Cytokine Panels and Pediatric Acute Respiratory Distress Syndrome: A Translational Investigation*

Daniel J. McKeone, MD[1,2]; Margaret Mathewson, MD[1,2]; Priti G. Dalal, MD[3]; Debbie Spear, RN[1]; Todd M. Umstead, BS[1,2]; Steven D. Hicks, MD, PhD[1]; Zissis C. Chroneos, PhD[1,2,4]; Ming Wang, PhD[5]; Neal J. Thomas, MD[1]; E. Scott Halstead, MD, PhD[1,2]

Objectives: To identify and compare serum and lower respiratory tract fluid biomarkers of lung injury using well-characterized mouse models of lung injury. To explore the relationship between these preclinical biomarkers and clinical outcomes in a discovery cohort of pediatric patients with acute respiratory failure from pneumonia.

Design: Prospective, observational cohort study.

Setting: A basic science laboratory and the PICU of a tertiary-care children's hospital.

Patients: PICU patients intubated for respiratory failure from a suspected respiratory infection.

Interventions: Prospective enrollment and collection of lower respiratory tract fluid samples.

Measurements and Main Results: C57BL6/J mice were intranasally inoculated with escalating doses of influenza A virus or toll-like receptor agonists to simulate varying degrees of lung injury. Serum and bronchoalveolar lavage fluid were measured for the presence of cytokines using commercially available multiplex cytokine assays. Elevated levels of C-C motif chemokine ligand 7 at the peak of inflammation in both bronchoalveolar lavage fluid and serum correlated with lethality, with the bronchoalveolar lavage fluid ratio of C-C motif chemokine ligand 7:C-C motif chemokine ligand 22 providing the best predic-

tion in the mouse models. These preclinical biomarkers were examined in the plasma and lower respiratory tract fluid of a discovery cohort of pediatric patients with acute respiratory failure from pneumonia. The primary clinical outcome measure was ventilator-free days, with secondary outcomes of pediatric acute respiratory distress syndrome severity and mortality. Elevation in peak lower respiratory tract fluid C-C motif chemokine ligand 7:C-C motif chemokine ligand 22 ratios demonstrated a significant negative correlation with ventilator-free days ($r = -0.805$; $p < 0.02$).

Conclusions: This study provides evidence that lung immune profiling via lower respiratory tract fluid cytokine analysis is feasible and may provide insight into clinical outcomes. Further validation of markers, including the C-C motif chemokine ligand 7:C-C motif chemokine ligand 22 ratio in this limited study, in a larger cohort of patients is necessary. (*Pediatr Crit Care Med* 2020; 21:e1084–e1093)

Key Words: acute respiratory distress syndrome; biomarker; bronchoalveolar lavage; chemokines; pediatric; pediatric acute respiratory distress syndrome; pneumonia

Respiratory infections leading to bronchiolitis or viral/bacterial pneumonia are the most common cause of admission to the PICU (1). Although some viruses, such as influenza A, are associated with worse outcomes, pediatric acute respiratory distress syndrome (PARDS) develops in patients from even mild viruses such as respiratory syncytial virus (RSV) (2, 3). New respiratory viruses, such as severe acute respiratory syndrome coronavirus 2, are especially challenging as they may not be detected on first-pass testing. A shift in focus from respiratory viral detection to host immune response profiling may be warranted. At the current time, pediatric intensive care physicians cannot objectively predict which patients are likely to deteriorate or to rationally administer specific immune-targeted therapies: needs that could be filled by pediatric biomarkers of PARDS (4). Immune biomarkers may be a way to gain insight into the underlying timing and pathophysiology of disease processes.

In lieu of identifying single biomarkers, another diagnostic strategy is to establish an immunologic profile of disease

using a panel of biomarkers. This approach worked with pediatric sepsis in the Pediatric Sepsis Biomarker Risk Model (PERSEVERE) study (5) and was recently described with plasma matrix metalloproteinase profiles in PARDS patients (6). Although serum is the most common site of biomarker measurement, a better location to retrieve biomarkers of alveolar lung injury may be the airways. There is a long history of examining biomarkers in the bronchoalveolar lavage (BAL) fluid of adult patients with acute respiratory distress syndrome (ARDS). Not unexpectedly, airway cytokine levels are elevated, but the levels of antagonists/receptors are often even higher, indicating the complex inflammatory/anti-inflammatory milieu during ARDS (7). However, clinical measurement of BAL biomarkers has not become standard practice, possibly due to the heterogeneity of disease and host factors in adult ARDS. In contrast, PARDS may represent a more homogenous condition, as it is usually triggered by viral respiratory infections (2).

We investigated mouse models of inflammation-induced acute lung injury in response to viral as well as gram-positive/negative bacterial antigenic challenges. Using an exploratory multiplex cytokine panel, we sought to identify patterns of cytokine expression correlating with mouse illness severity as measured by lethality. We then attempted to extrapolate findings from the mouse model to a cohort of pediatric patients intubated for respiratory failure due to infection. A similar broad multiplex cytokine panel was applied to a pediatric cohort and correlated with relevant clinical outcomes. Ventilator-free days (VFDs) were chosen as the primary clinical outcome. We secondarily examined mortality and PARDS severity.

MATERIALS AND METHODS

Influenza A Virus Preparation
The influenza strain A/Puerto Rico/8/34 virus was provided by Hartshorn et al (8). The virus was grown in the chorioallantoic fluid of 10-day-old specific-pathogen-free chicken eggs purchased from Charles River Laboratories (Wilmington, MA) and purified on a discontinuous sucrose gradient. Influenza A virus (IAV) infectivity was measured by the infection of Madin-Darby canine kidney cell monolayers, followed by the determination of fluorescent focus units (FFUs) as previously described (8). Serial dilutions of IAV in phosphate-buffered saline (PBS) were performed immediately prior to infections.

