected versus Uninfected Patients

To determine whether tracheal aspirates could provide a sampling of immune cells in the airway, we analyzed the cellular content

of airway fluid obtained from uninfected and infected patients. The majority of airway samples contained 1–10 million cells, and the total cell count did not vary significantly between uninfected and infected patients (median, 2,600,000, range, 150,000–164,000,000 versus median, 2,000,000, range, 61,000–40,400,000; $P = 0.09$; see Figure E1 in the online

Figure 1. Immune cell composition in airway aspirates. Representative flow cytometry plots from an uninfected (male, 3 mo of age after cardiac surgery, *left panel*) and an infected patient (female, 2.5 mo with respiratory syncytial virus [RSV] and rhino-/enterovirus coinfection, *right panel*). The *top row* shows large cells by HLA-DR (MHC class II molecule) versus CD177 expression; the neutrophil population is denoted by $\text{HLA-DR}^-/\text{CD177}^+$, with frequency noted in the *lower right quadrant*. The *middle row* shows large cells by HLA-DR versus CD14 expression; monocytes are denoted as $\text{HLA-DR}^+/\text{CD14}^+$, and frequency is shown. The *bottom row* displays all events by side scatter (SSC) versus CD3 expression; lymphocyte population is gated as $\text{SSC low}/\text{CD3}^+$, and frequency of CD3^+ lymphocytes of total events recorded is shown. CD, cluster of differentiation; HLA-DR, human leukocyte antigen DR.

supplement). Neutrophils were the majority cell population in blood and airway aspirates in both uninfected and infected patients (Figure 1, *top row*). Monocytes were also represented at significant frequencies in airway aspirates, comprising 5–10% of the total cellular content (Figure 1, *middle row*). Lymphocytes were present as low-frequency, but measurable, populations in airway aspirates, representing less than 5% of the total cells (Figure 1, *bottom row*) in both uninfected and infected patients. This cellular composition of airway aspirates is similar to that found in bronchoalveolar lavage fluid of infants (19), and therefore tracheal aspirates represent an accurate sampling of the airway environment using a noninvasive approach that is part of routine clinical care.

We further compared the overall cellular composition of innate cells and lymphocytes in airway aspirates (as in Figure 1) and blood from uninfected versus infected patients at discrete time points and during the course of their disease. Cellular composition in blood was obtained from the complete blood count information obtained from routine clinical blood draws taken at regular intervals from each patient, as consent for research blood draws was only obtained from a fraction of enrolled patients. The neutrophil content in the airways remained constant over the course of mechanical ventilation in both uninfected (median, 51% of airway cells; range, 18–85%) and infected (median, 48% of airway cells; range, 5–86%) patients (Figure 2A); however, the median absolute neutrophil count (ANC) in blood varied significantly between uninfected and infected groups at initiation of mechanical ventilation (8,318 cells/ μ L, range, 4,176–12,931 versus 3,815 cells/ μ L, range, 820–14,994; $P = 0.005$) and over the study period as a whole (6,350 cells/ μ L versus 4,874 cells/ μ L; $P = 0.02$) (Figure 2A). This change in circulating neutrophils could be due to an influx of neutrophils into the airway of infected patients (20, 21). Neutropenia (ANC < 1,500 cells/ μ L) occurred in only 6 out of the 178 clinical complete blood counts (RSV-infected [$n = 5$] and HMPV-infected [$n = 1$] patients) with no subject having severe neutropenia (ANC < 500 cells/ μ L). The fractional composition of monocytes in airways was low for both uninfected (median, 4.7%; range, 0.7–15.7%) and

Figure 2. Immune cell composition of aggregated blood and airway samples during study enrollment. Neutrophil (A), monocyte (B), and lymphocyte (C) composition of blood (*left*) and airway (*right*) samples from uninfected and infected patients depicted as aggregated data for each day of enrollment. Blood counts (*left*) derive from 134 complete blood counts sent from 43 enrolled patients as part of routine clinical testing, with the *shaded area* representing the normal range for that parameter. Airway cellular composition (*right*) represents aggregated data obtained by flow cytometry analysis of 178 airway aspirates from 53 enrolled patients. Percentage is calculated from the total number of airway cells analyzed. Individual patient characteristics are shown in Table E1.

infected (median, 3.3%; range, 0.4–23.5%) patients, but lowest for infected patients ($P = 0.04$) (Figure 2B). The median absolute monocyte count was 961 (range, 175–1,897) for uninfected patients and 760 (range, 32–3,696) for infected patients, and was not statistically significant ($P = 0.7$).

