Integrated plasma proteomic and single-cell immune signaling network signatures demarcate mild, moderate, and severe COVID-19

Graphical abstract

Highlights

- Proteomic analysis of plasma analytes and immune cells in blood during COVID-19
- Multi-variate stacked generalization LASSO model classifies COVID-19 severity
- Dampening of intracellular signaling responses with increasing COVID-19 severity
- Enrichment of plasma proteins with increasing severity

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In brief

Feyaerts et al. integrate plasma and single-cell proteomic analysis of patients with mild, moderate, and severe COVID-19 to reveal biological signatures that differentiate COVID-19 severity, including dysregulation of pro-inflammatory signaling networks.
Integrated plasma proteomic and single-cell immune signaling network signatures demarcate mild, moderate, and severe COVID-19

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SUMMARY

The biological determinants underlying the range of coronavirus 2019 (COVID-19) clinical manifestations are not fully understood. Here, over 1,400 plasma proteins and 2,600 single-cell immune features comprising cell phenotype, endogenous signaling activity, and signaling responses to inflammatory ligands are cross-sectionally assessed in peripheral blood from 97 patients with mild, moderate, and severe COVID-19 and 40 uninfected patients. Using an integrated computational approach to analyze the combined plasma and single-cell proteomic data, we identify and independently validate a multi-variate model classifying COVID-19 severity (multi-class area under the curve \( \text{AUC} \) \(_{\text{training}} = 0.799, p = 4.2e-6; \) multi-class \( \text{AUC} \) \(_{\text{validation}} = 0.773, p = 7.7e-6). Examination of informative model features reveals biological signatures of COVID-19 severity, including the dysregulation of JAK/STAT, MAPK/mTOR, and nuclear factor \( \kappa \)B (NF-\( \kappa \)B) immune signaling networks in addition to recapitulating known hallmarks of COVID-19. These results provide a set of early determinants of COVID-19 severity that may point to therapeutic targets for prevention and/or treatment of COVID-19 progression.

INTRODUCTION

The ongoing coronavirus disease 2019 (COVID-19) pandemic, caused by the novel and highly contagious severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2),1 has affected more than 240 million patients worldwide as of late 2021.2 A wide range of clinical manifestations exists for COVID-19, which require different intervention strategies. While the majority of
patients with COVID-19 experience mild or asymptomatic infections, nearly 20% of patients develop severe disease requiring hospitalization. A substantial portion (8%–30%) of those hospitalized patients ultimately succumbs to the disease, leading to a devastating global tally of COVID-19 fatalities.

Varying outcomes for COVID-19 depend on a set of risk factors and the interplay between viral replication and tissue damage, as well as a balance of beneficial and detrimental host immune responses. Several studies have provided evidence for profoundly altered immune responses caused by SARS-CoV-2 infection, including sustained functional changes in circulating immune cells. Lymphopenia, increased inflammatory plasma cytokine levels, dysregulated innate immune cell function, and abnormal T cell activation and exhaustion have been observed, particularly in hospitalized patients with severe COVID-19. However, prior studies have primarily focused on patients with severe COVID-19, while fewer studies have included non-hospitalized patients with mild and moderate COVID-19. In addition, while prior studies have reported on the distribution, phenotype, and transcriptional profile of peripheral immune cells, how SARS-CoV-2 infection alters immune cell signaling responses to inflammatory challenges (or immune signaling networks) has not been determined. As such, the immunological mechanisms that differentiate patients with mild, moderate, and severe COVID-19 are poorly understood. Unraveling the underlying immune pathogenesis across the spectrum of COVID-19 presentations is important to both understand the drivers of disease severity as well as to identify clinically relevant biological signatures that could inform therapeutic interventions.

High-dimensional mass cytometry immunoassays are uniquely adapted to the analysis of immune cell signaling networks as multiple intracellular signaling events (e.g., post-translational protein modifications) are simultaneously quantified in precisely phenotyped immune cells in their endogenous state and in response to ex vivo stimulations. The approach has previously enabled the identification of clinically relevant biological signatures predictive of patient outcomes in several clinical contexts, including infection, immunization, malignancies, stroke, and traumatic injury.

In this cross-sectional study, we combined the mass cytometry analysis of immune cell signaling responses with the high-content proteomic analysis of plasma analytes in blood samples from patients identified with mild, moderate, and severe COVID-19 to establish biological signatures that demarcate COVID-19 clinical manifestations. The integrated single-cell and plasma proteomic analysis allowed including an additional dimension in the characterization of immune signaling networks by accounting for the plasma environment of circulating immune cells.

RESULTS

Combined plasma and single-cell proteomic analysis of peripheral blood samples from patients with mild, moderate, and severe COVID-19

Ninety-seven SARS-CoV-2-positive patients with mild, moderate, or severe COVID-19 were enrolled in this cross-sectional study at Stanford University Medical Center (CA, USA) (Figure 1A). Patient characteristics can be found in Table 1. COVID-19 severity was determined using previously defined clinical National Institute of Health (NIH) criteria and assigned at the time of a SARS-CoV-2 qRT-PCR test (see STAR Methods). In brief, SARS-CoV-2-positive patients categorized as mild had zero or mild COVID-19 symptoms without any breathing issues. Moderate patients had signs of lower respiratory tract disease with an oxygen saturation above 94%. All severe patients were hospitalized due to respiratory distress. Importantly, none of the patients had received COVID-19 treatment at time of sample collection. Samples from SARS-CoV-2-positive patients were examined alongside those from 40 healthy controls collected at Stanford in 2019, before the detection of SARS-CoV-2 in the geographic region.

Blood samples were used to isolate both plasma and peripheral blood mononuclear cells (PBMCs). PBMCs were stimulated ex vivo to trigger pathogen sensing and cytokine signaling response pathways in innate and adaptive immune cells relevant during infection (TLR4 stimulant lipopolysaccharide [LPS] and TLR7/8 agonist CL097, interferon alpha [IFNα], interleukin-2 [IL-2], IL-4, and IL-6 cytokine cocktail, and cell stimulation cocktail consisting of phorbol 12-myristate 13-acetate [PMA] and...
The frequencies of 44 manually gated immune cell subsets representing major circulating innate and adaptive immune cells were determined using a 42-parameter single-cell phospho-mass cytometry immunoassay (Figure S1). For each immune cell subset, the frequency was determined, alongside endogenous signaling activity (unstimulated condition) and signaling response capacity of cells to ex vivo stimulation with inflammatory reagents, which were measured by examining the phosphorylation state of 15 intracellular signaling proteins and 5 markers that relate to immune-subset-specific functionality (see STAR Methods and Figure S2).

After penalization, the mass cytometry analysis generated a total of 2,662 immune cell response features per PBMC sample. To complement this single-cell analysis, 1,472 circulating plasma proteins were measured using the proximity extension assay (PEA) platform from Olink Proteomics. Five mass cytometry (cell frequency, endogenous signaling, IFNα + IL-2 + IL-4 + IL-6, LPS + ionomycin [PI], or left unstimulated [Unstim]) for endogenous signaling before barcoding, antibody staining, and analysis by single-cell mass cytometry.

Correlation networks demonstrate the existence of strong inter- and intra-layer correlations between features (Figures 1C and 1D).

To understand the relationships between the different data layers and COVID-19 severity, we first applied unsupervised...
Dimensionality reduction with multi-omics factor analysis (MOFA), which infers a set of factors that capture shared sources of variability across different datasets. We supplied all six data layers for the analysis, resulting in a MOFA model with 17 factors (Figure S3A, model trial 6). Both single-cell immune response (endogenous signaling and signaling response to IFNα/IL-2/IL-4/IL-6 and LPS/CL097; factor 10) and plasma proteome (factor 3) data contributed strongly to the variance observed in our samples (Figure S3B) and were significantly associated with COVID-19 severity (Figure S3C). A gradient with increasing disease severity across factors 3 and 10 (Figure 1E) was also observed, suggesting that single-cell immune response and plasma proteome data both contain clinically important biological events. The results prompted us to perform an integrated analysis to determine whether a classifier of COVID-19 severity could be derived from the combined plasma and single-cell proteomics data.