Animals, Infections, and Inoculations
All animal experiments were approved by the Penn State University College of Medicine Institutional Animal Care and Use Committee. Male and female, 9–11 weeks old, C57BL6/J wild-type mice were purchased from The Jackson Laboratory (Bay Harbor, ME). The mice were anesthetized with ketamine/xylazine and inoculated intranasally with 40 µL of PBS containing either varying doses of IAV (100, 1,000, or 10,000 FFU) or 50 µg of the toll-like receptor (TLR)-4 agonist, lipopolysaccharide (LPS) (*Escherichia coli* 0114:B4; Millipore-Sigma, St. Louis, MO), or 50 mg of the TLR2 agonist, Pam3CSK4

(Invivogen, San Diego, CA), to represent gram-negative and gram-positive bacterial infections, respectively. We also included two control groups of mice: an untreated group and a sham-infected group, which were anesthetized, administered vehicle control only (PBS), and harvested at 4 hours postinfection. All mice were infected or inoculated in a biosafety level 2 biosafety cabinet and housed within filter-top microisolator cages in the Pulmonary Immunology and Physiology core, a BSL2+ facility in the Department of Comparative Medicine's animal facility at the Penn State University College of Medicine. They were observed at least twice daily to assess morbidity and mortality.

Mouse Biomarker Fluid Collection
Mice (n = 4–7) per time point and per insult) were harvested at time points of 4 hours and 1, 3, 5, 7, 9, 12, and 16 days post-inoculation (dpi). These time intervals were chosen to capture the early effect of TLR agonists while also encompassing the duration of IAV infection. Blood was collected from the inferior vena cava and then allowed to clot at room temperature (RT) for 4 hours prior to centrifugation at $400 \times g$ for 5 minutes at 4°C. Serum supernatant was collected and two aliquots were stored at –80°C for subsequent batch analysis. BAL was performed by cannulation of the trachea, followed by instillation and aspiration of five aliquots of 500 µL of PBS with 1-mM EDTA. BAL fluid was centrifuged at $150 \times g$ for 10 minutes at 4°C. BAL supernatants were removed, aliquoted, and stored at –80°C for subsequent batch analysis.

Pediatric Pneumonia Discovery Cohort
We performed a single-center, prospective cohort study examining pediatric patients admitted to the Penn State Health Children's Hospital PICU with acute respiratory failure. Patients 0–17 years old who had an oral/nasal endotracheal tube in place were screened for enrollment in the study group and were only eligible if they had a proven viral or bacterial pneumonia as confirmed by a positive polymerase chain reaction-based respiratory pathogen panel (RPP). Patients were not eligible if they had a history of chronic lung disease prior to PICU admission. We also enrolled a small control group of patients to determine our lower limits of detection of cytokines in lower respiratory tract (LRT) fluid: pediatric patients 0–17 years old who were intubated with an oral/nasal endotracheal tube for airway control during elective surgical procedures. Informed consent was obtained from the patients' parents prior to enrollment. Patients were enrolled from April 2015 to March 2019. The Institutional Review Board of the Pennsylvania State University College of Medicine approved the study prior to any patient screening or data collection. Clinical data points including oxygen saturation (Spo_2), Fio_2, and mean airway pressure were obtained each day of intubation at 4:00 AM. The oxygen saturation index (OSI) was calculated (OSI = mean airway pressure [cm H_2O] × Fio_2 × 100/SpO_2) daily to categorize the severity of PARDS as defined by the Pediatric Acute Lung Injury Consensus Conference recommendations (3).

LRT Fluid Collection

LRT fluid was collected by blind lavage with a patient weight–adjusted amount of sterile saline (3 mL if < 10 kg, 5 mL if 10–20 kg, 8 mL if 20–40 kg, and 10 mL if > 40 kg) followed by immediate aspiration. Two sampling techniques, either direct tracheal aspiration or blind mini-BAL with a soft feeding tube, were performed. Recovery of the instillate was approximately 20% for both methods. LRT fluid was transferred to a 15-mL cone and centrifuged at $500 \times g$ for 10 minutes at RT. The supernatant was removed by aspiration and aliquoted into 1.5-mL cryovials, initially frozen at –20°C for 1–3 days, and then stored at –80°C for batch analysis.

Multiplex Analysis of Fluids

A multiplex panel of cytokines was designed based on both published animal and human data, as well as our own laboratory's preliminary data (**Supplemental Table 1**, Supplemental Digital Content 1, http://links.lww.com/PCC/B499). Analyte concentrations were measured in all the samples, both mouse and human, using bead-based fluorescent multiplex immunoassays. For analysis of mouse BAL and serum samples, we used a commercially available, custom 23-plex mouse magnetic multiplex bead assay (Bio-techne, Minneapolis, MN) to measure monocyte chemoattractant protein 1 (MCP-1 or chemokine [C-C motif] ligand [CCL] 2), macrophage inflammatory protein 1 alpha (MIP-1 alpha or CCL3), MIP-1 beta or CCL4, regulated upon activation, normal T cell expressed and presumably secreted (RANTES or CCL5), monocyte-chemotactic protein 3 (MCP3 or CCL7), CCL11/Eotaxin, CCL22/macrophage-derived chemokine (MDC), chemokine (C-X-C motif) ligand 1 (CXCL1 or GRO-alpha), interferon gamma-induced protein 10 (IP-10 or CXCL10), granulocyte/macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-gamma, interleukin (IL)-1 alpha, IL-1 beta, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17/IL-17A, IL-27, macrophage colony-stimulating factor (M-CSF), matrix metalloproteinase 12 (MMP-12), tumor necrosis factor alpha (TNF-alpha). For analysis of human BAL samples, we utilized a custom 26-plex human magnetic multiplex bead assay (Biotechne) to measure CCL3/MIP-1 alpha, CCL4/MIP-1 beta, CXCL10/IP-10, epithelial cell adhesion molecule (EpCAM), fatty acid binding protein 4 (FABP4), GM-CSF, IFN-gamma, IL-10, IL-18, IL-8/CXCL8, MCP-1/CCL2, MDC or CCL22, monokine induced by IFN-gamma (MIG or CXCL9), MMP-1, MMP-12, MMP-2, MMP-8, MMP-9, thymus and activation-regulated chemokine (TARC or CCL17), TNF-alpha, TNF receptor apoptosis-inducing ligand (TRAIL), MCP-3/CCL7, MIP-1 delta/CCL15, CXCL12, RANTES/CCL5, IL-17A. All sample measurements were acquired on a Luminex (Austin, TX) MAGPIX instrument running the xPONENT software.

Statistical Analysis

All data points are means ± sem unless otherwise stated. Graphs were created using Prism 8 for Mac OS X (GraphPad, La Jolla, CA). All statistical analysis was performed using the JMP 12.0.1 software (SAS, Cary, NC). The normality assumption for continuous variables was checked based on Shapiro-Wilk tests, and natural log-transformation was implemented if the assumption was violated. Group comparisons were performed using two-sample t tests or Wilcoxon rank-sum tests, as appropriate. Correlation analysis was conducted using Pearson correlation coefficient. Survival analysis was calculated by Kaplan-Meier curves, and the comparison was based on the log-rank test. The comprehensive web application MetaboAnalyst (https://www.metaboanalyst.ca) was used to generate receiver operating characteristic (ROC) curves with the area under the curves (AUCs) reported to evaluate the predictive accuracy of the biomarker function.