Examination of T cells revealed differences between uninfected and infected patients and between airway and blood samples. In the blood, CD3⁺ T cells comprised a significant fraction of total

mononuclear cells, which was higher in blood samples obtained from uninfected patients compared with infected patients (median, 8.9%, range, 3.9–23.6% versus median, 1.9%, range, 0.1–10.9%; $P = 0.0014$). By contrast, in the airways, CD3⁺ T cells accounted for a low percentage of the total cellular composition in all samples (Figure 2C), with median frequencies of 0.12% (range, 0.01–0.9%) for uninfected patients, and 0.11% (range, 0.01–4.8%) for infected patients ($P = 0.95$). Analysis of airway aspirates obtained

during the course of disease revealed an increase in the frequency of total $CD3^+$ T cells in airway aspirates from infected compared with uninfected patients at initiation of mechanical ventilation (median, 0.05%, range 0.01–0.3% versus median, 0.2%, range 0.02–4.3%; $P = 0.003$), which decreased during the study period to levels seen in uninfected patients (Figure 2C). The increase in airway $CD3^+$ T cell frequencies was coincident with a decrease in blood $CD3^+$ T cells observed in samples from infected patients. The absolute lymphocyte count (including T and B lymphocytes) was not significantly different in uninfected and infected patients, either at initiation of mechanical ventilation (median, 2,358 cells/ μ l, range, 1,152–5,916 cells/ μ l versus median, 3,066 cells/ μ l, range, 552–12,852 cells/ μ l; $P = 0.38$) or over the course of the study (median, 2,725 cells/ μ l, range, 805–5,962 cells/ μ l versus median, 2,660 cells/ μ l, range, 507–12852 cells/ μ l; $P = 0.84$)

(Figure 2C). These results suggest that the T lymphocyte response may be dynamically different in uninfected versus infected patients, and this difference is more strikingly observed when monitoring airway secretions at the site of infection compared with peripheral blood.

Distinct T Cell Subset Composition and Differentiation State in Airways of Infected Patients

The subset composition and activation/differentiation state of blood versus airway T cells was compared in uninfected and infected patients. In the blood, circulating T cells were predominantly $CD4^+$ (80%) with a correspondingly lower proportion of $CD8^+$ T cells (20%) in both infected and uninfected patients (Figure 3, *first and third columns*), as is typical of infants and young children (22). The corresponding low ratio of $CD8:CD4$ T cells in blood was similar between uninfected and infected patients (median,

0.45, range, 0.26–0.97 versus median, 0.45, range, 0.17–1.9; $P = 0.8$). In contrast, there was an elevated $CD8:CD4$ T cell ratio in airway aspirates, which was significantly higher in infected compared with uninfected patients, as shown in representative flow cytometry results (Figure 3, *second and fourth columns*). Moreover, the activation and differentiation state of airway T cells was distinct from circulating T cells. Although blood $CD4^+$ and $CD8^+$ T cells exhibited a predominant naive phenotype ($CD45RA^+$) with few memory ($CD45RO^+$) cells, airway T cells in these same patients exhibited a prevalent activated/memory phenotype ($CD45RO^+/CD45RA^-$) with few naive T cells present (Figure 3). These results demonstrate that airway T cells have a distinct T cell subset and differentiation state compared with peripheral blood T cells, and that the airways are sites for accumulation of activated/memory

Figure 3. Differential T cell subset composition in blood and airway aspirates between infected and uninfected patients. Representative flow cytometry from an uninfected (*left panel*) and an infected infant (*right panel*) also shown in Figure 1. Patients' samples are divided into two *columns*, with blood samples on the *left* and airway aspirates on the *right*. The *top row* depicts $CD3^+$ T cells graphed by CD4 versus CD8, with gates applied to $CD4^+$ and $CD8^+$ populations. The *middle row* displays $CD4^+$ T cells graphed by CD45RO versus CD45RA. The *bottom row* displays $CD8^+$ T cells graphed by CD45RO versus CD45RA.

CD8⁺ T cells during infection, even in very young infants.