Table 1. Patient characteristics

|                      | Control (N = 40) | Mild (N = 50; 2 asymptomatic) | Moderate (N = 21) | Severe (N = 26) |
|----------------------|-----------------|--------------------------------|-------------------|----------------|
| Age                  | 48 (23–73)      | 41.5 (23–78)                   | 45 (19–78)        | 52.5 (29–78)   |
| Gender               |                 |                                |                   |                |
| M                    | 16              | 40                             | 23                 | 8              |
| F                    | 24              | 60                             | 27                 | 13             |
| Race                 |                 |                                |                   |                |
| Asian                | 11              | 27.5                           | 12                 | 2              |
| Hispanic/Latino      | 0               | 0                              | 5                  | 10             |
| White                | 15              | 37.5                           | 23                 | 46             |
| Black                | 1               | 2.5                            | 3                  | 6              |
| Multi-racial         | 3               | 7.5                            | 0                  | 0              |
| Middle Eastern       | 1               | 2.5                            | 0                  | 0              |
| Not reported         | 9               | 22.5                           | 7                  | 14             |
| HispanicRace/Ethnicity |            |                                |                   |                |
| Yes                  | 0               | 0                              | 9                  | 18             |
| No                   | 31              | 77.5                           | 34                 | 68             |
| Not reported         | 9               | 22.5                           | 7                  | 14             |
| Comorbidities        |                 |                                |                   |                |
| Diabetes             |                 |                                |                   |                |
| Yes                  | 0               | 0                              | 4                  | 8              |
| Prediabetic          | 1               | 2.5                            | 3                  | 6              |
| No                   | 32              | 80                             | 34                 | 68             |
| Not reported         | 7               | 17.5                           | 9                  | 18             |
| Asthma               |                 |                                |                   |                |
| Yes                  | 4               | 10                             | 7                  | 14             |
| No                   | 29              | 72.5                           | 33                 | 66             |
| Not reported         | 7               | 17.5                           | 10                 | 20             |
| CV Condition         |                 |                                |                   |                |
| Yes                  | 5               | 12.5                           | 2                  | 4              |
| No                   | 28              | 70                             | 48                 | 96             |
| Not reported         | 7               | 17.5                           | 0                  | 0              |
| Obesity              |                 |                                |                   |                |
| Yes                  | 0               | 0                              | 3                  | 6              |
| No                   | 33              | 82.5                           | 32                 | 64             |
| Not reported         | 7               | 17.5                           | 15                 | 30             |
| Outcome, death       | 0               | 0                              | 0                  | 0              |
| Days between self-reported symptom onset and sample collection | 25.5 (-1–69) | 22 (-1–66) | 5 (0–75) |

Data are shown as a number and a percentage. Age is reported in median years (minimum to maximum), and the days between onset of self-reported COVID-19 symptoms and sample collection is reported in median days (minimum to maximum). The category multiracial includes the variables Multi-racial, Asian/Native American, White/Asian/Pacific Islander, and/or Mexican/Native American. The category White includes White, White/Hispanic, and White/non-Hispanic. The category Black includes both Black and Black/Hispanic/Latino. The category Hispanic/Latino includes the variables Hispanic, Hispanic/Latino, and Mexican/Hispanic. Ethnicity makes the distinction between those who reported as Hispanic and those who did not.
Integrated modeling of plasma and single-cell proteomic events differentiates COVID-19 severity

A high-dimensional computational analysis pipeline was applied to train and independently validate (training cohort: n = 74; 25 control, 20 mild, 10 moderate, and 19 severe patients; validation cohort: n = 63; 15 control, 30 mild, 11 moderate, and 7 severe patients) an integrated model of COVID-19 severity based on the combined proteomic and single-cell immune response data.35

In this approach, the six data layers were considered separately, and a two-step process was used to combine these data layers in a multi-omic fashion (Figure 2A). Cross-validated multi-variate regression models36 were first trained for each individual data layer of the training cohort, with disease severity used as a ranked order variable (i.e., control classed as 1 to severe classed as 4), and second, the individual LASSO models were integrated into a single model by stacked generalization (SG)35 (Figure 2A). The second step uses the estimations of disease severity of each LASSO model as predictors for a constrained regression model.

The analysis identified an SG model (“severity model”) that classified COVID-19-severity categories at time of sampling for patients in the training cohort (r = 0.61, p = 4.2e-6, n = 74) and validation cohort (r = 0.69, p = 7.7e-6, n = 73) (Figures 2B and 2C). The generalizability of the severity model was independently tested in patients from the validation cohort (r = 0.69, p = 7.7e-6, n = 63) (Figures 2D and 2E). The contribution of individual data layers to the overall severity model was highest for the plasma proteome and lowest for the signaling responses to LPS/CL097 stimulation, according to severity model coefficients (Table S1).

To estimate the performance of the severity model, a multi-class area under the curve receiver operating characteristic (ROC) analysis was performed for the training and validation cohort (Figures 2A). The multi-class ROC analysis showed that the severity model performed well at classifying patients across disease-severity categories (multi-class area under the curve [AUC] training = 0.799; multi-class AUC validation = 0.773).37,38 The most accurate classification regression models were first trained for each individual data layer before integration of all six data layers (proteome, frequency, endogenous signaling, LPS/CL097 signaling response, IFNα/IL-2/IL-4/IL-6 signaling response, and PI signaling response) using a stacked generalization (SG) method. (B and C) Outcome of predicted versus true disease severity derived from SG model for the (B) training (r = 0.61, p = 4.2e-6, n = 74) and (C) validation cohort (r = 0.69, p = 7.7e-6, n = 73).

(D and E) Multi-class area under the curve receiver operating characteristic (ROC) analysis of the training (D; AUC = 0.799, n = 74) and validation (E; AUC = 0.773, n = 63) severity model (see Table S2 for individual AUCs). 1 = control, 2 = mild, 3 = moderate, and 4 = severe.

For boxplots, the center line represents the median value; upper and lower box limits indicate first (Q1) and third (Q3) quartile, respectively; whiskers, minimum (Q1−1.5*IQR) and maximum (Q3+1.5*IQR). IQR, interquartile range. AUC, area under the curve. See also Figure S6 and Tables S1–S4.
Figure 3. An iterative bootstrapping method identifies robust informative features for the differentiation of mild, moderate, and severe COVID-19

(A) Workflow of the iterative bootstrap method used to identify informative features in the six data layers of the severity model. LASSO regression model was run 1,000 times on random sub-samples with replacement for each data layer, \( X_i \), then the number of times an individual feature was selected in one of the bootstrap iterations was counted, and the features were ranked according to the frequency of selection in the bootstrap models.

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was achieved when classifying severe patients from the other patient groups (Table S2), with a model performance similar to the classifier of severe disease described by Filbin et al.\textsuperscript{39} In addition, different regression strategies were tested showing similar results, with the best overall performance obtained using LASSO regression (Table S3). Among patients for whom infection-related clinical laboratory parameters were available, model values for signatures of COVID-19 severity significantly correlated with both CRP and D dimer (Spearman correlation: CRP, \(r = 0.803, p = 0.0002\); D dimer, \(r = 0.695, p = 0.0051\)).

Results from the severity model indicate that mild, moderate, and severe COVID-19 manifestations can be differentiated from the measurement of plasma proteins and single-cell immune signaling events in patient’s peripheral blood. Several clinical and (socio)demographic variables such as old age, male gender, high BMI, and Hispanic ethnicity have previously been shown to be risk factors for COVID-19 severity.\textsuperscript{12,39–44} In addition, recent studies have highlighted the dynamic changes of innate and adaptive immune responses over the course of COVID-19 disease,\textsuperscript{5,18} such that the timing of sample collection (Figure S4) may affect the detection of immune features related to disease severity. We observed significant correlations between the covariates of obesity, Hispanic ethnicity, and days since symptom onset with COVID-19 severity (Figure S5). To account for inter-patient variability in these key clinical, (socio)demographic, and experimental variables, we performed a confounder analysis. The results showed that the SG model remained significantly predictive of COVID-19 severity when accounting for the variables age, gender, obesity, Hispanic ethnicity, and time between reported symptom onset and sample collection (Table S4).