RESULTS

Mouse Pneumonia Models

We first analyzed the weight loss and survival of mice to various lung insults, either with TLR agonists or infectious IAV, as a preclinical model of PARDS. After LPS inoculation, mice lost weight immediately, reaching a nadir 3 dpi at 80% of original weight (**Fig. 1A**, purple diamonds). In contrast, Pam3CSK4 inoculation did not have any effect on body weight other than a transient loss at 1 dpi (Fig. 1*A*, orange circles). Infection with IAV led to dose-dependent differences in weight loss and survival. Mice infected with the lowest dose of IAV (100 FFU) did not lose weight until 7 dpi and recovered this mild weight loss by 10 dpi. In comparison, mice infected with larger dosages of IAV (1,000 [red triangles] and 10,000 FFU [green triangles]) lost weight more quickly, did not demonstrate any weight recovery, and did not survive the 16-day observation period (**Fig. 1**, *A* and *B*).

Descriptive Statistics of Mouse Biomarkers

We investigated our hypothesis that BAL biomarkers are more representative of the immune microenvironment of the lung and would show more dynamic range as opposed to serum markers that are distributed throughout the body. We first queried whether the protein levels of the individual analytes were within the detection limits of the assay for each of the sampling locations (serum and BAL fluid). In mouse serum, six analytes (CCL11, CCL3, CCL7, IL-4, M-CSF, and MMP-12) were able to be detected in all 195 samples regardless of lung insult or timing. In BAL fluid, only CCL22 and MMP-12 were able to be detected in all 195 samples (**Fig. 1C**).

We investigated the dynamic range of each analyte for both sample fluids. We defined "effective dynamic range" as being the highest measured value divided by the lowest measured value across all lung insults and timing, whereas values that were out of range were not included. The effective dynamic range was higher in BAL fluid than serum for all of the analytes except for CXCL10, GM-CSF, IL-4, IL-10, IL-17, and IL-27 (**Fig. 1D**). For some markers, such as CCL7, the effective dynamic range in BAL fluid was more than 1,000-fold higher than serum.

Temporal Expression of Common Cytokine Biomarkers in Mouse Pneumonia Models

Using commercially available multiplex bead assays that measured 23 individual mouse analytes, we examined the cytokine response to various insults in mouse serum and BAL fluid. Consistent with previous studies (9, 10), TNF-α peaked
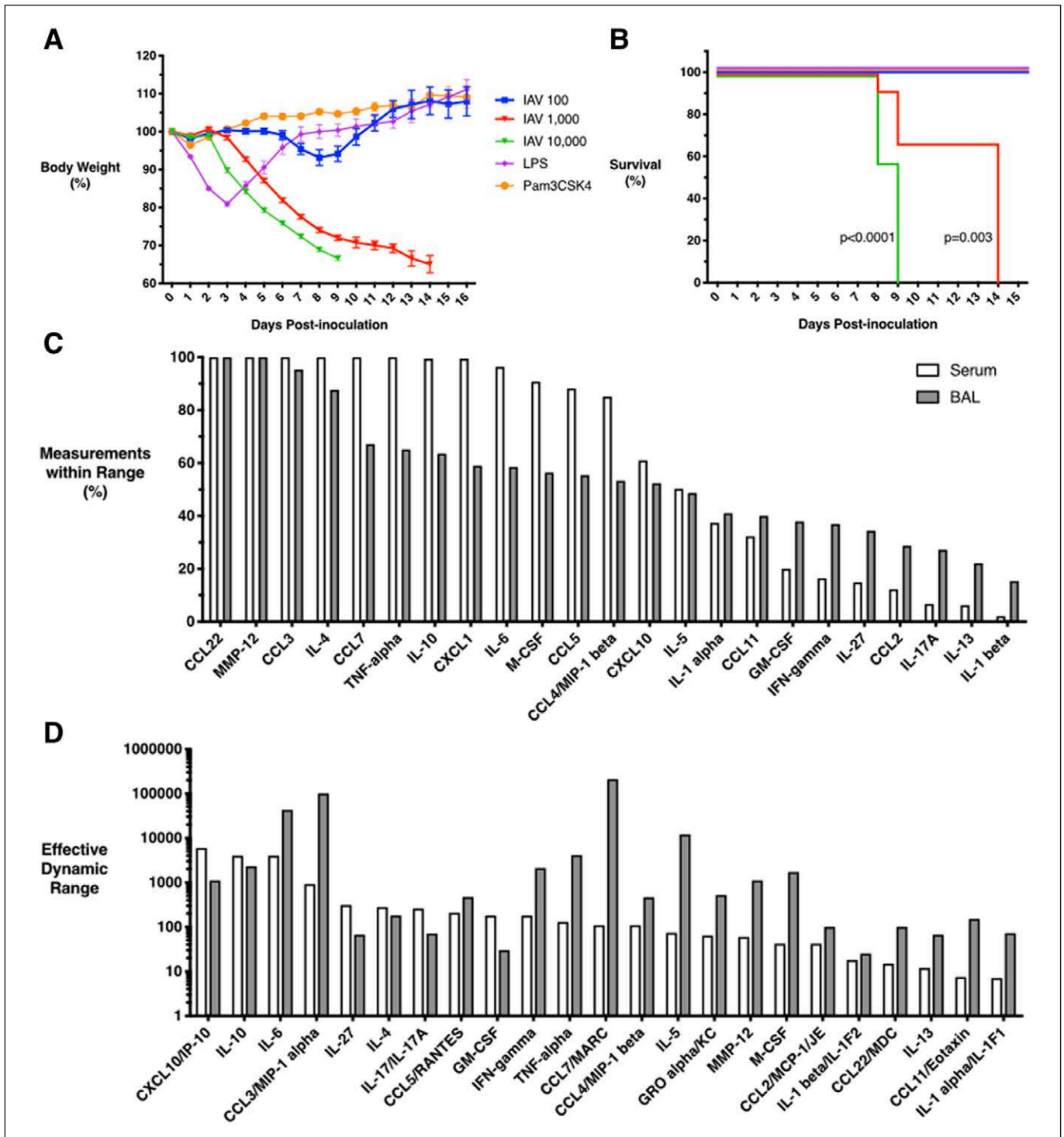
Figure 1. Mouse lung injury model after influenza A virus (IAV) infection and toll-like receptor (TLR)-agonist administration, and descriptive statistics of biomarkers. **A**, Wildtype C57BL/6J mice (male and female), 9–11 wk old, were either infected with 100 ($n = 48$ mice), 1,000 ($n = 35$), or 10,000 fluorescent focus units (FFUs; $n = 32$) of the Puerto Rico/8/34 strain of IAV, whereas other groups were inoculated with 50 µg of the TLR4 agonist, lipopolysaccharide (LPS) ($n = 34$), or the TLR2 agonist, Pam3CSK4 ($n = 34$), and weight loss and recovery were measured. **B**, Mice were harvested at 4 hr and 1-, 3-, 5-, 7-, 9-, 12-, and 16-d postinoculation for biomarker measurements. Mice infected with either 1,000 or 10,000 FFU of IAV did not survive past 14 d, whereas no mice died from the other lung insults. **C**, Biomarkers were more likely to be within the measurable range when obtained from serum (*white bars*) as opposed to bronchoalveolar lavage (BAL) fluid (*gray bars*). **D**, The effective dynamic range for each analyte for each body fluid was usually higher in BAL fluid than serum and was calculated as follows: (highest detectable value [pg/mL])/(lowest detectable value [pg/mL]). CCL = [C-C motif] ligand, CXCL = chemokine (C-X-C motif) ligand, GM-CSF = granulocyte/macrophage colony-stimulating factor, IFN-gamma = interferon gamma, IL = interleukin, IP-10 = interferon gamma-induced protein 10, KC = keratinocytes-derived chemokine, MCP-1 = monocyte chemoattractant protein 1, M-CSF = macrophage colony-stimulating factor, MDC = macrophage-derived chemokine, MIP-1 = macrophage inflammatory protein 1 (MIP-1), MMP-12 = matrix metalloproteinase-12, TNF-alpha = tumor necrosis factor-alpha.