To determine whether the increased CD8:CD4 T cell ratio varied over the course of infection and/or mechanical ventilation in both groups, we analyzed this parameter for all daily samples obtained. Analysis of peak CD8:CD4 T cell ratio from uninfected and infected groups for each patient revealed significant differences between these groups (Figure 4A);

Figure 4. Airway T cell response by infection status. (A) Peak CD8:CD4 T cell ratio obtained from each uninfected and infected patient over the enrollment period; the bar represents median for group. (B) Peak percentage of CD107⁺ CD8⁺ T cells per subject over the enrollment period; the bar represents median for group. (C) Mean CD8:CD4 T cell ratio for each group by enrollment day; error bars indicate $\pm$ SEM. Data are derived from 178 airway aspirates from 53 patients, and *P* values were obtained by Mann-Whitney comparison of medians.

Table 2. Infected Patients by Viral Type and Acute Lung Injury Status

Patient Characteristics	No Lung Injury	Lung Injury	<i>P</i> Value
<i>n</i>	14	20	
Male sex, <i>n</i>	5	13	0.16
Median age, mo	7.5	3.5	0.37
Virus			
Respiratory syncytial virus, <i>n</i>	4	9	
Rhinovirus/enterovirus, <i>n</i>	6	3	
Coinfection, <i>n</i>	2	3	
Human metapneumovirus, <i>n</i>	0	4	
Other, <i>n</i>	2	1	

uninfected, median, 0.4, range, 0.1–2.4 versus infected, median, 2.8, range, 0.1–25.2; *P* < 0.0001). CD8⁺ T cells in the airways of infected patients showed increased evidence of degranulation, as indicated by CD107 expression (23), compared with the airways of uninfected patients (Figure 4B; uninfected, median, 0%, range, 0–3.7% versus infected, median, 1%, range, 0–41.7%; *P* = 0.009). There was a further increase in CD8:CD4 T cell ratio in the airways during the course of hospitalization for infected compared with uninfected patients who did not exhibit an appreciable CD8⁺ T cell predominance at any time point during their respiratory failure (Figure 4C). These findings demonstrate an accumulation and predominance of cytotoxic CD8⁺ T cells into the airways of patients with respiratory virus infection that developed over the course of prolonged disease.

The CD8:CD4 T Cell Ratio Is Highest in Infected Patients with ALI

The variation in the peak CD8:CD4 T cell ratio within the infected group (ranging from 0.1 to 25.2; Figure 4A) suggested that additional parameters related to disease severity and/or the nature of infection might be associated with these differences in respiratory T cell composition. We therefore stratified the infected patients based on the presence of ALI, as defined by clinical criteria (17, 18; and see MATERIALS AND METHODS), and the type of viral pathogen (Table 2). Although ALI within the infected patients occurred with similar incidence in infants and children from 1–4 years of age, there was a significant difference in ALI as a function of specific viral pathogens (Table 2). ALI occurred more frequently in RSV- and HMPV-infected groups compared with the rhino-/enterovirus-infected group

(*P* = 0.04; Table 2), suggesting distinct responses to these pathogens. Importantly, infected patients with ALI had a higher median peak CD8:CD4 ratio compared with infected patients without ALI (5.9; range, 0.86–22.9 versus 0.8, range, 0.13–25.2; *P* = 0.002; Figure 5), whereas the CD3⁺ T cell response was not significantly different when controlling for infection and ALI status (Figure E2). Moreover, the CD8:CD4 T cell ratio in the ALI group increased during the course of disease, but did not change in infected patients who did not develop ALI (data not shown). These results indicate that CD8⁺ T cells in the respiratory environment in response to infection are associated with lung damage and severe illness in infants and young children with VRTI.

The presence of CD8⁺ T cells with cytotoxic potential in infected patients with ALI suggested a local inflammatory response in the respiratory tract. We

Figure 5. Peak CD8:CD4 ratio in airway associated with acute lung injury (ALI). Peak CD8:CD4 T cell ratio obtained from each subject over the enrollment period grouped by infection status and presence of ALI; the bar represents median for group (black plus symbols, uninfected, no ALI; green squares, uninfected + ALI; purple circles, infected, no ALI; red triangles, infected + ALI). *P* value obtained by Mann-Whitney comparison of medians.