Our SG severity model was built and independently tested in a cohort of patients recruited at a single center. A recent proteomic study\textsuperscript{45,46} of patients enrolled at the Mass General Hospital (MGH, Boston, MA, USA) that used an identical proteomic assay (the Olink Explore 1536) provided an opportunity to examine, at least partially, the generalizability of our findings to a broader patient population. A severity model built on the plasma proteomic data from the Stanford cohort accurately predicted the disease severity of patients included in the MGH cohort (Figure S6; \(r = -0.453541, p < 2e-16\)), providing independent validation of the plasma proteomic component of our predictive model in a second patient cohort. Notably, a significant correlation was found between the two studies for the set of 195 proteomic features associated with COVID-19 severity described in Filbin et al.\textsuperscript{39} (\(r = 0.40, p < 0.0001\), Pearson, -log-adjusted p value; Data S1B).

**Biological signatures of COVID-19 severity**

To facilitate biological interpretation of the high-dimensional severity model, the contribution of individual plasma and single-cell proteomic features to the severity model performance was quantified by measuring repeated selection during a 1,000-iteration bootstrap procedure\textsuperscript{45,46} (Figure 3). This bootstrap procedure recreates the dataset 1,000 times by sampling from the original dataset with replacement, and a new cross-validated LASSO model is trained for each iteration.\textsuperscript{45,46} The relative importance of each feature to the model is based on frequency of selection for a given feature (Data S1). We examined in detail the top 10% of total features ranked by the bootstrap procedure and that thus contributed strongly to the overall model (Figures 3B and 3C; Data S1).

Single-cell (Figure 3B) and plasma (Figure 3C) proteomic features were visualized with two correlation networks, highlighting the correlation between individual features (edges) and between individual features and COVID-19 severity (node size/color). Features within top 10% of bootstrap selection segregated into correlated communities, which were annotated according to the cellular attribute most commonly represented within each community (immune cell frequency or signaling response for single-cell features, Figure 3B; protein name for plasma proteomic features, Figure 3C). To complement the analysis of “interomic” correlations within omic datasets, “interomic” correlations between features from different single cell or plasma proteomic data layers contributing the most to the severity model were visualized on a chord diagram (Figure 3D). The chord diagram highlighted multiple interomic correlations, 29% of which occurred between plasma proteome and single-cell proteomic features, with the most correlations between plasma proteome components and endogenous phosphorylated (p)S6 and pCREB signal in Ki67\textsuperscript{+} CD8 T cells and frequency of plasmacytoid dendritic cells (pDCs). Plasma proteins correlating with these cellular features were enriched for those involved in cytokine signaling (Reactome pathway identification). This analysis highlighted the interconnected nature of single-cell and plasma proteomic features of the severity model and underscored the need for an integrated approach to characterize the inflammatory state of patients with varying COVID-19 severity.

With respect to cell frequency features contributing the most to the severity model, we observed changes in immune cell distribution that are reminiscent of recent immunophenotyping studies in patients with severe COVID-19. For instance, in our study and prior reports, pDCs and CD161\textsuperscript{+}CD8\textsuperscript{+} T cell frequencies were negatively correlated, while Ki67\textsuperscript{+}CD8\textsuperscript{+} T cell and granulocyte frequencies were positively correlated with COVID-19 severity\textsuperscript{17,19,47,48} (Figure S7). Furthermore, increased plasmablast frequencies in severe patients complemented prior reports\textsuperscript{19,47} (Figure S7). While changes in frequencies of monocyte subsets were not among the most informative features of our severity model, a secondary analysis focused on monocyte subsets recapitulated previously reported monocytic changes associated with disease severity, including increased frequency of classical monocytes, decreased frequency of non-classical monocytes, and decreased HLA-DR expression by (classical, non-classical) monocytes in severe patients (Figure S8).\textsuperscript{16–18,22,47,49–51} In particular, we observed a reduction in frequency of CD16\textsuperscript{+} non-classical

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**(B and C) Correlation network depicting single-cell (B) or plasma (C) proteomic features. Edges represent the correlation between features (Spearman cor > 0.9). Blue/orange nodes highlight positive/negative correlation with disease severity. Node size reflects -log10 of \(p\) value (Spearman). Communities containing the bootstrap-selected informative single-cell (B) or plasma (C) proteomic features are highlighted and annotated.**

**(D) Interomic correlations between features of the six data layers are visualized in a chord diagram. Interomic correlations of the top 10% features ranked by bootstrap with absolute Spearman correlation coefficients between 0.5 and 1.0 are shown.**

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monocytes, which was recently suggested to be mediated by antibody-mediated SARS-CoV-2 infection and pyroptosis.52

Assays examining endogenous and stimulation-dependent cell signaling markers revealed alterations in cell states associated with varying disease severity. A negative correlation was observed between endogenous signaling in CD8+ T cell subsets and natural killer T (NKT) cells and COVID-19 severity, notably for the pS6, total IkBa, and pCREB signals (Figures 4A and S8A). Negative correlations with COVID-19 severity were also observed for endogenous p4EBP1 and total IkBa signals in

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Figure 4. Severity model features reveal biological signatures that demarcate patients with mild, moderate, and severe COVID-19

Boxplots, classified by disease severity, showing features informative to the model. r and p indicate Spearman coefficient and p value of Spearman correlation of the feature with disease severity.

(A) Endogenous immune cell signaling (arcsinh transformed values; see STAR Methods).

(B) Immune cell signaling response to PI stimulation is reported as the arcsinh transformed ratio over the endogenous signaling response (see STAR Methods).

(C) Immune cell signaling responses to IFNα/IL-2/IL-4/IL-6 (IFN/IL) stimulation are reported as the arcsinh transformed ratio over the endogenous signaling response (see STAR Methods).

(D) Plasma protein levels are reported as the normalized protein expression, an arbitrary unit provided by the Olink assay. Tmem, memory T cell; MERTK, tyrosine-protein kinase Mer.

For boxplots, the center line represents the median value; upper and lower box limits indicate first (Q1) and third (Q3) quartile, respectively; whiskers, minimum (Q1−1.5*IQR) and maximum (Q3+1.5*IQR). IQR, interquartile range. AUC, area under the curve.

See also Figure S7–S11.
granulocytes, while endogenous granulocyte pERK1/2 signal was positively correlated with disease severity (Figure S9A). Overall, these results suggest that alterations in endogenous immune cell signaling markers exist between COVID-19-severity categories. In particular, endogenous signaling activities of key elements of the mTOR, MAPK, and NF-κB pathway (pS6, pERK1/2, pCREB, and total IkBα) were diminished with increasing disease severity in immune cell populations that play important roles in the defense against viral pathogens, such as CD8+ T, NKT, and granulocyte cell subsets.

Additionally, immune cell signaling responses to PI stimulation were largely diminished with increasing disease severity, especially for innate immune cell populations. Specifically, PI-stimulated signaling responses in granulocytes (pS6, pERK1/2, and p38), myeloid dendritic cells (mDCs) (pERK1/2 and pSTAT3), and CD56brightCD16− NK cells (p38) were negatively correlated with disease severity (Figures 4B and S9B). Negative correlations with disease severity were also observed for the pSTAT3 signal in regulatory T cells (Tregs) and pMAPKAPK2 signal in CD161hiCD8+ T cells in response to PI stimulation (Figures 4B and S9B). Decreased responsiveness to PI stimulation may be an indication of reduced effector function of circulating innate and adaptive immune cells during severe COVID-19, driven by cell-intrinsic effects and/or changes in cytokines and other modulating factors present in the circulation.

