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Comprehensive Immunologic Evaluation of Bronchoalveolar Lavage Samples from Human Patients with Moderate and Severe Seasonal Influenza and Severe COVID-19

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Infection with severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) or seasonal influenza may lead to respiratory failure requiring intubation and mechanical ventilation. The pathophysiology of this respiratory failure is attributed to local immune dysregulation, but how the immune response to viral infection in the lower airways of the human lung differs between individuals with respiratory failure and those without is not well understood. We used quantitative multiparameter flow cytometry and multiplex cytokine assays to evaluate matched blood and bronchoalveolar lavage (BAL) samples from control human subjects, subjects with symptomatic seasonal influenza who did not have respiratory failure, and subjects with severe seasonal influenza or SARS-CoV-2 infection with respiratory failure. We find that severe cases are associated with an influx of nonclassical monocytes, activated T cells, and plasmablast B cells into the lower airways. Cytokine concentrations were not elevated in the lower airways of moderate influenza patients compared with controls; however, 28 of 35 measured cytokines were significantly elevated in severe influenza, severe SARS-CoV-2 infection, or both. We noted the largest elevations in IL-6, IP-10, MCP-1, and IL-8. IL-1 family cytokines and RANTES were higher in severe influenza infection than severe SARS-CoV-2 infection. Interestingly, only the concentration of IP-10–correlated between blood and BAL during severe infection. Our results demonstrate inflammatory immune dysregulation in the lower airways during severe viral pneumonia that is distinct from lower airway responses seen in human patients with symptomatic, but not severe, illness and suggest that measurement of blood IP-10 concentration may predict this unique dysregulation. *The Journal of Immunology*, **2021, 207: 1229–1238.**

Infection with both seasonal influenza and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which causes coronavirus disease 2019 (COVID-19), are associated with substantial morbidity and mortality (1–5). Manifestations of both diseases range from asymptomatic infection (4, 6) to severe illness leading to acute respiratory failure. Current treatment of severe COVID-19 and severe influenza is primarily supportive with the administration of oxygen and mechanical ventilation when necessary (7–9). There is a more limited role for antiviral therapies during severe disease (10, 11) likely because of ongoing immunopathology that is less dependent on high viral replication during the later stage of illness at which respiratory failure develops. Indeed, recent evidence (12, 13) suggests a mortality benefit in a subset of severely ill COVID-19 patients administered high-dose corticosteroids. Despite this advance, mortality in severe influenza- and COVID-19–associated respiratory failure remains high and the absolute risk reduction for mortality in steroid-treated, mechanically ventilated COVID-19 patients is somewhat low at ∼8–12% (12, 13)

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Abbreviations used in this article: BAL, Bronchoalveolar lavage; COVID-19, coronavirus disease 2019; IQR, interquartile range; SAR-CoV-2, severe acute respiratory syndrome coronavirus 2.

suggesting only a subset of severe COVID-19 patients respond to anti-inflammatory treatment.

A complete understanding of the immunologic response in the human lung during severe SARS-CoV-2 and influenza infection is essential to determine the ideal management of these patients, including determining who may benefit from anti-inflammatory medications such as steroids. Unfortunately, only a small number of studies have evaluated lower airway responses in human subjects with severe influenza (14) or COVID-19 (15–18) and very few evaluate individuals with acute symptomatic viral infection who do not have severe disease requiring mechanical ventilation. Numerous studies have shown that subjects with severe influenza and COVID-19 have elevated levels of proinflammatory cytokines in the blood (3, 19, 20). For this reason, there has been significant interest in modulating the immune system to blunt the effects of proinflammatory cytokines during severe COVID-19 (21, 22). However, our previous analysis of plasma samples from subjects with COVID-19 and influenza found a relatively low incidence of dysregulated blood cytokine expression among COVID-19 patients (23) despite generally modest increases in the concentration of circulating cytokines in patients with increasingly severe COVID-19. Other investigators have also reported lower concentrations of inflammatory cytokines in the blood of COVID-19 patients when compared with other groups of critically ill patients (24, 25). Identifying individuals with elevated and dysregulated cytokine expression in the bronchoalveolar space rather than the blood may help to more precisely stratify those at increased risk of severe respiratory failure or death. The bronchoalveolar space serves as a small window into the broader immune response in the lung. Unfortunately, sampling the bronchoalveolar space is complex. The identification of blood markers that correlate with excessive lung inflammation would be helpful. Such markers would allow the physician to target steroids and other novel immunosuppressive therapies to virus-infected individuals with documented severe inflammation in the lung, avoid side-effects in individuals without hyperinflammatory respiratory failure, and potentially increase response rates to targeted anti-inflammatory therapies that have so far proven ineffective in clinical trials (26–28).

To further understand the lower airway mucosal immune response to severe influenza and SARS-CoV-2 infection, we analyzed plasma and bronchoalveolar lavage (BAL) samples from subjects with severe influenza and COVID-19 and compared them to samples collected with identical methods from healthy controls and subjects with moderate symptomatic influenza infection that did not have respiratory failure or require hospitalization.

Materials and Methods

Study design

This is a prospective observational cohort study of subjects with viral respiratory illness who presented to Barnes Jewish Hospital in Saint Louis, Missouri. COVID-19 patients were enrolled between May 8, 2020, and December 17, 2020 after a positive clinical test for SARS-CoV-2. We also report findings from a subset of healthy control subjects and influenza-infected subjects enrolled in the ongoing prospective observational EDFLU cohort study (29) who underwent sampling of blood and BAL fluid. Subjects with influenza were enrolled in the 2018–2019 and the 2019–2020 influenza seasons.

For subjects with severe COVID-19 or influenza, BAL fluid was obtained via bronchoscopy that was performed for a clinical reason, most frequently to evaluate for bacterial coinfection, at the discretion of the patient's treating physician. The BAL samples analyzed in this study were excess material collected during that clinical bronchoscopy with BAL procedure. For severe influenza and severe COVID-19 bronchoscopy procedures, the BAL was collected most frequently by wedging the bronchoscope within the right middle lobe bronchus. One severe influenza patient had collection performed in the left lower lobe for clinical purposes. Each clinical procedure involved instilling 100 ml of sterile saline and collecting all returned fluid. The majority of each sample was sent to the clinical laboratory for analysis, but ~10–15 ml of the BAL sample was provided to a member of the study team present for the procedure and kept on ice until samples were processed in the laboratory.

For EDFLU subjects with moderate illness and control subjects, samples were obtained during a scheduled elective outpatient research bronchoscopy. Briefly, following subject consent and safety screening with blood coagulation studies and a screening chest X-ray, outpatient subjects received i.v. conscious sedation along with application of lidocaine to the upper airway and vocal cords as the bronchoscope was passed into the airways. A brief visual inspection of the airways was performed to select an appropriate location for BAL collection; however, all outpatient research BAL samples were collected in the right middle lobe bronchus. BAL samples were collected by the instillation of 100–150 ml of sterile saline and collection of all returned lavage fluid.