immediately (4 hr) in BAL fluid after LPS challenge, without any appreciable induction in serum (**Supplemental Fig. 1**, Supplemental Digital Content 2, http://links.lww.com/PCC/B500). TNF-α also briefly increased in BAL fluid at 4 hour after Pam3CSK4, but the measured level was approximately 10-fold lower than that of LPS. A spike in IL-6 was detected in both serum and BAL fluid at 1 dpi with LPS, but no increase was noted with Pam3CSK4 (**Supplemental Fig. 2**, Supplemental Digital Content 3, http://links.lww.com/PCC/B501). In lethally infected IAV-exposed mice, an early rise in IL-6 was starting at 3 dpi, whereas the increase in IL-6 was only seen at 7 dpi in the 100 FFU IAV-infected group. A large single spike of IFN-γ in BAL fluid was noted at 7 dpi only in the IAV-infected mice, corresponding to the influx of antigen-specific T cells (11). This corresponded to a small peak of IFN-γ in the serum of lethally IAV-infected mice (**Supplemental Fig. 3**, Supplemental Digital Content 4, http://links.lww.com/PCC/B502).

Mouse Biomarkers of Clinical Outcomes

We identified mouse body weight and survival as two objective clinical markers of illness severity and impartially investigated their correlations with a broad panel of biomarkers using linear regression analysis. We first examined all biomarkers' concentrations against the percentage (%) of original body weight gained or lost (**Supplemental Table 2**, Supplemental Digital Content 5, http://links.lww.com/PCC/B503). Although many biomarkers demonstrated a significant correlation with body weight, three biomarkers demonstrated high correlations ($r > 0.7$). The biomarker that demonstrated the best correlation with body weight was serum TNF-α ($r = -0.746$; $p < 0.0001$), followed by BAL CCL7 ($r = -0.736$; $p < 0.0001$) and BAL CCL11 ($r = -0.721$; $p < 0.0001$). Based on these body weight correlations, we proceeded to investigate the kinetics of select markers. CCL7 demonstrated a robust increase in both serum and BAL fluid with high dose (1,000 and 10,000 FFU) IAV infection, peaking at 7 dpi in BAL and around 9 dpi in serum, as opposed to the other insults (**Fig. 2A**). Interestingly, serum levels of the IFN-inducible chemokine, CCL11, were substantial (> 400 pg/mL) regardless of timing and treatment (**Fig. 2B**).

Next, we examined the correlation of individual biomarkers with lethality. Given that no mice in the high-dose 1,000 and 10,000 FFU IAV dosage groups survived past 14 dpi, we grouped these mice together as "lethal" insults, whereas the other groups (IAV 100 FFU, LPS, and Pam3CSK4) were grouped together as "nonlethal" insults. We performed an unbiased evaluation of biomarkers at the peak of inflammation (1–3 dpi for LPS and Pam3CSK4 and 7 dpi for IAV) against lethal infection (yes/no) by classical univariate ROC curve analysis (**Table 1**).

Elevated levels of CCL7 demonstrated high predictive ability of lethality (AUC > 0.99) when measured in either BAL fluid or serum. CCL11 demonstrated high predictive ability when measured in BAL fluid (AUC = 0.994) but less so when measured in serum (AUC = 0.902). Other markers that demonstrated a high correlation with lethality were BAL M-CSF (**Supplemental Fig. 4**, Supplemental Digital Content 6, http://links.lww.com/PCC/B504), BAL IL-10 (**Supplemental Fig. 5**, Supplemental Digital Content 7, http://links.lww.com/PCC/B505), and BAL MMP-12 (**Supplemental Fig. 6**, Supplemental Digital Content 8, http://links.lww.com/PCC/B506).