Figure 6. Elevated IL-6 in airway samples of infected patients is associated with severity of lung injury. Airway samples were analyzed for cytokine content by Luminex. (A) Scatter plot of IL-6 concentrations measured in airway aspirates of infected patients by day of study enrollment, grouped by presence of ALI. (B) Scatter plot of IFN- γ concentration measured in airway aspirates of infected patients by day of study enrollment, grouped by presence of acute lung injury. (C) Graph illustrates the inverse relation between minimum arterial oxygen pressure/fraction of inspired oxygen (PF) ratio and average IL-6 in logarithmic terms, shown by the negative slopes of the regression lines. The solid line portrays the relation for infected patients at the median value of immune response (median per diem area under the curve of the CD8:CD4 ratio). The two dashed lines fit the relation for patients at the upper quartile of immune response (bottom line) and at the lower quartile (top line). All lines assume age fixed at the mean age among the infected patients.

assayed for multiple cytokines, including IFN- γ , IL-4, IL-6, IL-10, IL-17, and TNF- α in airway aspirates of infected and uninfected patients, also stratified by ALI. Only IL-6 showed significant increases in infected patients with ALI compared with infected patients without ALI (Figure 6A). IFN- γ , a key T cell-derived cytokine, was elevated in some infected patients with lung injury, but overall did not show a significant difference between groups (Figure 6B). Further analysis revealed a significant correlation in infected patients between a lower minimum PF ratio, indicative of ALI, and a higher IL-6 content in airway aspirates (Figure 6C), indicating that the cytokine was associated with increased inflammation. A similar correlation to lung injury severity was not seen with other inflammatory cytokines, including IFN- γ and TNF- α (data not shown).

Statistical Model for Dependence of Age, Viral Infection, and ALI on CD8:CD4 Immunophenotype

We used statistical modeling to determine the interaction of patient age, virus infection, and ALI on the resultant CD8:CD4 immunophenotypes, based on a per diem AUC for normalization between groups (see MATERIALS AND METHODS). Patient age was not a significant predictor in any model, but was retained in the models as an adjustment factor. In additive models, including viral agent and ALI status (Figure E3), patients infected with RSV and HMPV had significantly increased per diem AUC compared with uninfected patients (RSV, $P < 0.0001$; HMPV, $P = 0.001$), whereas patients infected with rhino-/enterovirus did not have significantly different per diem AUC compared with uninfected patients ($P = 0.33$). However, models that included an interaction between viral infection and presence of ALI fit significantly better than additive models (Figure 7). Considering infection (yes/no) and ALI (yes/no), neither variable when present alone was significantly associated with per diem AUC (infection, $P = 0.18$; ALI, $P = 0.95$), but, for patients with both infection and ALI, the per diem AUC was significantly increased (interaction $P = 0.006$; Figure 7). The best fitting model (adjusted $r^2 = 0.67$) included viral types, ALI, and their interaction. In this model, only the interaction between RSV and ALI retained statistical significance ($P = 0.002$), although the

Figure 7. Increased CD8:CD4 ratio in airways is associated with ALI. Per diem area under curve statistical model. Graph depicts the log adjusted per diem area under the curve for CD8:CD4 T cell ratio on the y-axis versus age on the x-axis (black plus symbols, uninfected, no ALI; green squares, uninfected + ALI; purple circles, infected, no ALI; red triangles, infected + ALI). Patients were grouped based on infection status and presence of ALI, similar to Figure 5. Best fit line applied to each group is shown.

interaction between HMPV and ALI was numerically large and similar to its value in the additive model (data not shown). Together, this statistical analysis reveals interaction of an increased CD8:CD4 T cell ratio with ALI and viral agent, but not with age during the early years of life, as both infants and children have similar CD8:CD4 immunophenotypes associated with ALI due to VRTI.

Discussion

Infants experience disproportionate susceptibility to severe disease from VRTI, although the underlying causes remain unknown. In this study, we examined the local immune response in the airways compared with circulating blood over the course of VRTI-induced disease in infants and young children to identify immune-based correlates to disease. While innate cells comprised the majority of the cellular composition of airway aspirates, the T-cell subset composition varied significantly in infected patients, which was further impacted by ALI and viral agent independent of age. Infants who develop ALI during infection exhibit a skewed airway CD8:CD4 T cell ratio dominated by effector-memory CD8⁺ T cells compared with uninfected patients and infected patients without ALI, where CD4⁺ T cells outnumber CD8⁺ T cells in the airways. We also found increased IL-6 in airway samples that correlated with the severity of

lung injury. Persistence of this CD8⁺ T cell imbalance and abnormal cytokine profile was associated with an increased inflammatory environment in the airways and increased clinical disease severity.