Informed consent was obtained from all subjects or their legally authorized representatives. The Institutional Review Board at Washington University in Saint Louis approved these studies (approval numbers 2018-08-115, 2019-10-011, 2020-03-085, and 2020-06-151).

We obtained matched blood and BAL samples from two severe influenza subjects, MC-68 and MC-77, during the 2018–2019 influenza season as part of an ongoing prospective observational cohort study at the National Institute of Respiratory Diseases Ismael Cosío Villegas in Mexico City, Mexico. This study was approved by the National Institute of Respiratory Diseases Institutional Review Board (approval number B28-16).

Cytokine quantification

Blood was collected into EDTA-anticoagulated tubes and plasma was frozen at −80°C until analysis. BAL samples were kept on ice and prepared within 4 h of collection. BAL samples were centrifuged at 300 × g for 15 min at 4°C and the collected supernatant was frozen at −80°C.

Plasma and BAL samples were analyzed using a human magnetic cytokine panel providing parallel measurement of 35 cytokines and chemokines (Thermo Fisher Scientific). The assay was performed according to the manufacturer's instructions with the following modification: the samples were fixed with 100 μL of 1% paraformaldehyde at room temperature for 60 min on a shaker at 700 RPM and washed once before the final resuspension. The samples were analyzed on a Luminex FLEXMAP 3D instrument.

Multiparameter flow cytometry

Absolute cell counts in BAL were determined by flow cytometry with Precision Count Beads (BioLegend) by staining 100 μL of BAL fluid with pretitrated amounts of CD45 FITC (clone HI30), CD14 PerCP-Cy5.5 (clone M5E2), CD3 PE-Cy7 (clone UCHT1), CD8 BV421 (clone RPA-T8), CD4 allophycocyanin-Cy7 (clone OKT4), and CD19 allophycocyanin (clone HIB19). 1 ml of FACS Lysing Solution (BD Biosciences) was added to each sample. One hundred microliters of Precision Count Beads were added immediately prior to flow cytometry to allow quantification of absolute volume analyzed on the cytometer.

In a separate experiment, fresh BAL cells were analyzed using a panel of Abs directed against the following Ags: CD3 FITC (clone UCHT1), CD4 allophycocyanin -Cy7 (clone OKT4), CD8 BV421 (clone RPA-T8), CD14 allophycocyanin (clone M5E2), CD16 BV570 (clone 3G8), CD19 BV750 (clone HIB19), CD20 Pacific Blue (clone 2H7), CD38 PE-Cy7 (clone HIT2), CD45 Alexa Fluor 532 (clone HI30), CD56 PE/Dazzle 594 (clone HCD56), and HLA-DR BV605 (clone L243). 500,000–1,000,000 fresh BAL cells were stained in BD Brilliant Buffer (BD Biosciences) with Zombie NIR Fixable Viability Marker (BioLegend). Samples were run on a Cytek Aurora spectral flow cytometer using SpectroFlo software (version 2; Cytek) before final analysis in FlowJo software (version 10; BD Biosciences).

Analysis

Absolute cell counts were calculated by measuring the frequencies of each subset in terms of percent of total $CD45^+$ cells or other subsets with known concentration from the Precision Count Bead experiment. Absolute cell count comparisons were made using Kruskal–Wallis ANOVA with Dunn posttest.

Individual cytokine concentrations were compared using Kruskal–Wallis ANOVA with Dunn posttest. Plasma and BAL correlations were performed with simple linear regression. We used a Bonferroni-corrected p value of 0.0015 to adjust for the 35 comparisons.

For the cytokine clustering analysis, cytokine values were processed in R (v4.0.2) and rescaled from 0 to 1 based on the maximum protein level measured for each cytokine. Data were then clustered and visualized using the pheatmap (v1.0.12) R package using Euclidean distances for both row and column clustering.

Plasma IP-10 concentrations measured in a large cohort of COVID-19 patients had been previously reported (23). For the analysis in this study, we separated the cohort into the groups from the earlier report–uninfected

controls and individuals who were intubated or died of COVID-19. We then performed additional chart review to classify all other COVID-19 subjects who did not require intubation or die into groups based upon the presence or absence of organ dysfunction. Those with organ dysfunction experienced one or more of the following: 1) respiratory failure with a new or increasing oxygen requirement caused by COVID-19, 2) acute kidney injury defined by elevated creatinine requiring hospitalization, 3) cardiac dysfunction classified by a new elevation of troponin in the blood, 4) hypotension, or 5) acute delirium that the medical record documented was due to COVID-19. All Kruskal–Wallis ANOVA tests, linear regression analyses and receiver operating curve analyses were performed in Prism software, version 9 (GraphPad Software).

Results

Demographic and clinical characteristics of human subjects

We prospectively enrolled 17 subjects with severe viral pneumonia who experienced respiratory failure requiring intubation and mechanical ventilation (Table I). Our cohort included 13 individuals with severe COVID-19 and four individuals with severe influenza. We also prospectively enrolled seven individuals who presented for medical attention with moderate symptomatic acute influenza infection that did not require hospitalization (Table I). These individuals returned to the research clinic for an outpatient bronchoscopy with BAL. We further recruited two healthy individuals who had not experienced an influenza-like illness in the 60 d prior to enrollment for outpatient bronchoscopy with BAL. Two of the seven moderate influenza subjects (1920B004 and 1920B005) returned to the clinic more than 45 d after the start of their illness and more than 30 d after the initial bronchoscopy procedure for repeat bronchoscopy with BAL sampling, allowing us to analyze linked samples from these same individuals to make comparisons between their long-term recovery phase (control samples) and their acute symptomatic phase.