For immune cell signaling responses to IFNα/IL-2/IL-4/IL-6 stimulation, we also observed a negative correlation with disease severity for the pSTAT4/5/6 signals in both adaptive and innate immune cells (Figures 4C and S9C), suggestive of an impaired signaling response to IFN and cytokines in those individuals with more severe COVID-19. Indeed, others have also observed an impaired type I IFN activity in peripheral immune cells of patients critically ill with COVID-19, shown by downregulation of IFN-stimulated genes upon whole-blood IFNα stimulation.53

Among the most robust plasma proteomic features identified by the severity model were several features that positively correlated with disease severity and which overlapped with prior descriptions of the cytokine storm syndrome described in patients with severe COVID-19.5,11 For instance, plasma levels of the cytokines IFN-γ, IL-1β, and IL-33, showed a positive correlation with disease severity (Figure 4D). IL-6, one of the first plasma cytokines recognized as elevated during COVID-19,54 also exhibited a positive correlation with disease severity in our cohort (Figure S10). Although this feature contributed to the severity model, it ranked well below the top 10% of bootstrap-selected informative features (Data S1). A similar positive correlation was observed for lung-related proteins in circulation, such as pulmonary surfactant-associated protein A2 (SFTPA2) and cathepsin H (CTSH), which are involved in surfactant homeostasis (Figure S10).55,56

Angiotensin-converting enzyme 2 (ACE2) is used as a viral entry receptor by SARS-CoV-2 and is released from the epithelial cell surface upon viral binding.57 ACE2 ranked within the top 10% of informative features, and levels showed a positive correlation with severity (Figure S10). The severity model also identified the protease MME (nephrilysin), another key player of the renin-angiotensin system (RAS),58 as a positive correlate of COVID-19 severity (Figure S10). MME is also implicated in neutrophil degranulation,59 and our list of informative model features contained multiple other proteins involved in neutrophil degranulation (protein pathway identified by Reactome; see STAR Methods; Reactome gene set identifier R-HSA-6798695.2; Figure S11A), which were mainly positively correlated with severity as well. This dysregulation of neutrophil degranulation in severe patients is in agreement with recent plasma proteome findings.50

Our analysis revealed tyrosine-protein kinase Mer (MERTK) as the most robust feature in the proteome dataset contributing to the severity model (Data S1). Levels of MERTK, an immunosuppressive tyrosine kinase receptor,61 were positively correlated with disease severity (Figure 4D). MERTK is found on the surface of macrophages, where it mediates phagocytosis of apoptotic cells.62 Activation of MERTK has an immunosuppressive effect by downregulating the production of cytokines and type I IFNs.61,62 Increased MERTK shedding could result in reduced surface expression and loss of MERTK signaling,63 which could play a central role in the hyper inflammation observed in severe COVID-19. MERTK also plays a role in platelet aggregation63 and endothelial barrier integrity.64 Plasma MERTK levels were not positively correlated with most available markers of activation of monocyte subsets (Data S3). Plasma levels of several other proteins involved in primary hemostasis (Reactome gene set identifier R-HSA-109582) were also found to be informative in our model of disease severity (Figure S11B), with the majority of them displaying positive correlations with increasing disease severity.

To gain further insight into the dynamic behavior of plasma and immune cell events in patients with COVID-19, the most informative severity model features (top 10%) were correlated with time since symptom onset for each patient severity category and data layer (Figure S12; Data S1). Only 5 out of 374 model features examined showed correlations with p values below a false discovery rate (FDR)-adjusted value of 0.05, indicating that the large majority of model features were not significantly correlated with time since symptom onset in this dataset (Figure S12A; Data S1C). Among those 5 features with significant time association, ITIH3 and LGALS9 levels appear elevated near symptom onset in severe COVID-19 but decline precipitously thereafter, unlike more stable trajectories in mild and moderate patients (Figure S12B). These features may therefore have important diagnostic value in distinguishing severe cases soon after infection.

In summary, informative features of the severity model revealed biological signatures that progressed from mild to moderate and severe COVID-19. Salient characteristics of this biological progression included cellular elements of immune signaling networks implicated in defensive immunity against viral pathogens (such as the progressive dampening of NF-κB, MAPK/mTOR, and JAK/STAT signaling in multiple innate and adaptive immune cell subsets) and sentinel proteomic pathways involved in lung and RAS homeostasis, primary hemostasis, neutrophil degranulation, and inflammation.

**DISCUSSION**

This cross-sectional study combined high-content plasma proteomics with the single-cell analysis of immune signaling
responses to identify biological determinants of severity across the spectrum of COVID-19 manifestations. Using a two-step analytical approach that accounts for the dimensionality of different data layers, we built and independently validated an integrated model that classifies COVID-19 severity. The biological underpinnings of the severity model consisted of coregulated plasma and single-cell proteomic elements that progressed with COVID-19 severity, including the inflammatory cytokine response to SARS-CoV-2, the mobilization of the RAS and primary hemostasis system, and the dysregulation of the JAK/STAT, NF-κB, and MAPK/mTOR immune signaling responses. The identification of biological signatures progressing with COVID-19 severity provides a set of sentinel events detectable in the early phase of infection that may be possible therapeutic targets for the prevention and/or treatment of severe COVID-19.

The ongoing pandemic has fueled major research efforts toward understanding the host immune response against SARS-CoV-2 infection. Previous efforts have been particularly focused on hospitalized patients with severe COVID-19, which, while of paramount importance, excludes the majority of SARS-CoV-2-infected patients who suffer from mild or moderate COVID-19 and do not require hospitalization. The comparative analysis of samples from patients with mild, moderate, and severe disease afforded a more exhaustive characterization of immune responses related to COVID-19 severity. Our approach dovetails with prior studies identifying an immunological shift distinguishing the spectrum of COVID-19-infection states. Consistent with our results, this switch included an increase in inflammation, the emergence of CD4 and CD8 T cells with a proliferative-exhausted phenotype, and a distinct increase in inflammation, the emergence of CD4 and CD8 T cells as well as their capacity to respond to inflammatory stimulation.

Two major biological signatures associated with the progression from mild to moderate and severe disease emerged from our integrated analysis: (1) the dampening of NF-κB, MAPK/mTOR, and JAK/STAT intracellular signaling responses in multiple innate and adaptive immune cell subsets, and (2) the mobilization of a proteomic network enriched for elements of the RAS, lung homeostasis, and hemostasis pathways, alongside canonical elements of the cytokine storm signature of severe COVID-19.

Several of the features informative to our severity model resonate with previous findings in patients suffering from severe COVID-19. For example, plasma levels of cytokines such as IFNγ, IL-1β, IL-33, and IL-6 increased with increasing severity, consistent with the cytokine storm observed in patients with severe COVID-19, and with circulating IL-33 levels as a potential indicator of damaged lung tissue. In addition, changing frequencies of circulating immune cells also aligned with prior reports in severe patients. Interestingly, an increased percentage of granulocytes was observed in the PBMC fraction of patients with severe COVID-19. Low-density granulocytes also appear in the PBMC fraction of patients with inflammatory diseases and severe infection. In addition to previously reported features of COVID-19 disease severity, the functional analysis of intracellular signaling events in this study revealed intriguing new biology, notably with respect to immune cell responses to inflammatory ligands. The aforementioned low-density granulocytes can display dysfunctional immune responses, which supports our observation of a decreased immune cell signaling response by the granulocytes present in the PBMC fraction of patients with severe disease. In addition, in agreement with Overmyer et al., increasing plasma levels of several proteins involved in neutrophil degranulation were correlated with disease severity. Excessive release of granules can result in tissue damage and is a feature of acute lung injury and septic shock. In addition to granulocytes, other cell types such as mDCs, NK cells, NKT cells, Tregs, and CD4 and CD8 T cells also showed an inverse relationship between capacity to respond to cytokine stimulation and disease severity, suggestive of overall diminished effector functions of circulating innate and adaptive immune cells with increasing severity. These results are consistent with several other studies that have shown decreased functional responses and exhausted phenotypes in peripheral innate and adaptive immune cells in severe patients as well.

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The key proteomic pathways that were mobilized with disease severity, including pathways related to lung homeostasis, RAS homeostasis, and hemostasis. Notably, the plasma levels of two proteins implicated in the production of lung surfactant (SFTPA2 and CTSH) markedly increased with disease severity. Both SFTPA2 and CTSH are synthesized in type II pneumocytes, which are primary targets for SARS-CoV-2 infiltration. As such, these proteins may be early markers of type II pneumocyte dysfunction, impaired surfactant synthesis, and lung damage, as SFTPA2 plasma levels are elevated in patients suffering from acute respiratory failure. These results are consistent with recent transcriptomics analyses of lung biopsies showing impaired surfactant production in patients with severe COVID-19. Elevated SFTPA2 and CTSH could be important markers for clinicians to differentiate severe patients.
Key proteomic features of the COVID-19 severity model also included two components of the RAS (ACE2 and MME), a complex hormonal system that regulates blood pressure and fluid homeostasis as well as pulmonary inflammation.58 The involvement of the RAS in the pathogenesis of SARS-CoV-2 infection is well established, since the interaction between the viral spike protein and ACE2 is a primary mechanism of viral entry into host cells.66 High ACE2 and MME plasma levels in severe patients suggest increased shedding from the cell surface, which may result in loss of function of these proteins.82 A loss of function of ACE2 and MME will likely result in a decrease in angiotensin I (Ang I) and Ang II degradation, which aligns with the multi-organ injuries observed in severe patients.62,83 Interestingly, increased plasma ACE2 concentration is associated with an increased risk for subsequent cardiovascular events in patients with COVID-19.84 As such, our analysis points at mechanistic markers of disease severity that may also be implicated in the clinical manifestations of patients recovering from COVID-19, such as cardiovascular or neurological complications.