Mouse BAL Biomarker Ratios and Lethality

One of the problems with BAL fluid analysis in patients is the variability in sample concentration due to dilution error. We investigated whether
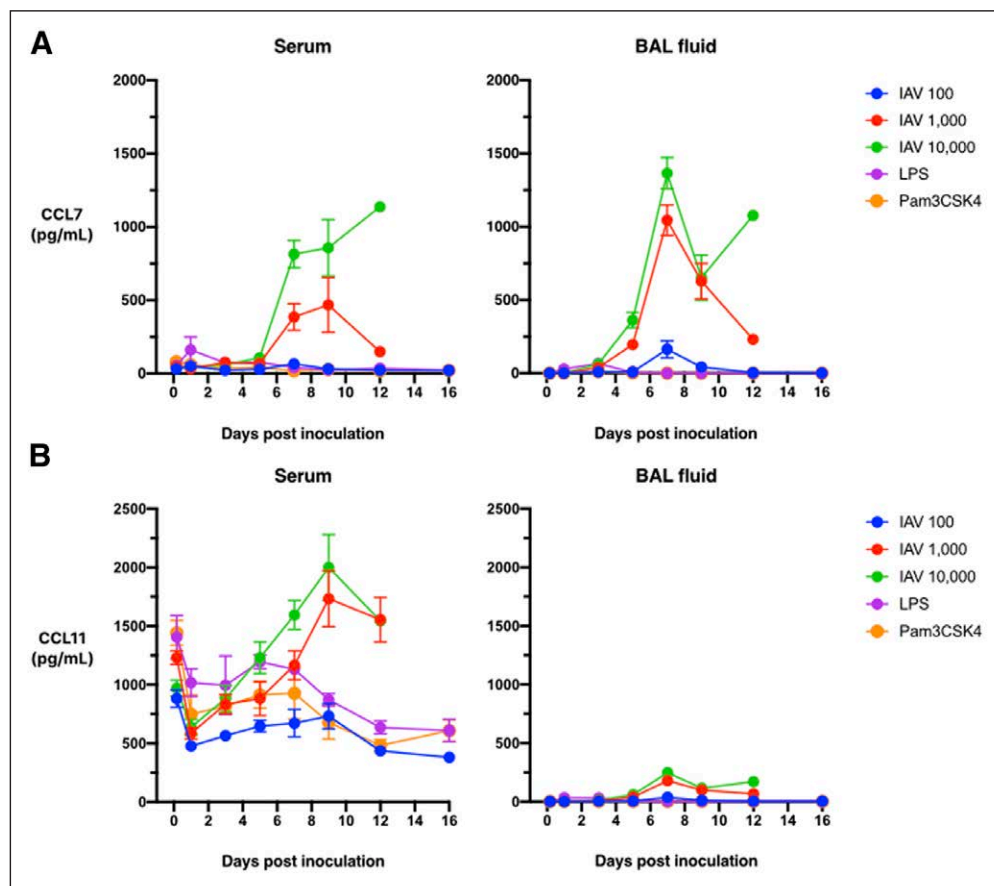

Figure 2. Kinetics of cytokine/chemokines that demonstrated high correlation with weight loss. Levels of cytokines/chemokines were measured in serum and bronchoalveolar lavage (BAL) fluid at 4 hr and 1, 3, 5, 7, 9, 12, and 16 d after lung insults. Levels of [C-C motif] ligand (CCL) 7 (**A**) and CCL11 (**B**) in both serum and BAL fluid following lung insults. IAV = influenza A virus, LPS = lipopolysaccharide.

TABLE 1. Biomarkers During Peak of Inflammation and Correlation With Mouse Lethality

Analyte	Area Under the Curves (95% CI)	Log2-Fold Change	p
BAL CCL7	0.999 (0.994–1)	5.4	2.8×10^{-18}
BAL CCL11	0.994 (0.978–1)	4.4	1.2×10^{-13}
Serum CCL7	0.991 (0.964–1)	3.3	5.6×10^{-12}
BAL M-CSF	0.969 (0.925–0.997)	4.1	7.6×10^{-12}
BAL interleukin-10	0.955 (0.901–0.993)	4.2	8.9×10^{-10}
BAL matrix metalloproteinase-12	0.954 (0.89–0.996)	2.3	1.9×10^{-10}
Serum CCL11	0.902 (0.815–0.974)	1.0	4.9×10^{-08}
BAL CCL2	0.891 (0.799–0.964)	5.6	2.7×10^{-07}
Serum CXCL10	0.890 (0.792–0.967)	2.9	8.1×10^{-07}
BAL CXCL10	0.860 (0.765–0.955)	3.8	1.5×10^{-05}

BAL = bronchoalveolar lavage, CCL = [C-C motif] ligand.

TABLE 2. Bronchoalveolar Lavage Biomarker Ratios During Peak of Inflammation and Correlation With Mouse Lethality

Ratio	Area Under the Curves (95% CI)	Log2-Fold Change	p
BAL CCL7/CCL22	1.000 (1–1)	10.2	1.9×10^{-16}
BAL CCL7/IL-1 alpha	0.999 (0.991–1)	9.8	2.7×10^{-13}
BAL CCL7/IL-1 beta	0.999 (0.991–1)	10.1	4.8×10^{-16}
BAL CCL11/CCL22	0.997 (0.987–1)	12.7	2.0×10^{-10}
BAL CCL11/IL-1 beta	0.997 (0.986–1)	11.8	2.4×10^{-11}
BAL M-CSF/CCL22	0.996 (0.978–1)	2.7	6.3×10^{-17}
BAL CCL7/CCL3	0.996 (0.985–1)	9.6	7.7×10^{-10}
BAL matrix metalloproteinase-12/CCL22	0.994 (0.976–1)	3.1	7.7×10^{-10}
BAL M-CSF/IL-1 alpha	0.993 (0.97–1)	3.5	7.5×10^{-13}
BAL M-CSF/IL-1 beta	0.987 (0.961–1)	4.5	1.4×10^{-12}

BAL = bronchoalveolar lavage, CCL = [C-C motif] ligand, CXCL = chemokine (C-X-C motif) ligand, IL = interleukin, M-CSF = macrophage colony-stimulating factor.

analysis of the multiplex protein ratios further improved our prediction models. BAL cytokine ratios at peak inflammation demonstrated very good correlation with lethality, with the top 10 ratios demonstrating AUCs greater than 0.98 (**Table 2**). The three "drivers" of separation to discriminate lethality appeared to be CCL7, CCL11, and M-CSF. Interestingly, the

MetaboAnalyst program impartially identified three proteins to be the denominators for the ratio: CCL22, IL-1α, and IL-1β. These cytokines, because of their low variation across time and insults, likely serve as "normalization factors": surrogate markers of total protein content.