The mean age of the COVID-19 cohort (68, interquartile range [IQR]: 61–74) was significantly older than the mean age of the control (27, IQR: 24–29) and moderate influenza (31, IQR: 27–40) cohorts ($p < 0.005$ in both comparisons by Kruskal–Wallis ANOVA with Dunn posttest), and was also higher than the mean age of the severe influenza cohort (41, IQR: 30–49) although this difference was not significant ($p = 0.13$ by Kruskal–Wallis ANOVA with Dunn posttest). We found no difference in the ratio of female/male subjects between the four cohorts. The mean interval between the start of illness symptoms and BAL sample collection was significantly longer in the moderate influenza cohort (14.6 d, IQR: 13–18 d) when compared with the severe influenza cohort (7.5 d, IQR: 7–8 d; $p = 0.007$ by Kruskal–Wallis ANOVA with Dunn posttest); however, there were

Table I. Cohort demographics

Subject ID No.[a]	Age	Sex	Infection Status[b]	Days after Symptom Onset for Blood/BAL Collection[c]	VFD[d]	Died?[e]	CLD?[f]	CHF?[g]	IS?[h]	DM?[i]	ESRD?[j]
1819B001	28	F	Control	N/A	28	N	N	N	N	N	N
1920B002	29	M	Control	N/A	28	N	N	N	N	N	N
1819B004	40	F	Moderate influenza - IAV	D18	28	N	N	N	N	N	N
1819B007	27	M	Moderate influenza - IAV	D9	28	N	N	N	N	N	N
1920B004	29	F	Moderate influenza - IBV	D13, D48	28	N	N	N	N	N	N
1920B005	23	F	Moderate influenza - IBV	D14, D59	28	N	N	N	N	N	N
1920B007	28	F	Moderate influenza - IBV	D20	28	N	N	N	N	N	N
1920B008	31	M	Moderate influenza - IAV	D14	28	N	N	N	N	N	N
1920B013	41	M	Moderate influenza - IBV	D14	28	N	N	N	N	N	N
1920B011	26	F	Severe influenza - IBV	D7	18	N	Y	N	N	Y	N
1920B014	50	M	Severe influenza - IAV	D8	0	N	N	N	N	N	N
MC-68	46	M	Severe influenza - IAV	D8	0	Y	Y	N	N	Y	N
MC-77	43	F	Severe influenza - IAV	D7	8	N	N	N	N	N	N
350-174	74	M	Severe COVID-19	D10	0	N	N	N	Y[k]	Y	N
350-428	59	F	Severe COVID-19	D11, D25	0	N	Y	N	N	Y	N
350-441	79	M	Severe COVID-19	D11	0	Y	N	N	N	Y	N
350-472	67	M	Severe COVID-19	D10	0	Y	N	N	N	Y	N
C2	78	F	Severe COVID-19	D9	0	Y	N	N	Y[l]	N	N
C3	62	M	Severe COVID-19	D14	0	Y	N	N	N	Y	N
C7	61	F	Severe COVID-19	D17	0	Y	Y	N	N	Y	Y
C9	72	F	Severe COVID-19	D32	14	N	Y	N	Y[m]	N	N
C11	60	F	Severe COVID-19	D14	0	Y	N	N	Y[n]	Y	N
C12	53	M	Severe COVID-19	D11	0	N	N	N	N	N	N
C14	74	F	Severe COVID-19	D14	0	Y	N	N	N	Y	N
C15	66	F	Severe COVID-19	D6	0	N	Y	N	N	Y	N
C16	73	F	Severe COVID-19	D13	0	Y	N	N	Y[o]	Y	N

[a]Assigned study subject identification number.
[b]Subject severity classification grouping and infecting virus: Control, healthy individuals who did not exhibit an influenza-like illness for at least 60 d prior to outpatient bronchoscopy and BAL procedure; moderate influenza, IAV/IBV, healthy individuals with symptomatic influenza A (IAV) or influenza B (IBV) virus infection that did not require hospitalization prior to outpatient bronchoscopy and BAL procedure; severe influenza, IAV/IBV, individuals with severe IAV or IBV virus infection who were intubated for acute respiratory failure and BAL sampling was performed as a part of standard clinical care; severe COVID-19, individuals with severe COVID-19 who were intubated for acute respiratory failure and BAL sampling was performed as part of standard clinical care.
[c]The number of days following the onset of symptoms of viral respiratory illness before each BAL sample was collected.
[d]Ventilator-free days (VFD), 28 d minus the number of days the subject required mechanical ventilation in the intensive care unit; individuals who expired on mechanical ventilation are set at 0.
[e]Yes (Y) or No (N) that patient died during the index hospitalization.
[f]Subject had a history of chronic lung disease (CLD) of any type including asthma or chronic obstructive pulmonary disease prior to infection.
[g]Subject had a history of congestive heart failure (CHF) prior to infection.
[h]Subject had a history of immunosuppression (IS) prior to infection.
[i]Subject had a history of diabetes mellitus (DM) prior to infection.
[j]Subject had a history of end stage renal disease (ESRD) requiring dialysis prior to infection.
[k]Subject 350-174 had a history of autoimmune disease and was on prednisone.
[l]Subject C2 had a history of solid organ transplantation and was on mycophenolate and sirolimus.
[m]Subject C9 had a history of solid organ transplantation and was on tacrolimus, mycophenolate, and prednisone.
[n]Subject C11 had a history of solid organ transplantation and was on tacrolimus.
[o]Subject C16 had a history of autoimmune disease and was on prednisone and azathioprine.

no differences between the moderate influenza cohort and the COVID-19 cohort or the severe influenza cohort and the COVID-19 cohort (COVID-19 cohort mean 13.2 d, IQR: 10–14 d).

Increased CD14^{low}CD16^{+} nonclassical monocytes, activated T cells, and plasmablast B cells in the bronchoalveolar space of individuals with severe viral pneumonia

We evaluated the absolute number of major adaptive and innate immune subsets in the blood and bronchoalveolar space in our cohort. Changes in the population size of peripheral blood immune cell subsets and their activation status during acute influenza and acute COVID-19 have been extensively described by our group (23, 29) and by others (30–32); therefore, for this analysis, we focused on differences in cells found within the BAL fluid. Using quantitative flow cytometry methods (flow cytometry gating strategies are outlined in Supplemental Fig. 1), we discovered that the total immune cellularity, as represented by the number of CD45^{+} cells in BAL, was increased in the individuals with severe viral pneumonia compared with those with moderate influenza and controls (Fig. 1). The absolute number of CD14^{+} monocytic-lineage cells and CD19^{+} B cells were elevated during severe disease; however, the absolute number of total T cells and the CD4/CD8 subpopulations were not consistently different between individuals with severe viral pneumonia and those without (Fig. 1). The large increase in CD14^{+} cell counts in the lower airways of individuals with severe viral pneumonia appeared to be driven primarily by significantly elevated CD14^{high}CD16^{-} cell counts and, most prominently, CD14^{low}CD16^{+} cell counts (Fig. 2A, 2B).

Interestingly, we found that the absolute numbers of activated CD8^{+} and activated CD4^{+} T cells were significantly increased in BAL fluid during severe COVID-19 (Fig. 2C, 2D, and 2F). In addition, we noted increased populations of CD19^{+} B cell plasmablasts (Fig. 2E, 2F). We did not have cellular components from the BAL fluid available for analysis from two of four severe influenza subjects; however, increased CD14^{low}CD16^{+} cells and high absolute numbers of activated T cells and B cells were also noted in the BAL of our two included severe influenza subjects (Fig. 2B, 2F).