The most informative protein feature in our severity model was soluble (s) MERTK, for which plasma levels increased with severity. sMERTK can be produced by proteolytic ectodomain shedding of membrane-bound (mb) MERTK, a member of the Tyro-Axl-MerTK (TAM) family of receptor tyrosine kinases, the activation of which leads to immunosuppression and macrophage-mediated apoptotic cell phagocytosis.61–63,85 sMERTK can act as a competitive inhibitor of MERTK signaling by sequestering ligands that could otherwise bind to mbMERTK.61–63 In the context of COVID-19, impaired MERTK signaling has been proposed as a link between the hyperinflammatory and hypercoagulative state observed in severe patients with disease.57,62,85–88 In the model proposed by Lemke et al., sequestration of MERTK-ligand protein spike (S) by developing blood clots results in impaired MERTK signaling in neighboring macrophages and increased pro-inflammatory cytokine production.61 Sequestration of protein S by sMERTK may also contribute to this effect. In addition, loss of endothelial mbMERTK has been shown to exacerbate lung inflammation in the context of acute respiratory distress syndrome by enhancing endothelial permeability and leukocyte transendothelial migration at the site of infection.64 The pleiotropic roles of MERTK in the regulation of lung endothelial integrity, coagulation, and inflammation suggest that impaired MERTK signaling may be a central component of the pathogenesis of severe COVID-19. sMERTK levels were not positively correlated with markers of monocyte activation in this study, raising the possibility that sMERTK could instead be driven by other factors such as pyroptosis of circulating monocytes, by activation of monocyte/macrophages in the tissues, or by loss of endothelial mbMERTK in the lung. Strategies to regulate ADAM-17-dependent shedding of mbMERTK may be worth investigating in the context of COVID-19.89–91

Assessing the dynamic behavior of model features (top 10%) over the course of disease showed that, consistent with our confounder analysis, the majority of features of the severity model (98.66%) did not correlate with time since symptom onset. The levels of only 5 single-cell or plasma proteomic features correlated with the timing of symptom onset. Notably, ITIH3 and LGALS9 (galectin-9) levels were elevated early after symptom onset in severe patients and declined over the course of disease, suggesting that these proteomic features may be important early indicators of patients with severe disease. Elevated plasma galectin-9 levels have been proposed to be a contributor to the cytokine storm observed in SARS-CoV-2-infected patients, while serum ITIH3 levels have been found to be more abundant in fatal COVID-19 cases upon intensive care unit (ICU) admission, with levels decreasing over the course of disease in both survivors and non-survivors.93

Determining the underlying immune pathogenesis across the spectrum of COVID-19 severity remains an important clinical challenge. Our integrated analysis of plasma and single-cell proteomics in patients with mild, moderate, and severe COVID-19 identified a multi-variate model that differentiates COVID-19 severity. The observations identified by this model contribute clinically relevant insights into the status of patient’s immune responses during SARS-CoV-2 infection and provide promising severity-specific biological signatures for future validation that may inform decision-making on potential therapeutic targets for the prevention of disease progression.

**Limitations of the study**

This study has certain limitations. First, in this cohort, we only assessed peripheral blood samples of patients affected by COVID-19. If available from future cohorts, it would be highly informative to assess the local immune and proteome perturbations in parallel by analyzing lung biopsies or bronchoalveolar lavage fluid to investigate whether the same trends and dampened immune cell responses are observed locally in the lung as well. Second, there are discrepancies in the definition of COVID-19-severity categories—especially for the definition mild—that can hinder the comparison of different studies. In this study, we defined mild patients as those SARS-CoV-2-infected patients that only experience mild symptoms without any breathing issues and that do not require hospitalization, which is similar to the work done by Chevrier et al.16 and Silvin et al.23 Others, on the other hand, defined mild as those SARS-CoV-2-infected patients that were hospitalized but had no or only low oxygen requirements.16,94–96 Despite these differences, the tremendous effort of the research community to rapidly make research available is an enormous advantage for much-needed fast-paced research into COVID-19, and the availability of public datasets will empower future meta-analyses. Thirdly, while it is becoming clearer that those severe patients that die versus those that survive can be phenotypically distinct, the presence of only 3 non-survivors in our cohort precludes an adequately powered analysis to decouple severity-related factors from survival-related factors. Fourthly, our study is cross-sectional in nature. While a time-dependent representation of cross-sectional data provides insights into disease evolution, serial blood sample collection and longitudinal molecular monitoring will be necessary in future studies to discern cause versus effect and identify predictive features that precede severe COVID-19 disease or the development of post-acute sequelae. While several longitudinal studies have indeed shown that fluctuations exist over the disease course, these studies have mainly been conducted in...
hospitalized patients, and a longitudinal study into the disease dynamics of non-hospitalized patients with only mild and/or moderate disease has not been studied rigorously. Finally, while the results of the plasma proteomic model were validated in an independent cohort from a different center, further validation with orthogonal methods (i.e., ELISA, flow cytometry, etc.) will be imperative in the development of future diagnostic tests.

STAR METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Conceptualization, B.G., K.C.N., D.R.M., and G.P.N.; data curation, K.C.N., G.K.R.D., J.F., M.M., J.H., and D. Feyaerts; formal analysis, J.H., J.G., and D. Feyaerts; funding acquisition, B.G., K.C.N., D.R.M., and G.P.N.; investigation/data acquisition, D. Feyaerts, H.C., E.S.T., D.R.M., L.S.P., K.A., M.M., E.D., G.K.R.D., J.F., I.C., T.T.S., C.M.W., J.G.M., E.A.G., I.A.S., X.H., F.V., D.K.G., A.S.T., K.K.R., S.J., S.I.V.F., J.D.K., and D. Furman; methodology, B.G., D.R.M., G.P.N., N.A., and J.H.; project administration, B.G., D.R.M., and K.C.N.; resources, K.C.N., D.R.M., B.G., N.A., B.A.P., S.D.B., M.S.A., M.A., R.S.C., and R.W.; software, N.A., J.H., and J.G.; supervision, B.G., D.R.M., K.C.N., and G.P.N.; visualization, D. Feyaerts, J.H., H.C., J.G., and A.S.T.; writing – original draft, D. Feyaerts, D.R.M., and B.G.; writing – review & editing, B.G., D.R.M., K.C.N., D. Feyaerts, J.H., J.G., N.A., and all authors.

DECLARATION OF INTERESTS

S.D.B. has consulted for Regeneron, Sanofi, Novartis, and Janssen on topics unrelated to this study and owns stocks in AbCellera Biologics. The other authors declare no competing interests.

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REFERENCES

1. Lu, R., Zhao, X., Li, J., Niu, P., Yang, B., Wu, H., Wang, W., Song, H., Huang, B., Zhu, N., et al. (2020). Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. Lancet 395, 565–574.
2. Dong, E., Du, H., and Gardner, L. (2020). An interactive web-based dashboard to track COVID-19 in real time. Lancet Infect. Dis. 20, 533–534.
3. Wu, Z., and McGoogan, J.M. (2020). Characteristics of and important lessons from the coronavirus disease 2019 (COVID-19) outbreak in China: summary of a report of 72314 cases from the Chinese center for disease control and prevention. J. Am. Med. Assoc. 323, 1239–1242.
4. CDC. COVIDView: A weekly surveillance summary of U.S. COVID-19 activity. Key Updates for Week 4, ending January 30, 2021. [www.cdc.gov/covid2019]2021
5. Piroh, L., Cottenet, J., Mariet, A.S., Bonniaud, P., Blot, M., Tubert-Bitter, P., and Quantin, C. (2021). Comparison of the characteristics, morbidity, and mortality of COVID-19 and seasonal influenza: a nationwide, population-based retrospective cohort study. Lancet Respir. Med. 9, 251–259.
6. Horwitz, L.I., Jones, S.A., Cefiro, R.J., Francois, F., Greco, J., Rudy, B., and Petrelli, C.M. (2021). Trends in COVID-19 risk-adjusted mortality rates. J. Hosp. Med. 16, 90–92.