The infant innate and adaptive immune responses are largely biased toward antiinflammatory responses (4). The overall functional capacities of human infant T cells as being skewed toward Th2 or antiinflammatory responses (8) is largely derived from studies of umbilical cord blood (24–26). Measuring the *in vivo* infant immune response to VRTI has been more challenging, and has involved characterization of cells and cytokines in peripheral blood (6, 21, 27, 28), nasopharyngeal washes (29, 30), or tracheal aspirates (31, 32). However, sampling remains limited, and unifying immune parameters related to disease outcome and severity has not emerged. Our results show that daily sampling of the local site of infection provides a more dynamic view of the ongoing immune response and correlates more with disease severity than peripheral blood. We show that, whereas circulating T cells in infants remain overtly naive, infants as young as 2 weeks old have predominant effector memory T cells in the airway during VRTI, and these were mostly cytotoxic CD8⁺ T cells in patients with the most severe VRTI-induced disease. Previous studies have identified virus-specific CD8⁺ T cells in infants and an association with viral clearance and/or recovery (19, 21, 31). However, by integrating our results on airway T cell content from multiple VRTI with disease severity and ALI, we reveal an association of the persistence of airway CD8⁺ T cells with ALI and disease progression.

We propose that the elevated CD8⁺ T cell response in infant VRTI, which was associated with lung damage, may promote immunopathology, or could indicate a dysregulated CD8⁺ T cell response to prolonged infection. In mouse models,

CD8⁺ T cells recruited to the lungs can promote lung damage through cytotoxic mediators or production of proinflammatory cytokines (33), and, in humans, CD8⁺ T cells have been found in lungs of infants who succumbed to severe influenza or RSV infection (34, 35), suggesting an end-stage association of CD8⁺ T cells and lung damage. We found that CD8⁺ T cells in the airways expressed CD107, a marker of degranulation, indicating their cytotoxic potential, which protects by clearing virally infected cells, but can also promote tissue damage. Interestingly, although IFN- γ produced by CD8⁺ T cells also plays an important role in protection (36), only a few infected patients with lung injury exhibited elevated levels of IFN- γ in the airways. As infant T cells are known to have reduced capacities for IFN- γ production (4, 8), this functional impairment could lead to incomplete viral clearance, resulting in prolonged infection and lung damage. Persisting virus can lead, in turn, to chronic CD8⁺ cell activation, resulting in functional exhaustion or hyporesponsiveness (37), provoking increased presence of new effector CD8⁺ T cells into airways, resulting in immunopathology.

We found that elevated levels of IL-6 correlated to the severity of lung injury in infected patients. IL-6 in the airway samples could derive from monocytes, T cells, and/or airway cells (38). The role of IL-6 in ALI is not defined; however, IL-6 has been associated with increased disease severity in enteroviral (39) and human influenza infections (40, 41), and could contribute to the overall inflammatory process, leading to lung injury as a result of the virus infection.

Pediatric ALI accounts for almost 10% of all mechanically ventilated patients admitted to pediatric intensive care units, with infection being the most frequent cause (42). Although RSV has been associated with significant lung pathology in the pediatric population, other viruses also

cause significant morbidity and mortality, as seen in the cohort studied here. We found significant ALI associated with RSV, HMPV, and coinfections, whereas fewer patients with rhino-/enterovirus developed ALI. Clinically significant rhinovirus infections are more common in patients with underlying immunosuppressive conditions (43). We found that the airway immune response in rhino-/enterovirus-infected patients with no ALI was indistinguishable from uninfected patients in terms of T cell composition and cytokine content. The different pathophysiologic mechanisms of distinct viral pathogens is likely a contributing factor to developing ALI.

Our findings provide insight into the local response to infection in the youngest of patients, revealing dynamic changes related to clinical outcome that cannot be inferred from assessing similar parameters in blood. Future studies should focus on delineating lung damage that results from viral factors versus CD8⁺ T cell damage mediated by either direct cytopathic effect or secreted proinflammatory cytokines. Delineating viral versus immune cell effects will be useful for potential diagnostic and therapeutic applications to improve treatment for respiratory disease at the most vulnerable stages of life. ■

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