Bronchoalveolar concentration of multiple cytokines and chemokines is increased in severe viral pneumonia leading to respiratory failure

We next measured the concentration of 35 individual cytokines and chemokines in the blood and temporally matched samples of BAL fluid from each study subject. We found that many cytokines and chemokines were elevated in the plasma of individuals with moderate or severe influenza and severe COVID-19 when compared with the four control samples (Supplemental Fig. 2A), suggesting that, despite the longer interval between symptom start and collection, there was still ongoing systemic inflammation in some of our subjects with moderate influenza infection. Some cytokines were selectively higher in plasma when compared with BAL fluid regardless of infection status, including IL-12, RANTES, and EOTAXIN

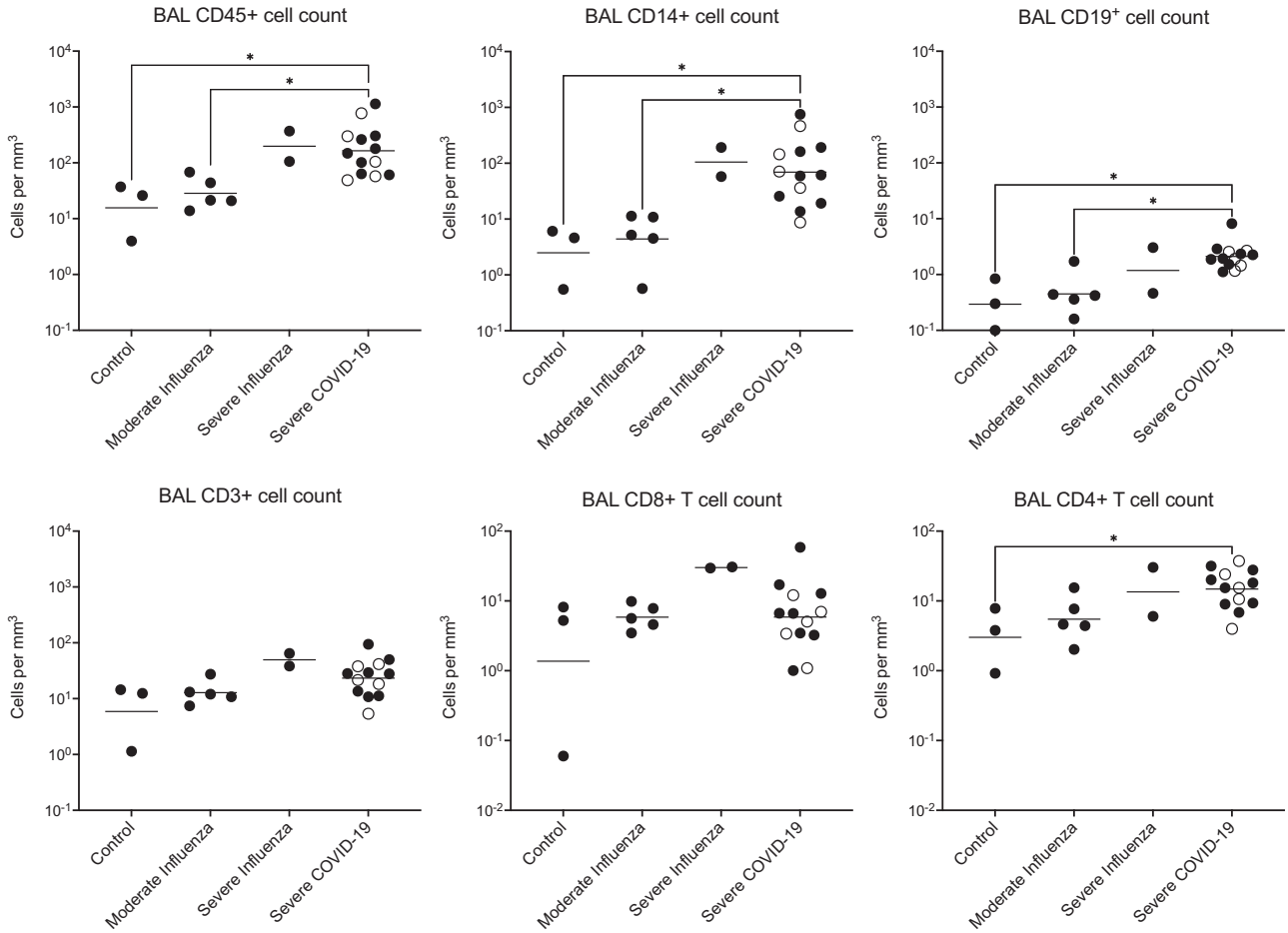

FIGURE 1. Absolute number of various immune cell subsets in BAL. The indicated cell populations were quantified by flow cytometry using counting beads. Comparisons were made using Kruskal–Wallis ANOVA with Dunn multiple comparisons posttest. Significance is indicated by $*p < 0.05$. Open symbols represent COVID-19 patients on chronic immunosuppressive therapy. There were no significant differences between the COVID-19 patients on chronic immunosuppressive therapy and those who were not in any of the measured variables.