7. Rosenthal, N., Cao, Z., Gundrum, J., Sianis, J., and Safo, S. (2020). Risk factors associated with in-hospital mortality in a US national sample of patients with COVID-19. JAMA Netw. Open 3, e2029058.

8. Cao, X. (2020). COVID-19: immunopathology and its implications for therapy. Nat. Rev. Immunol. 20, 269–270.

9. Rodriguez, L., Pekkarinen, P.T., Lakshminikanth, T., Tan, Z., Consiglio, C.R., Poc, C., Chen, Y., Mugabo, C.H., Nguyen, N.A., Novlan, K., et al. (2020). Systems-level immunomonitoring from acute to recovery phase of severe COVID-19. Cell Rep. Med. 1, 100078.

10. Zhang, J.J., Dong, X., Cao, Y.Y., Yuan, Y.D., Yang, Y.B., Yan, Y.Q., Akdis, C.A., and Gao, Y.D. (2020). Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. Allergy 75, 1730–1741.

11. Tang, L., Yin, Z., Hu, Y., Mei, H., van de Veen, W., Bruggen, M.C., O’Mahony, L., Gao, Y., Nadeau, K., and Akdis, C.A. (2020). Immune response to SARS-CoV-2 and mechanisms of immunopathological changes in COVID-19. Allergy 75, 1564.

12. Gaudilliere, B., Fragiadakis, G.K., Bruggner, R.V., Nicolau, M., Finck, R., Tingle, M., Silva, J., Gao, E.A., Yeh, C.G., Maloney, W.J., et al. (2020). Clinical recovery from surgery correlates with single-cell immune signatures. Sci. Transl. Med. 12, 255ra131.

13. Tsai, A.S., Berry, K., Beneyto, M.M., Gaudilliere, D., Gao, E.A., Culos, A., Ghaemi, M.S., Choisy, B., Djebali, K., Einhaus, J.F., et al. (2019). A year-long immune profile of the systemic response in acute stroke survivors. Brain 142, 978–991.

14. Good, Z., Samo, J., Jager, A., Samusik, N., Aghaeepour, N., Simonis, E.F., White, L., Lacayo, N.J., Farrit, W.J., Fazli, G., et al. (2018). Single-cell developmental classification of B cell precursor acute lymphoblastic leukemia at diagnosis reveals predictors of relapse. Nat. Med. 24, 474–483.

15. Myklebust, J.H., Brody, J., Kohrt, H.E., Kolstad, A., Czerwinski, D.K., Wäichi, S., Green, M.R., Treen, G., Liestøl, K., Beiske, K., et al. (2017). Distinct patterns of B-cell receptor signaling in non-Hodgkin lymphomas identified by single-cell profiling. Blood 129, 759–770.

16. Irish, J.M., and Doxie, D.B. (2014). High-dimensional single-cell cancer biology. Curr. Top. Microbiol. Immunol. 377, 1–21.

17. McIlvain, D.R., Chen, H., Rahil, Z., Bidoki, N.H., Jiang, S., Bjornson, Z., Kolhatkar, N.S., Martinez, C.J., Gaudilliere, B., Hedou, J., et al. (2021). Human influenza virus challenge identifies cellular correlates of protection for oral vaccination. Cell Host Microbe 29, 1828–1837.e5.

18. Chen, G., Wu, D., Guo, W., Cao, Y., Huang, D., Wang, T., Zhang, X., Chen, H., Yu, H., et al. (2020). Clinical and immunological features of severe and moderate coronavirus disease 2019. J. Clin. Invest. 130, 2620–2629.

19. NIH (2020). NIH COVID-19 clinical spectrum guidelines. https://www.covid19treatmentguidelines.nih.gov/overview/clinical-spectrum/.

20. Angelaguet, R., Veiten, B., Arndt, D., Dietrich, S., Zenz, T., Marioni, J.C., et al. (2020). Distinct immunological signatures discriminate severe COVID-19 from non-SARS-CoV-2-driven critical pneumonia. Immunity 55, 366–375.

21. Su, Y., Chen, D., Yuan, D., Lausted, C., Choi, J., Dai, C.L., Voillet, V., Duvvuri, V.R., Scherler, K., Troisch, P., et al. (2020). Multi-omics resolves a sharp disease-state shift between mild and moderate COVID-19. Cell 183, 1479–1495.

22. Slivin, A., Chapuis, N., Dunsmore, G., Goubet, A.G., Dubuisson, A., Derosa, L., Almire, C., Hénon, C., Kosmider, O., Droin, N., et al. (2020). Elevated capsaicin and abnormal myeloid cell subsets discriminate severe from mild COVID-19. Cell 182, 1401–1418.e18.

23. Leelatian, N., Diggins, K.E., and Ishii, J.M. (2015). Characterizing phenotypes and signaling networks of single human cells by mass cytometry. Methods Mol. Biol. 1346, 99–113.

24. Rahil, Z., Leylék, R., Schürch, C.M., Chen, H., Bjornson–Hooper, Z., Christensen, S.R., Gherardiní, P.F., Bhat, S.S., Spitzer, M.H., Fragiadakis, G.K., et al. (2020). Landscape of coordinated immune responses to H1N1 challenge in humans. J. Clin. Invest. 130, 5800–5816.

25. Gao, E.A., Stanley, N., Lindberg-Larsen, V., Einhaus, J., Tsai, A.S., Verdonk, F., Culos, A., Ghaemi, S., Rumer, K.K., Stelzer, I.A., et al. (2020). Author Correction: preferential inhibition of adaptive immune system dynamics by glucocorticoids in patients after acute surgical trauma. Nat. Commun. 11, 4495.

26. Arunachalam, P.S., Wimmers, F., Mok, C.K.P., Perera, R.A.P.M., Scott, J.H., Brody, J., Kohrt, H.E., Kolstad, A., Czerwinski, D.K., Wäichi, S., Green, M.R., Treen, G., Liestøl, K., Beiske, K., et al. (2017). Distinct patterns of B-cell receptor signaling in non-Hodgkin lymphomas identified by single-cell profiling. Blood 129, 759–770.

27. Myklebust, J.H., Brody, J., Kohrt, H.E., Kolstad, A., Czerwinski, D.K., Wäichi, S., Green, M.R., Treen, G., Liestøl, K., Beiske, K., et al. (2017). Distinct patterns of B-cell receptor signaling in non-Hodgkin lymphomas identified by single-cell profiling. Blood 129, 759–770.

28. Irish, J.M., and Doxie, D.B. (2014). High-dimensional single-cell cancer biology. Curr. Top. Microbiol. Immunol. 377, 1–21.

29. McIlvain, D.R., Chen, H., Rahil, Z., Bidoki, N.H., Jiang, S., Bjornson, Z., Kolhatkar, N.S., Martinez, C.J., Gaudilliere, B., Hedou, J., et al. (2021). Human influenza virus challenge identifies cellular correlates of protection for oral vaccination. Cell Host Microbe 29, 1828–1837.e5.

30. Chen, G., Wu, D., Guo, W., Cao, Y., Huang, D., Wang, T., Zhang, X., Chen, H., Yu, H., et al. (2020). Clinical and immunological features of severe and moderate coronavirus disease 2019. J. Clin. Invest. 130, 2620–2629.

31. NIH (2020). NIH COVID-19 clinical spectrum guidelines. https://www.covid19treatmentguidelines.nih.gov/overview/clinical-spectrum/.

32. Angelaguet, R., Veiten, B., Arndt, D., Dietrich, S., Zenz, T., Marioni, J.C., et al. (2020). Distinct immunological signatures discriminate severe COVID-19 from non-SARS-CoV-2-driven critical pneumonia. Immunity 55, 366–375.

33. NIH (2020). NIH COVID-19 clinical spectrum guidelines. https://www.covid19treatmentguidelines.nih.gov/overview/clinical-spectrum/.