The BAL fluid CCL7 level was the predominant biomarker, as it was represented in four of the top 10 ratios. CCL22 was represented in four of the top 10 ratios, and expression in BAL fluid was relatively stable across times and treatments with the only peak being at 1 dpi with LPS (**Fig. 3A**). We examined the kinetics of the BAL CCL7:CCL22 ratio after the various insults and chose to represent a peak cutoff ratio of 5:1, which demonstrated a sensitivity of 1 (95% CI, 1–1) and a specificity of 0.969 (0.89–1) for lethal infection. This cutoff of 5:1 provided good discrimination of lethality, as graphed on both linear (**Fig. 3B**) and logarithmic (**Fig. 3C**) scales.

Pediatric Respiratory Infection Cohort

We next used the large multiplex cytokine panel approach to the pulmonary immune microenvironment of humans. We used a discovery cohort of pediatric patients intubated for respiratory failure related to respiratory infection (n = 27, infection group). We also enrolled healthy pediatric patients (n = 12, control group) who were intubated for airway protection during routine procedures. This group served to test the sensitivity of our biomarker measurements in LRT fluid. The primary clinical outcome variable was VFDs within the infection group.

Respiratory viruses were identified by RPP in 26 of the 27 infected patients. RSV was the most common virus identified (n = 11; 41%), with rhinovirus being the second-most common virus (n = 8; 30%). Two participants tested positive for two respiratory viruses: RSV and adenovirus. Only one participant in the infected cohort was not virus-positive; instead, *Mycoplasma pneumoniae* was identified by RPP. Bacterial pathogens were codetected by respiratory bacterial culture in 11 of the 27 infected patients (41%), with *Haemophilus influenza* the most common pathogen isolated (**Table 3**).

The patients' numbers of VFDs (19.9±7.9) were relatively high, suggesting an only mildly sick sample of patients with acute respiratory failure. Of the 27 respiratory infection patients, only three patients qualified as being moderate/severe PARDS based on oxygen OSI. Both patients with severe PARDS (patients 007 and 036) were escalated to venovenous extracorporeal membrane oxygenation (ECMO) 3 days postintubation due to poor oxygenation but ultimately succumbed to CNS complications.

LRT Fluid Biomarkers

We analyzed cytokine levels in a total of 72 LRT fluid samples from our 39 patients using a commercially available multiplex bead array. Control patients only had one sample collected, whereas respiratory infection patients had samples every 2–3 days following enrollment until the end of invasive mechanical ventilation. We examined the peak of each soluble factor during mechanical ventilation to coincide with our analysis of
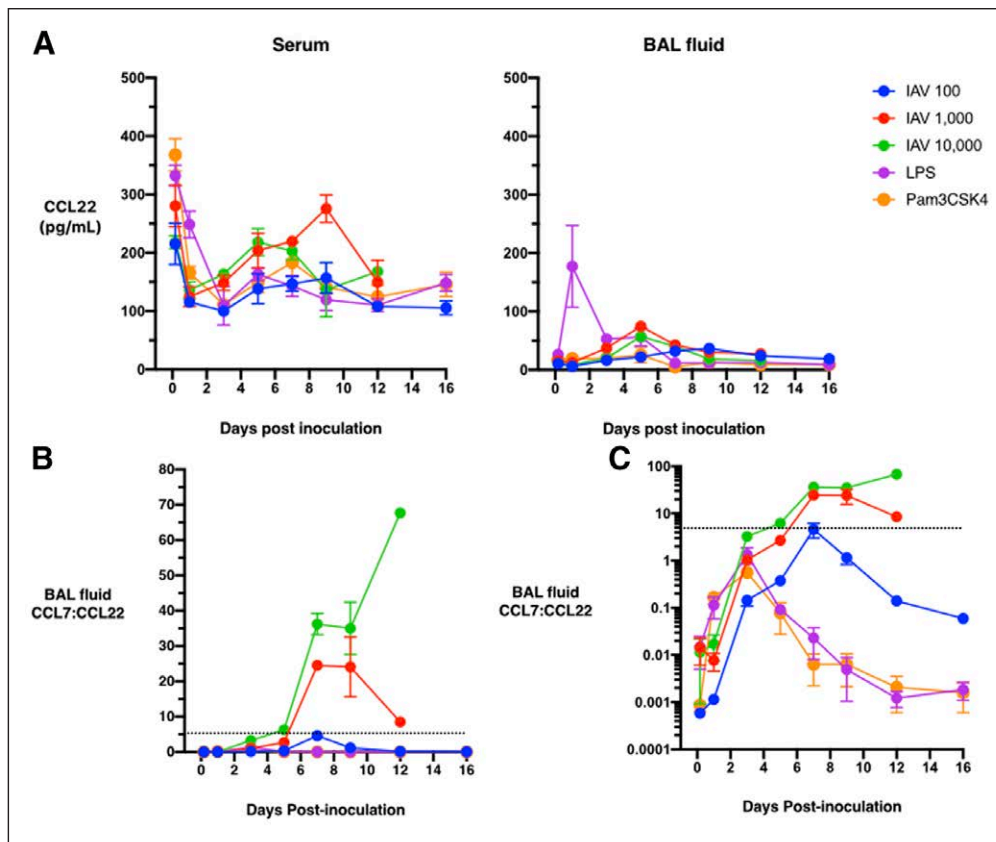
Figure 3. Kinetics of [C-C motif] ligand (CCL) 22 and the ratio of CCL7:CCL22, following lung insults. **A**, Bronchoalveolar lavage (BAL) fluid levels of CCL22 demonstrated only a transient increase at 1-d postinoculation after lipopolysaccharide (LPS), whereas CCL22 demonstrated constitutive expression in serum and transient elevations at 4 hr especially after toll-like receptor agonist treatments before stabilization. Kinetics of CCL7:CCL22 after lung insults plotted on (**B**) linear and (**C**) $\log_{10}$ scales with a chosen cutoff ratio of 5:1 (*dotted line*). IAV = influenza A virus.