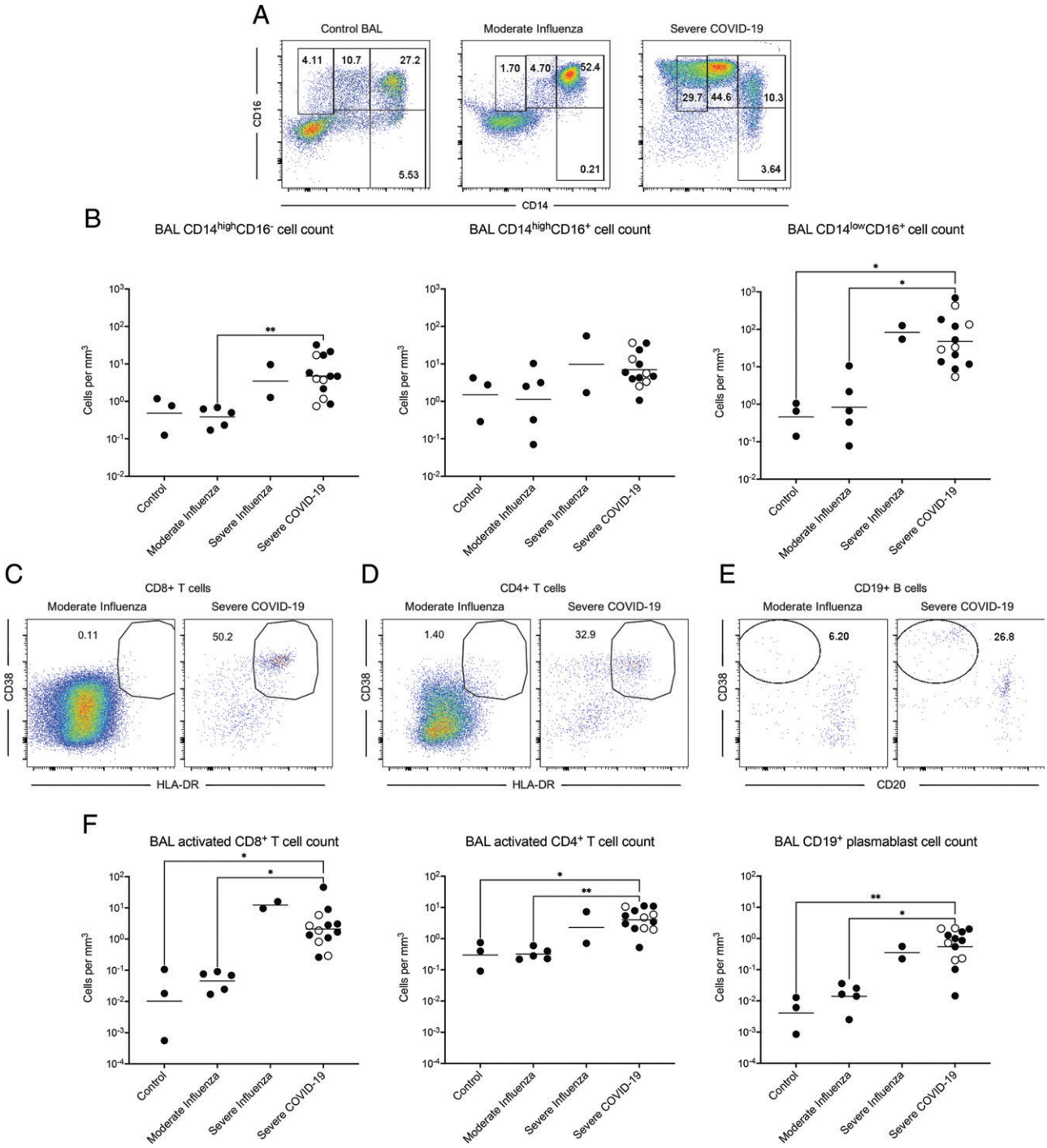

FIGURE 2. Evaluation of BAL cell populations by flow cytometry. (**A**) Representative flow cytometry plots for a control, moderate influenza, and severe COVID-19 individual illustrating the increasing $CD14^{low}CD16^{+}$ population in the severe viral pneumonia subjects. Presented dot plots were gated on single live $CD45^{+}CD19^{-}CD3^{-}CD56^{-}$ cells (see Supplemental Fig. 1 for full gating strategies). (**B**) Absolute cell numbers of the $CD14^{+}$ cell subsets illustrated in the previous panel. (**C–E**) Representative flow cytometry plots illustrating a moderate influenza subject and a severe COVID-19 subject for (C) activated $CD8^{+}$ T cells gated on single live $CD3^{+}CD8^{+}CD4^{-}$ cells, (D) activated $CD4^{+}$ T cells gated on single live $CD3^{+}CD8^{-}CD4^{+}$ cells and (E) plasmablast B cells gated on single live $CD45^{+}CD19^{+}$ cells. (**F**) Absolute cell numbers of activated $CD8^{+}$ T cells, activated $CD4^{+}$ T cells, and plasmablast B cells in the BAL of the indicated groups. Numbers on all flow cytometry dot plots indicate the percentage of positive events found within the indicated gate. Activated T cell gates were initially drawn on matched PBMC populations where more events were available for establishing the gates. Significance is indicated by $*p < 0.05$, $**p < 0.01$ with all testing performed using Kruskal–Wallis ANOVA with Dunn multiple comparisons posttest. Open symbols represent COVID-19 patients on chronic immunosuppressive therapy. There were no significant differences between the COVID-19 patients on chronic immunosuppressive therapy and those who were not in any of the measured variables.

(Supplemental Fig. 2A). Notably, lower relative concentrations of nearly all cytokines were found in the BAL fluid of control and moderate influenza subjects (Fig. 3A and Supplemental Fig. 2A). These subjects clustered together into one group when analyzing all of the BAL samples with Euclidean hierarchical clustering (Fig. 3A). We also found a large and diverse number of cytokines expressed in the BAL of all severe influenza and COVID-19 subjects, which clustered into three distinct groups (Fig. 3A). A group