34. Angelaguet, R., Veiten, B., Arndt, D., Dietrich, S., Zenz, T., Marioni, J.C., et al. (2020). Distinct immunological signatures discriminate severe COVID-19 from non-SARS-CoV-2-driven critical pneumonia. Immunity 55, 366–375.

35. NIH (2020). NIH COVID-19 clinical spectrum guidelines. https://www.covid19treatmentguidelines.nih.gov/overview/clinical-spectrum/.

36. Tibshirani, R. (1996). Regression shrinkage and selection via the lasso. J. R. Stat. Soc. B 58, 267–288.

37. Hosmer, D.W., Lemeshow, S., and Sturdivant, R.X. (2013). Applied Logistic Regression, 3rd ed. (New York: John Wiley & Sons), pp. 35–47.

38. Kremer, H.C., Morgan, G.A., Leech, N.L., Gliner, J.A., Vaske, J.J., and Harmon, R.J. (2003). Measures of clinical significance. J. Am. Acad. Child. Adolesc. Psychiatry 42, 1524–1529.

39. Filbin, M.R., Mehta, A., Schneider, A.M., Kays, K.R., Guess, J.R., Gentili, M., Fenyes, B.G., Charland, N.C., Gonye, A.L.K., Gushterova, I., et al. (2021). Longitudinal proteomic analysis of severe COVID-19 reveals survival-associated signatures, tissue-specific cell death, and cell-cell interactions. Cell Rep. Med. 2, 100287.
40. Sohrabi, M.R., Amin, R., Maher, A., Bahadorimofared, A., Janbazid, S., Hannani, K., Kolahi, A.A., and Zali, A.R. (2021). Sociodemographic determinants and clinical risk factors associated with COVID-19 severity: a cross-sectional analysis of over 200,000 patients in Tehran, Iran. BMC Infect. Dis. 21, 474.

41. Zhou, F., Yu, T., Du, R., Fan, G., Liu, Y., Liu, Z., Xiang, J., Wang, Y., Song, B., Gu, X., et al. (2020). Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. Lancet 395, 1054–1062.

42. Stefan, N., Birkenfeld, A.L., and Schulze, M.B. (2021). Global pandemics interconnected - obesity, impaired metabolic health and COVID-19. Nat. Rev. Endocrinol. 17, 135–149.

43. Vahidy, F.S., Nicolas, J.C., Meeks, J.R., Khan, O., Pan, A., Jones, S.L., Masud, F., Sostman, H.D., Phillips, R., Andrieni, J.D., et al. (2020). Racial and ethnic disparities in SARS-CoV-2 pandemic: analysis of a COVID-19 observational registry for a diverse US metropolitan population. BMJ Open 10, e039949.

44. Brodin, P. (2021). Immune determinants of COVID-19 disease presentation and severity. Nat. Med. 27, 28–33.

45. Bach, F.R. (2008). Bolasso: model consistent Lasso estimation through the bootstrap. ICML ’08. In Proceedings of the 25th International Conference on Machine Learning, pp. 33–40.

46. Chatterjee, A., and Lahiri, S.N. (2011). Bootstrapping lasso estimators. J. Am. Stat. Assoc. 106, 688–625.

47. Kuri-Cervantes, L., Pampena, M.B., Meng, W., Rosenfeld, A.M., Ittner, L.A., et al. (2020). Comprehensive mapping of immune perturbations associated with severe COVID-19. Sci. Immunol. 5, eaat7114.

48. Parrot, T., Gorin, J.B., Seuninck, P., Diego, D., Hertwig, L., Palma Medina, L.M., et al. (2020). MAIT cell activation and dynamics associated with COVID-19 disease severity. Sci. Immunol. 5, eabf1670.

49. Laing, A.G., Lorenc, A., Del Molino Del Barrio, I., Das, A., Fish, M., Monin, L., Munoz-Ruiz, M., McKenzie, D.R., Hayday, T.S., Francos-Quijorna, I., Kury-Ladner, M.E., Katsaounou, P., et al. (2020). Large-scale multi-omic analysis of COVID-19 severity. Cell 181, 27–30.

50. Giampietro, P., Antoniadou, A., Antonakos, N., Damoraki, G., Gkavogianni, T., Adami, M.E., Katsaounou, P., et al. (2020). Complex immune dysregulation in COVID-19 patients with severe respiratory failure. Cell Host Microbe 27, 992–1000.

51. Kvedaraite, E., Kusnadi, A., Ramírez-Sustegui, C., Fajardo, V., Chee, S.J., Meckiff, J., and Floros, J. (2018). Differential effects of innate immune variants and ethnic disparities in SARS-CoV-2 pandemic: analysis of a COVID-19 observational registry for a diverse US metropolitan population. BMJ Open 10, e039949.

52. Junqueira, C., Crespo, A., Ranjabar, S., de Lacerda, L.B., Lewandrowski, K., Ingber, J., Parry, B., Ravid, S., Clark, S., Schrimpf, M.R., et al. (2022). FcgammaR-mediated SARS-CoV-2 infection of monocytes activates inflammation. Nature, 1–9. https://doi.org/10.1038/s41586-022-04702-4.

53. Hadjadji, J., Yatim, N., Barnabei, L., Corneau, A., Boussier, J., Smith, N., Péret, H., Charbit, B., Bonnet, V., Chenevet-Gobeaux, C., et al. (2020). Impaired type I interferon activity and inflammatory responses in severe COVID-19 patients. Science 369, 718–724.

54. Ruan, Q., Yang, K., Wang, W., Jiang, L., and Song, J. (2020). Clinical predictors of mortality due to COVID-19 based on an analysis of data of 150 patients from Wuhan, China. Intensive Care Med. 46, 846–848.

55. Thorennoor, N., Zhang, X., Umstead, T.M., Scott Halstead, E., Phelps, D.S., and Floros, J. (2018). Differential effects of innate immune variants of surfactant protein-A1 (SFTPA1) and SP-A2 (SFTPA2) in airway function after Klebsiella pneumoniae infection and sex differences. Respir. Res. 19, 23.
coronavirus, respiratory syncytial virus and influenza virus. Rev. Med. Virol. 31, e2234.

76. Mettelman, R.C., and Thomas, P.G. (2021). Human susceptibility to influenza infection and severe disease. Cold Spring Harb. Perspect. Med. 11, a038711.

77. Florindo, H.F., Kleiner, R., Vaskovich-Koubi, D., Acúrcio, R.C., Carreira, B., Yeini, E., Tiram, G., Liobomirski, Y., and Satchi-Fainaro, R. (2020). Immune-mediated approaches against COVID-19. Nat. Nanotechnol. 15, 630–645.

78. Tang, Y., Liu, J., Zhang, D., Xu, Z., Ji, J., and Wen, C. (2020). Cytokine storm in COVID-19: the current evidence and treatment strategies. Front. Immunol. 11, 1708.

79. Remy, K.E., Brakenridge, S.C., Francois, B., Daix, T., Deutschman, C.S., Monneret, G., Jeannot, R., Laterre, P.F., Hotchkiss, R.S., and Moldawer, L.L. (2020). Immunotherapies for COVID-19: lessons learned from sepsis. Lancet Respir. Med. 8, 946–949.

80. Doyle, I.R., Bersten, A.D., and Nicholas, T.E. (1997). Surfactant proteins-A and -B are elevated in plasma of patients with acute respiratory failure. Am. J. Respir. Crit. Care. Med. 156, 1217–1229.

81. Islam, A.B.M.M.K., and Khan, M.A.A.K. (2020). Lung transcriptome of a COVID-19 patient and systems biology predictions suggest impaired surfactant production which may be druggable by surfactant therapy. Sci. Rep. 10, 19395.

82. Lu, R., Sui, J., and Zheng, X.L. (2020). Elevated plasma levels of cytokines in COVID-19 patients allow early prediction of disease progression. Life Sci. 1072, 1336–1345.

83. Peng, Y., Mentzer, A.J., Liu, G., Yao, X., Yin, Z., Dong, D., Dejirartisatt, W., Rostron, T., Supasa, P., Liu, C., et al. (2020). Broad and strong memory CD4(+) and CD8(+) T cells induced by SARS-CoV-2 in UK convalescent individuals following COVID-19. Nat. Immunol. 21, 1336–1345.