DISCUSSION

In this exploratory analysis of cytokines and chemokines in mouse and human pediatric models of ARDS secondary to infection, we found CCL7 elevation to be the most promising biomarker of illness severity. Furthermore, the use of chemokine ratios seemed to strengthen the correlations, perhaps by partially correcting the dilution errors commonly seen with airway fluid sampling. To our knowledge, this is the first study to implicate elevated levels of CCL7 as a biomarker for respiratory infection severity. The rationale for our study was partially based on the system analysis of lethal influenza infection in mice performed by Brandes et al (12). With escalating dosages of IAV, they showed that lethal influenza infection caused an early engagement of neutrophils, which instigated a damaging feedforward innate inflammatory circuit. Brandes et al (12) also noted an increased CCL7

the mice at the peak of inflammation; therefore, each patient only had one "peak" value. Our best performing solitary biomarker in the mouse model, BAL CCL7, was only measurable in the LRT fluid of seven patients (lower limit of detection 24.2 pg/mL); however, the peak CCL7 levels trended with increasing PARDS severity (**Fig. 4A**). In contrast, CCL22 was measurable in all 27 infection group patients, but only one of the 12 healthy control patients (**Fig. 4B**). Of interest, FABP4, a protein expressed by alveolar macrophages, was detectable in nine of the 12 healthy control patients and may serve as a normalization factor in future studies (**Supplemental Fig. 7**, Supplemental Digital Content 9, http://links.lww.com/PCC/B507).

Examination of the LRT fluid CCL7:CCL22 ratio revealed that while the two patients with severe PARDS exhibited peak ratios above 10:1, the one patient with moderate PARDS displayed a CCL7:CCL22 ratio near 5:1 (**Fig. 4C**), similar to the cutoff ratio we chose based on the mouse model (Fig. 3C). We correlated LRT fluid CCL7:CCL22 ratios to VFDs on all patients (**Fig. 4D**), and peak CCL7:CCL22 demonstrated a strong negative association with VFDs ($r = -0.805$; $p < 0.02$). Finally, for the two participants with severe PARDS (patient 007 [red squares] and patient 036 [orange squares]), the CCL7:CCL22 ratio remained at or above the threshold of 5:1 through 10 dpi and gradually declined afterward (**Fig. 4E**).

signature in the whole lung homogenate extracts through microarray analysis of messenger RNA transcripts. Although our study was exploratory in nature, transcriptomic evidence of CCL7 up-regulation supports our findings via direct assessment of protein concentrations in LRT fluid. Importantly, our approach may lend itself to practical clinical application, as direct protein measurement can now be performed at bedside using novel microfluidic enzyme-linked immunosorbent assay-based technology (13).

Depending on the model, CCL7 has been shown to be important for the chemotaxis of multiple cell types including monocytes, neutrophils, and eosinophils (14). In a BALB/c mouse rhinovirus model, CCL7 was necessary for maximal development of airway hyperreactivity. In contrast, CCL7 was elevated in BAL fluid from adult volunteers exposed to LPS and was chemotactic for neutrophils (15). Clinically, CCL7 was described as being elevated in lung biopsies from adult patients with interstitial pneumonia (16). The cellular source of CCL7 within LRT fluid is unclear. Previous RNAseq analysis of airway macrophages in mice demonstrated increased expression of CCL7 in inflammatory exudative or interstitial macrophages as opposed to alveolar macrophages (17). This is supported by the RNA microarray data from Brandes et al (12) that demonstrated increased CC7 expression

TABLE 3. Patient Demographics–Respiratory Infection Cohort

Patient	Age (yr)	Sex (Male/Female)	Respiratory Pathogen Panel	Microbial Respiratory Culture	Ventilator-Free Days
001	17.5	Male	RSV	*Pseudomonas aeruginosa*	25
007	0.53	Male	RSV	Yeast	0
008	6.1	Male	RSV, adenovirus	*P. aeruginosa*	16
010	0.2	Male	RSV	*Haemophilus influenzae*	24
011	0.3	Male	RSV, adenovirus		23
012	0.2	Male	RSV		24
013	0.28	Female	Rhinovirus	*H. influenzae*	17
017	7.14	Male	Rhinovirus	Yeast	0
018	0.21	Male	RSV	*H. influenzae*	21
021	4.72	Female	Rhinovirus		21
023	3.95	Male	Human metapneumovirus		17
024	3.85	Male	Parainfluenza	*Staphylococcus aureus*	25
025	0.11	Male	RSV		23
026	16.0	Female	Rhinovirus	*H. influenzae*	26
027	0.19	Female	Influenza B		24
028	17.9	Male	Human metapneumovirus	Yeast	20
029	2.87	Female	Parainfluenza		26
030	14.8	Female	Parainfluenza	*H. influenzae*	26
031	2.36	Female	Adenovirus	*Streptococcus pneumoniae*	27
032	0.17	Male	Rhinovirus		23
033	0.52	Male	Rhinovirus		17
034	0.21	Male	Rhinovirus		*
035	0.7	Male	Rhinovirus		24
036	10.9	Male	*Mycoplasma pneumoniae*		0
037	5.89	Male	RSV		25
038	0.12	Male	RSV	*H. influenzae*	21
039	0.11	Male	RSV	*S. pneumoniae*	23

RSV = respiratory syncytial virus.
*Patient transferred to another institution, lost to follow-up.

by monocytes during lethal influenza infection in mice. However, other studies have demonstrated alternative, CD45-negative, sources of CCL7 (18). Immunohistochemical staining of mouse lungs after LPS instillation demonstrated that CCL2 was localized to alveolar macrophages and CCL7 was restricted to the pulmonary epithelium (19). In a *Schistosoma mansoni* lung granuloma model, endothelial cells were an important in situ source of CCL7 (20). CCL7 is up-regulated in psoriatic lesions, with endothelial cells and keratinocytes being the likely cellular sources (21). In our mouse model, given the near-simultaneous increase of CCL7 in both BAL fluid and serum in lethal IAV-infected mice (Fig. 2*A*), we

surmise that the most likely sources of CCL7 in our model are either lung endothelial or epithelial cells. In is noteworthy that serum levels of CCL7 were almost as predictive as BAL levels (Table 2). Future research should focus on validating CCL7 as a biomarker of lung injury and to define the cellular source of CCL7 to provide insight into the pathophysiology of acute respiratory failure.