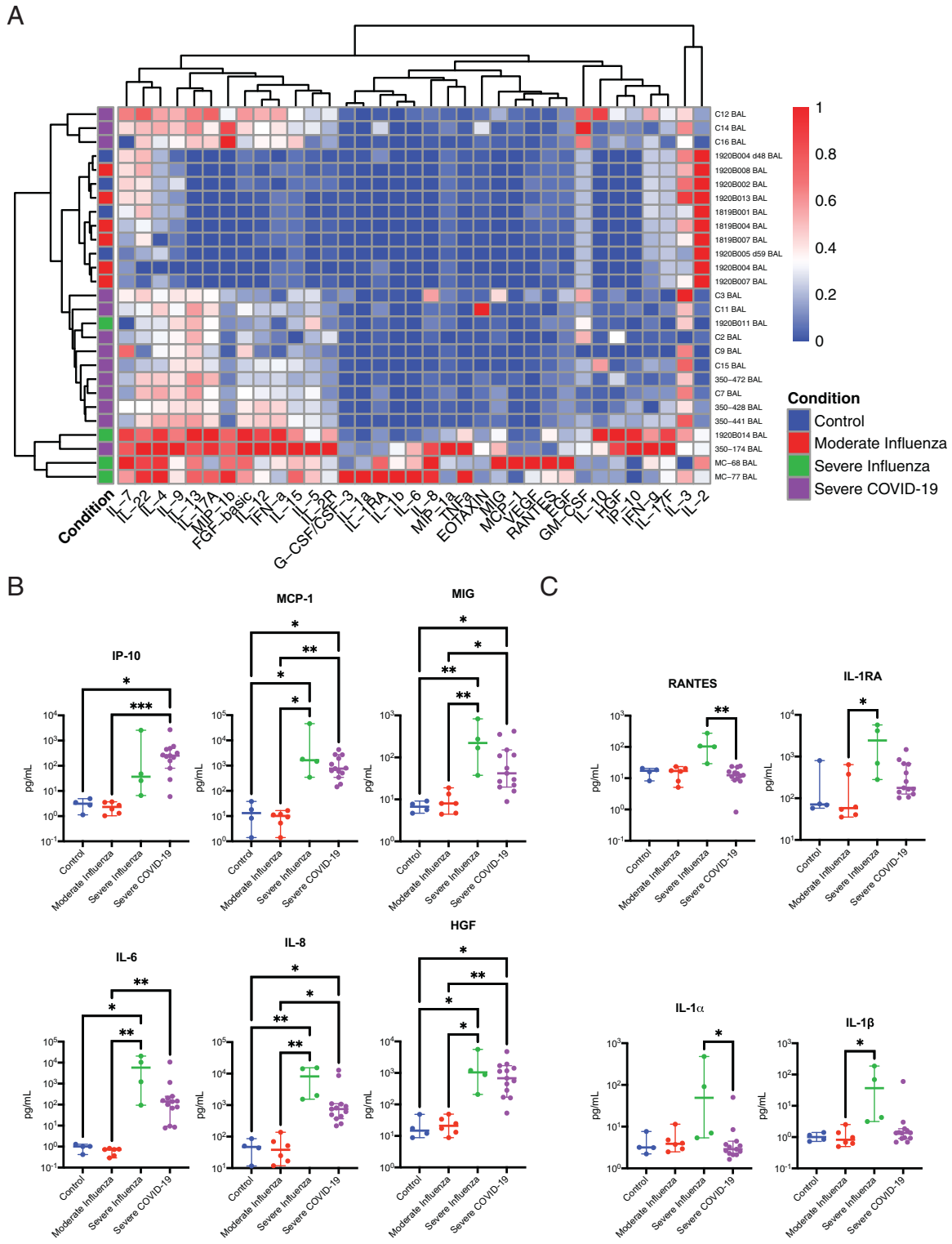

FIGURE 3. Elevated BAL cytokines in patients with severe viral pneumonia. (**A**) Euclidean hierarchical clustering analysis of all BAL cytokine measurements. The concentration of each cytokine column was normalized by setting the highest value to 1 and the lowest value to 0. (**B**) Representative BAL cytokine analysis graphs for 6 of 35 measured cytokines with the highest magnitude increases during severe disease. (**C**) Selective elevation of IL-1 family cytokines and RANTES in severe influenza but not in severe COVID-19. In (B) and (C) cytokine concentrations are measured in picograms per milliliter and plotted on a logarithmic scale. Individual subject values are plotted (each point) along with the group median value (line) and the 95% confidence interval. Significance is indicated by $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$, with all testing performed using Kruskal–Wallis ANOVA with Dunn multiple comparisons posttest.

of four individuals had some of the highest relative expression levels of multiple cytokines among the BAL samples and clustered together: three individuals with severe influenza and one with severe COVID-19 (Fig. 3A). The five subjects with severe COVID-19 on chronic immunosuppressive therapy equally segregated into the three distinct severe illness clusters composing one-third of the first cluster, three-tenths of the second cluster, and a quarter of the third cluster; therefore based upon the expression level of these 35 cytokines, the

immunosuppressed COVID-19 subjects did not exhibit a different pattern of cytokine expression during severe illness when compared with the other severe viral respiratory failure subjects.

When comparing the expression level of individual cytokines in the BAL between the four cohorts of subjects, we found that 28 of the 35 cytokines and chemokines were significantly elevated in the severe influenza cohort, the severe COVID-19 cohort or both (Fig. 3B, 3C and Supplemental Fig. 2). Many of the highest magnitude increases we noted between the control or moderate influenza cohorts and the two severe viral pneumonia cohorts included cytokines and chemokines typically expressed by monocytic-lineage cells that enhance proinflammatory Th1-type immune responses, including IL-6, IL-8, MCP-1, MIG, IP-10, IL-12, and MIP-1β (Fig. 3B and Supplemental Fig. 2). IL-1 signaling was noted to be significantly increased in BAL samples from the severe influenza cohort, but not the severe COVID-19 cohort (Fig. 3C). Also, the chemokine RANTES appeared to be selectively increased in BAL from individuals with severe influenza but not in individuals with severe COVID-19 (Fig. 3C), suggesting small but present differences in the lower airway inflammatory environment during severe infection with these two viruses.

The measured plasma concentration of IP-10 correlates with the measured BAL concentration

Given the difficulty in sampling and measuring lower airway inflammation in human patients during acute viral infection, we sought to use our dataset to determine if individual circulating blood cytokine concentrations correlate with hyperinflammatory bronchoalveolar cytokine expression. To do this, we performed linear regression of the plasma versus the BAL concentration of each measured cytokine. We performed our initial analysis in a combined cohort that included the 13 subjects with severe COVID-19 and the four subjects with severe influenza (Fig. 4A and Supplemental Fig. 3). IP-10, also known as CXCL10, was the only cytokine or chemokine of the 35 measured which significantly correlated between the blood and BAL in this combined cohort of individuals with severe viral pneumonia (Fig. 4A and Supplemental Fig. 3). Furthermore, the strength of the correlation between the blood and bronchoalveolar concentration of IP-10 as measured by the R^2 value was quite robust. Interestingly, many inflammatory cytokines commonly described as peripheral blood markers of severe influenza and COVID-19, including IL-6, IL-8, MCP-1, IL-1RA, G-CSF, IL-1β, and IL-10 (3, 23, 33), did not correlate between blood and BAL (Fig. 4A and Supplemental Fig. 3) despite the significantly increased concentration of many of these molecules in the BAL fluid of individuals with severe viral pneumonia (Fig. 3 and Supplemental Fig. 2).

To evaluate if a disease-specific rather than a general hyperinflammatory viral pneumonia phenotype drives the IP-10 correlation between blood and BAL that we observe, we evaluated the correlation separately in the severe COVID-19 cohort and in the combined moderate and severe influenza cohort. The IP-10 correlation remained significant in each individual disease (Fig. 4B).

Circulating plasma IP-10 concentration predicts organ dysfunction, intubation, or death in an independent cohort of patients with COVID-19

We next wanted to determine if the correlation we report between elevated plasma concentrations of IP-10 and elevated BAL concentrations could signal that plasma IP-10 might predict organ dysfunction in human patients, including respiratory failure, intubation or death. To accomplish this, we reanalyzed our previously published dataset of measured plasma cytokine and chemokine concentrations obtained within 48 h of hospital admission in a cohort of 168 prospectively sampled subjects with COVID-19 (23). We reasoned that if circulating IP-10 were able to serve as a marker of a hyperinflammatory bronchoalveolar environment during respiratory failure in COVID-19 as we observe in our small cohort in the current study, then increasing circulating concentrations in the blood might predict hyperinflammatory respiratory failure in a larger cohort of patients. Indeed, we found that elevated IP-10 was significantly associated with COVID-19 severity and elevated levels predicted organ dysfunction, intubation, or death (Fig. 4C). Furthermore, circulating plasma IP-10 concentrations alone could serve as a diagnostic test to predict organ dysfunction, intubation, or death in the cohort of 168 COVID-19 patients with an area under the receiver operating curve of 0.747 (Fig. 4D). Using an IP-10 concentration cutoff of 418 pg/ml such a test would have 20% sensitivity and 98% specificity for organ dysfunction, intubation, or death. A plasma IP-10 concentration cutoff of 89 pg/ml would provide 75% sensitivity and 73% specificity for organ dysfunction, intubation, or death. Our previously reported dataset (23) included 26 influenza-infected subjects. We detected elevated plasma IP-10 in the influenza patients compared with uninfected individuals (data not shown) as others have reported (14). However, this dataset was not large enough to evaluate IP-10 associations with respiratory failure in influenza patients.

Discussion

In this study, we comprehensively characterize the lower airway immune response in four cohorts of human subjects: 1) subjects without acute infection, 2) influenza-infected subjects who are symptomatic but have no evidence of respiratory failure, 3) influenza-infected subjects with acute respiratory failure, and 4) COVID-19 subjects with respiratory failure. We observe increased absolute numbers of activated T cells, B cells, and inflammatory-type monocyte-lineage cells in the bronchoalveolar space of subjects with respiratory failure. Furthermore, we find high magnitude elevations of the cytokines and chemokines IL-6, IL-8, MCP-1, MIG, IP-10, IL-12, and MIP-1β in the lower airways of the individuals with severe disease and respiratory failure. These cytokines are known to drive M1 monocyte, activated Th1, and cytotoxic T cell recruitment. Our findings are in line with a recent report in 23 subjects with severe COVID-19 that demonstrated an increased frequency of inflammatory transitional and nonclassical monocytes in bronchial washings of individuals with respiratory failure but did not quantify the absolute number of this subset nor compare it to individuals without acute infection (17). The observations in our COVID-19 cohort also comport with another recent study demonstrating broad expansion of monocyte-derived alveolar macrophages in severe COVID-19 and associated increased recruitment of activated T cells to the bronchoalveolar space (34). Indeed, our limited findings in two individuals with severe influenza suggest that the model proposed by Grant and colleagues for COVID-19 (34), which our present observations support, may also apply to severe influenza pneumonia that leads to hyperinflammatory respiratory failure. In fact, previously published literature reported increased frequencies of activated T cells in BAL fluid from two individuals with severe influenza (14) further supporting this position. Finally, we did not observe any of these cytokine elevations or cellular immune changes in our moderate influenza cohort arguing that the inflammatory cytokine and cellular changes we observe are unique to dysregulated disease, which leads to respiratory failure. However, this finding may be limited by the prolonged interval between illness onset and sample acquisition in our moderate influenza cohort when compared with our severe influenza cohort, despite continued elevated peripheral blood cytokine levels in a subset of our moderate influenza patients during BAL sampling.