The custom multiplex bead array panel that we designed focused on chemokines rather than other cytokines. We hypothesized that the role of chemokines in leukocyte chemotaxis would enhance their ability to serve as biomarkers of severe respiratory infection. This finding is consistent with
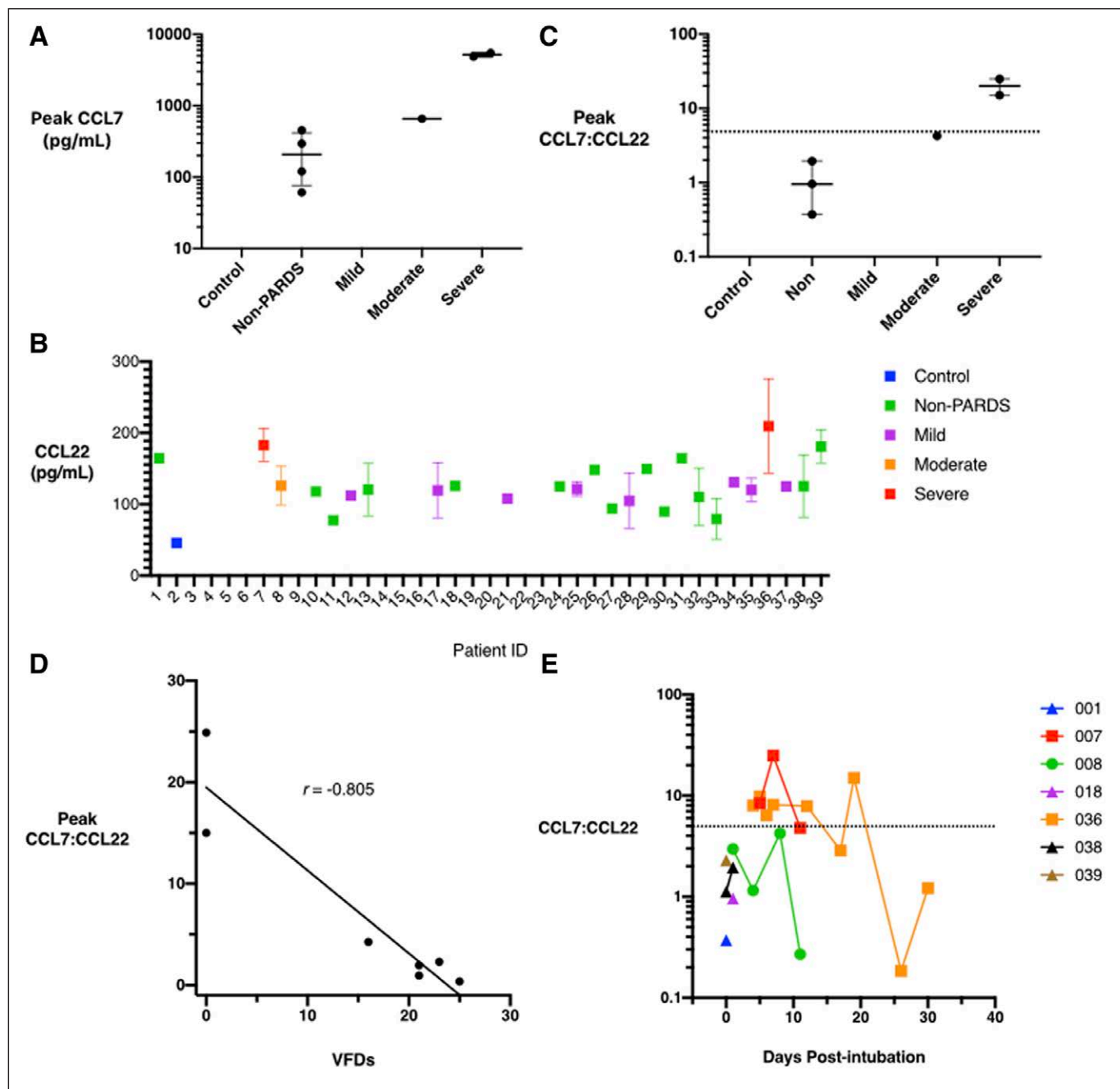
Figure 4. Lower respiratory tract (LRT) levels of chemokine [C-C motif] ligand (CCL) 7, CCL22, and CCL7:CCL22 ratios from infected patients. **A**, Peak LRT fluid levels of CCL7 trended with pediatric acute respiratory distress syndrome (PARDS) severity. **B**, Levels of CCL22 ($n = 1-9$ samples per patient) were similar among patients, regardless of their PARDS severity. Peak CCL7:CCL22 ratio also (**C**) trended with PARDS severity and (**D**) demonstrated a robust ($r = -0.805$; $p < 0.02$) negative correlation with ventilator-free days (VFDs). **E**, Longitudinal analysis of the CCL7:CCL22 ratio in infected patients with varying degrees of PARDS severity.

results from the OPPORTUNITY trial, which used CXCL10, TRAIL, and C-reactive protein to discriminate viral from bacterial infections in preschool children (22). Here, we show that the dynamic range of these chemokines in BAL may extend their clinical utility beyond traditional blood measurements (Fig. 1D). Furthermore, our findings support the idea that LRT fluid, an easily accessible biofluid with clinical relevance in pulmonary infections, is a reliable source of physiologically relevant chemokine biomarkers.

This study demonstrates that LRT measurement of protein biomarkers during infectious pediatric acute respiratory failure is feasible and the CCL7:CCL22 ratio may be a predictor of PARDS severity. Our study has several clear limitations. It is generally less desirable to use ratios as a correction factor for variations in the sample-collection technique. The ideal approach would guarantee similar sample volumes and protein concentrations, and use proper statistical normalization. However, the current clinical procurement of LRT fluid

is fraught with variability, for example, saline lavage could inconsistently yield 1–5 mL after a standard 10-mL instillation. Establishing a protein reference standard and creating ratios may be the best path forward. Our sample size is quite low, especially the number of patients with moderate or severe PARDS. The two patients with severe viral pneumonia and the highest CCL7 levels had been cannulated for ECMO prior to study enrollment; therefore, it is difficult to discern whether the increased inflammation in these patients was related to the underlying viral infection or the known inflammatory effects of an extracorporeal circuit. However, the specific increase in CCL7 seen with lethal influenza virus infection in mice, as opposed to the lack of CCL7 in response to LPS administration, suggests that the marker may be specific to severe viral pneumonia. Another limitation is the single-center design. Finally, because samples were only acquired while patients were intubated, and many patients were not mechanically ventilated for long durations, we lack strong longitudinal data. However, all of these concerns can be addressed with a large, multicenter, prospective, observational study of pediatric patients with a wide breadth of PARDS severity.

CONCLUSIONS

This study demonstrates that LRT fluid cytokine measurements are a promising way to assess the pulmonary immune microenvironment in PARDS. CCL7 may be a predictor of PARDS severity that could be used to improve care by identifying patients at high risk of deterioration and the use of cytokine ratios may assist in the analysis of LRT fluid for immune profiles. These results will need to be validated with a larger, prospective study of pediatric patients with pneumonia.

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