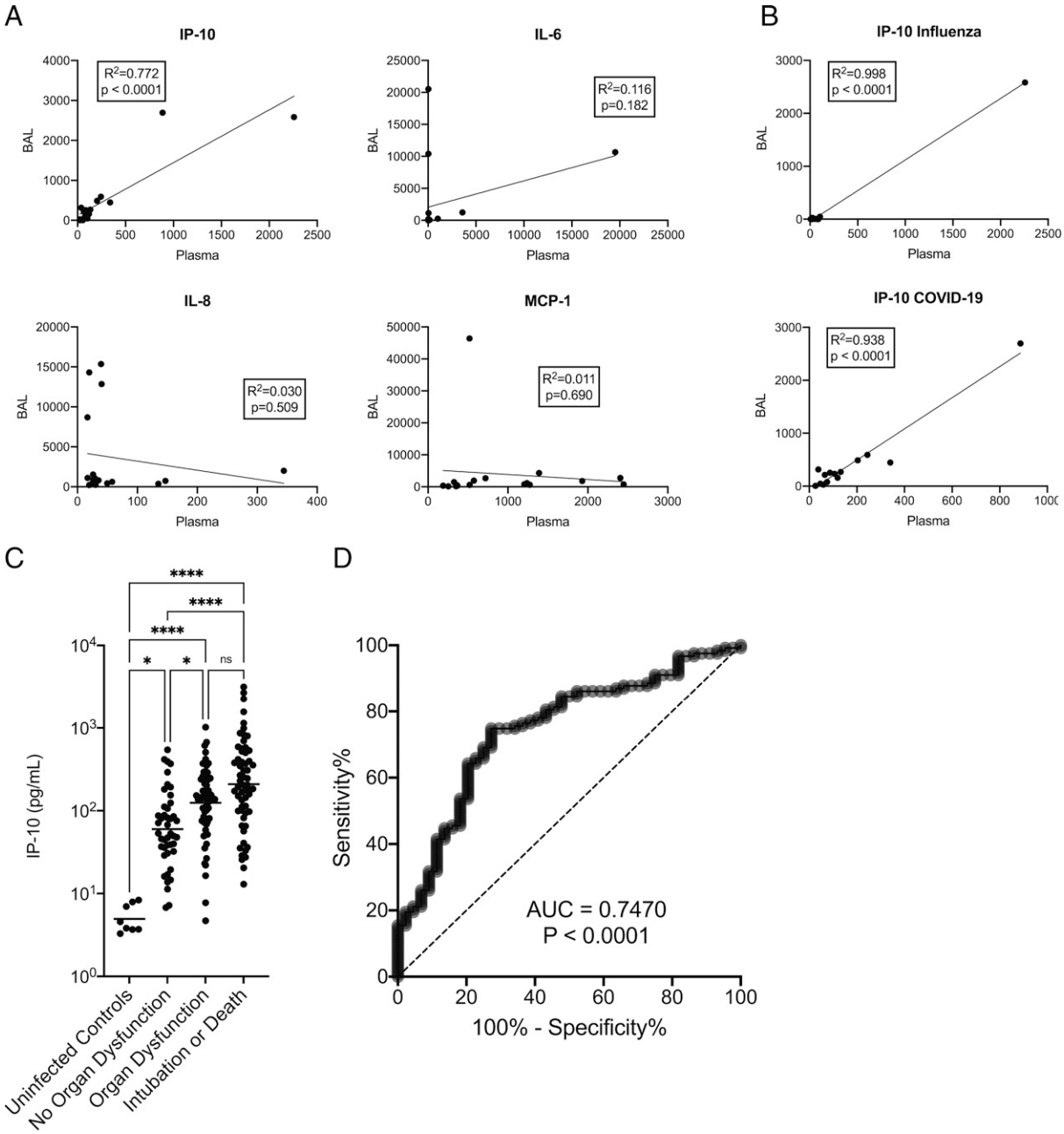

FIGURE 4. Plasma IP-10 concentrations serve as a marker of lower airway inflammation and predict severe disease outcomes in subjects with COVID-19. (**A**) Representative linear regression analysis plots for 4 of the 35 cytokines comparing blood plasma on the *x*-axis and BAL on the *y*-axis. All cytokine concentrations are in picograms per milliliter. This analysis was performed using the first matched blood and BAL samples from each of the individuals with severe COVID-19 and severe influenza ($n = 17$). The p value threshold for significance was corrected to <0.0015 using the Bonferroni method. (**B**) Linear regression analysis of IP-10 in the combined moderate influenza and severe influenza cohort (top panel, $n = 10$) and the severe COVID-19 cohort [bottom panel, $n = 14$, includes the 350–425 day-28 sample that was excluded in panel (A)]. (**C**) Blood plasma IP-10 concentrations in 168 individuals with PCR-diagnosed SARS-CoV-2 infection evaluated in hospital and eight control uninfected individuals. This data set has been previously published (23), but this is a new analysis of the earlier data. Groups were compared using Kruskal–Wallis ANOVA with Dunn multiple comparisons posttest. Significance is indicated by $*p < 0.05$, $****p < 0.0001$. (**D**) Receiver operating characteristic curve evaluating the ability of various concentrations of plasma IP-10 (in picograms per milliliter) to predict the composite outcome of organ dysfunction, intubation, or death in the cohort of 168 patients with COVID-19. AUC, area under the curve. ns, not significant.

We also demonstrate an association between increased circulating peripheral blood IP-10 concentration and bronchoavleolar inflammation. Furthermore, we show that blood IP-10 concentration can predict organ failure, intubation, and death in a separate large COVID-19 patient cohort. Previous studies have reported associations between increasing plasma or serum IP-10 concentration and poor outcomes in patients with COVID-19 (19, 35). Increasing blood IP-10 concentrations have also been associated with increased illness severity in seasonal (36) and avian (20, 37) influenza infection. To the best of our knowledge, our work is the first to link these IP-10 elevations in circulating blood to elevated levels found within the bronchoalveolar space of human patients with severe seasonal influenza and COVID-19. Our findings suggest that measurement of peripheral blood IP-10 in individuals with severe influenza or COVID-19 may identify those with hyperinflammatory respiratory failure that may respond to immunosuppressive therapies such as corticosteroids and perhaps other targeted therapies.

Several peripheral blood cytokines have been put forward as markers of severe influenza and COVID-19 including IL-6, IL-8, MCP-1, IL-1RA, G-CSF, IL-1β, and IL-10 (3, 23, 33). The circulating concentrations of all of these cytokines correlate with poor disease outcomes in larger cohorts of patients. Interestingly, none of these cytokines correlated between the blood and BAL in our present study, illustrating the relative weakness of these cytokines as peripheral blood biomarkers of hyperinflammatory respiratory failure when compared with the measurement of IP-10 in COVID-19. We believe that this point is best illustrated by the IL-6 cytokine. IL-6 was significantly elevated in the BAL of all subjects with severe viral pneumonia in our study by more than a 10-fold increase above values found in the BAL of controls or moderate influenza subjects. However, plasma IL-6 did not consistently serve as a marker for this bronchoalveolar elevation: two of the three individuals with the highest BAL concentration of IL-6 and values greater than 10,000 pg/ml in the BAL had plasma values of 38 and 39 pg/ml. These two values were in the lower half of the plasma measurements in all 17 of the severe viral pneumonia subjects in this study and fall within the range of healthy-uninfected individuals in other cohorts (23). Therefore, despite the importance of profound elevations of IL-6 in the BAL of individuals with severe viral pneumonia and the strong association between circulating plasma or serum levels of this cytokine and disease severity, there are still individuals with severe hyperinflammatory viral pneumonia who do not have remarkably elevated blood levels of IL-6.

Recent in vitro work has shown that human nasal epithelial cells selectively produce IP-10 on the basolateral surface following SARS-CoV-2 infection or seasonal influenza infection (38). Therefore, the IP-10 increases we observe may be due to the recruitment of inflammatory immune cells that express this chemokine in the lung or to the direct expression of IP-10 by respiratory epithelial cells following infection. If further work demonstrates that the IP-10 increases are related to direct viral infection of respiratory epithelial cells in vivo, it would suggest a mechanism whereby blocking IP-10 (a chemoattractant for pathogenic monocytes and activated T cells) may inhibit a key triggering event in the development of hyperinflammatory respiratory failure. Indeed, a mouse study found that genetically eliminating IP-10 or blocking IP-10 activity with a mAb during H1N1 influenza infection reduced lung inflammation, animal weight loss and mortality in two models of severe influenza (36). Targeted therapies blocking IP-10 chemokine activity in human patients with hyperinflammatory respiratory failure as classified by elevated peripheral blood concentrations of IP-10 may hold promise as a therapy for individuals with severe influenza and COVID-19.

Our study does have limitations. Older age has been associated with increased inflammatory cytokine responses and diminished development of adaptive immune responses (39, 40). However, recruitment for our study was limited to the collection of samples from individuals with severe illness, and the known association of severe COVID-19 with increasing age (4) limited our ability to collect samples from comparable cohorts of younger subjects. This reflects the intrinsic nature of the populations affected by severe influenza and severe COVID-19 rather than sampling bias. Furthermore, the quicker deterioration of the clinical condition in severe influenza patients when compared with COVID-19 patients has been noted in larger cohorts (23), reflecting the differences, once again, between the time-course and pathophysiology of these two viral diseases. Our significantly longer interval between illness onset and sampling in the moderate influenza and severe influenza cohorts reflects the rapid onset of disease in individuals with severe influenza and may limit direct comparisons between our severe and moderate influenza cohorts. Finally, the association we describe between circulating IP-10 and severe COVID-19, while also observed by other scientific groups in various human cohorts, will require formal validation in a large, multicenter prospective study prior to being applied in the clinical setting.

In conclusion, we find increased absolute numbers of inflammatory-type nonclassical monocytes and activated T and B cells in the bronchoalveolar space of individuals with severe viral pneumonia that are not observed in individuals with acute influenza who do not experience respiratory failure. These cellular changes are associated with significant increases in many different cytokines and chemokines in the bronchoalveolar space, most notably those expressed by epithelial cells and innate immune cells, which are known to drive Th1-type immune responses. In addition, we demonstrate that plasma IP-10 serves as a marker of severe hyperinflammatory respiratory failure. Measurement of circulating IP-10 predicts respiratory failure in COVID-19 and may allow more precise targeting of anti-inflammatory therapies to those who will benefit the most.

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Disclosures

P.A.M. is listed as inventor on a patent application based on the findings of this study. The other authors have no financial conflicts of interest.

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