84. Thompson, E.A., Cascino, K., Ordenze, A.A., Zhou, W., Vaghisia, A., Hamacher-Brady, A., Brady, N.R., Sun, I.H., Wang, R., Rosenberg, A.Z., et al. (2021). Metabolic programs define dysfunctional immune responses in severe COVID-19 patients. Cell Rep. 34, 108863.

85. Lucas, C., Wong, P., Klein, J., Castro, T.B.R., Silva, J., Sundaram, M., Elmingston, M.K.O., Mao, T., Oh, J.E., Israelow, B., et al. (2020). Longitudinal analyses reveal immunological misfiring in severe COVID-19. Nature 584, 483–489.

86. Bernardes, J.P., Casano, J.R., Vorks, C.K., Singh, H., Morales, A., DeSimone, R.A., Ellsworth, G.B., Soave, R., Kapadia, S.N., Saito, K., et al. (2021). Profiling of immune dysfunction in COVID-19 patients allows early prediction of disease progression. Life Sci. Alliance 4, e20020955.

87. Zheng, H.Y., Xu, M., Yang, C.X., Tian, R.R., Zhang, M., Li, J.J., Wang, X.C., Ding, Z.L., Li, G.M., Li, X.L., et al. (2020). Longitudinal transcriptome analyses show robust T cell immunity during recovery from COVID-19. Signal Transduct. Target. Ther. 5, 294.

88. Bergamaschi, L., Mescia, F., Turner, L., Hanson, A.L., Kotagiri, P., Dunmore, B.J., Ruffieux, H., De Sa, A., Huhn, O., Morgan, M.D., et al. (2021). Longitudinal analysis reveals immunological misfiring in severe COVID-19 patients. Cell Rep. 34, 108268.

89. Bjornson-Hooper, Z.B., Fragiadakis, G.K., Spitzer, M.H., Chen, H., Madhireddy, D., Hu, K., Lundsten, K., Mcllwain, D.R., and Nolan, G.P. (2022). A comprehensive atlas of immunological differences between humans, mice, and non-human primates. Front. Immunol. 13, 867015.

90. Syed, A., Garcia, M.A., Lly, S.U., Bucayu, R., Kohli, A., Ishida, S., Berglund, J.P., Tsai, M., Maecher, H., O’Riordan, G., et al. (2014). Peanut oral immunotherapy results in increased antigen-induced regulatory T cell function and hypomethylation of forkhead box protein 3 (FOXP3). J. Allergy Clin. Immunol. 133, 500–510.

91. Bjornson-Hooper, Z.B., Fragiadakis, G.K., Spitzer, M.H., Chen, H., Madhireddy, D., Hu, K., Lundsten, K., Mcllwain, D.R., and Nolan, G.P. (2022). A comprehensive atlas of immunological differences between humans, mice, and non-human primates. Front. Immunol. 13, 867015.

92. Bozorgmehr, N., Mashhouri, S., Perez Rosero, E., Xu, L., Shahbáz, S., Sligl, W., Osman, M., Kutsogiannis, D.J., MacIntyre, E., O’Neil, C.R., and Elahi, S. (2021). Galectin-9, a player in cytokine release syndrome and a surrogate diagnostic biomarker in SARS-CoV-2 infection. mBio 12, e00384–21.
machine learning pipeline improves predictions. Nat. Mach. Intell. 2, 619–628.

108. Assarsson, E., Lundberg, M., Helmgquist, G., Björkesten, J., Thorsen, S.B., Ekman, D., Eriksson, A., Rennel Dickens, E., Ohlsson, S., Edfeldt, G., et al. (2014). Homogenous 96-plex PEA immunoassay exhibiting high sensitivity, specificity, and excellent scalability. PLoS One 9, e95192.

109. Wong, T.T. (2015). Performance evaluation of classification algorithms by k-fold and leave-one-out cross validation. Pattern Recogn. 48, 2839–2846.

110. Hand, D.J., and Till, R.J. (2001). A simple generalisation of the area under the ROC curve for multiple class classification problems. Mach. Learn. 45, 171–186.

111. van der Maaten, L.J.P., and Hinton, G.E. (2008). Visualizing high-dimensional data using t-SNE. J. Mach. Learn. Res. 9, 2579–2605.

112. Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. B 57, 289–300.
## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Anti-human 4E-BP1 pT37/46 (clone 236B4) | CST | Cat# 2855; RRID: AB_560835 |
| Anti-human BDCA3 (clone 1A4) | BD Biosciences | Cat# 559780; RRID: AB_397321 |
| Anti-human CCR7 (clone 150503) | R&D Systems | Cat# MAB197-100; RRID: AB_2072803 |
| Anti-human CD11b (clone ICRF44) | BioLegend | Cat# 301302; RRID: AB_314154 |
| Anti-human CD11c (clone Bu15) | BioLegend | Cat# 337202; RRID: AB_1236381 |
| Anti-human CD123 (clone 3G8) | BioLegend | Cat# 301802; RRID: AB_314184 |
| Anti-human CD1A4 (clone 1A4) | BD Biosciences | Cat# 559780; RRID: AB_397321 |
| Anti-human CD1A6 (clone 3G8) | BioLegend | Cat# 302033; RRID: AB_2104002 |
| Anti-human CD1A9 (clone 3A1-19) | Beckman Coulter | Cat# IM1313; RRID: AB_131613 |
| Anti-human CD2 (clone HI2) | BioLegend | Cat# 306602; RRID: AB_314620 |
| Anti-human CD27 (clone O232) | BioLegend | Cat# 302802; RRID: AB_314294 |
| Anti-human CD3 (clone SP34.2) | BD Biosciences | Cat# 551916; RRID: AB_394293 |
| Anti-human CD3 (clone AC104.3E3) | Miltenyi | Cat# Custom Carrier Free; RRID: AB_615078 |
| Anti-human CD38 (clone HIT2) | BioLegend | Cat# 303502; RRID: AB_314354 |
| Anti-human CD4 (clone OKT4) | BioLegend | Cat# 317402; RRID: AB_571963 |
| Anti-human CD45 (clone HI30) | BioLegend | Cat# 304002; RRID: AB_314390 |
| Anti-human CD45RA (clone HI100) | BioLegend | Cat# 304102; RRID: AB_314406 |
| Anti-human CD56 (clone NCAM16.2) | BD Biosciences | Cat# 559043; RRID: AB_397180 |
| Anti-human CD61 (clone VI-PL2) | BioLegend | Cat# 336402; RRID: AB_1227584 |
| Anti-human CD66 (clone YTH71.3) | Pierce | Cat# MA1-36189; RRID: AB_1073288 |
| Anti-human CD7 (clone M-T701) | BD Biosciences | Cat# 555359; RRID: AB_395762 |
| Anti-human CD8 (clone RPA-T8) | BioLegend | Cat# 301002; RRID: AB_314120 |
| Anti-human CREB pS133 (clone 87G3) | CST | Cat# 9198; RRID: AB_2561044 |
| Anti-human Erk1/2 pT202/Y204 (clone D13.14.4E) | CST | Cat# 4370; RRID: AB_2315112 |
| Anti-human Foxp3 (clone PCH101) | Thermo Fisher | Cat# 14-4776-82; RRID: AB_467554 |
| Anti-human HLA-DR (clone Immu357) | Beckman Coulter | Cat# Immu357; RRID: AB_131284 |
| Anti-human IgM (clone G20-127) | BD Biosciences | Cat# 555780; RRID: AB_396115 |
| Anti-human IkBa amino-terminal (clone L35A5) | CST | Cat# 4814; RRID: AB_390781 |
| Anti-human K67 (clone SoIA15) | Thermo Fisher | Cat# 14-5698-82; RRID: AB_2688057 |
| Anti-human MAPKAPK2 pT334 (clone 27B7) | CST | Cat# 3007; RRID: AB_490936 |
| Anti-human P38 pT180/Y182 (clone 36/p38) | BD Biosciences | Cat# 612289; RRID: AB_396906 |
| Anti-human PLCγ2 pY759 (clone K86-689.37) | BD Biosciences | Custom Carrier Free; RRID: AB_647226 |
| Anti-human S6 pS235/236 (clone 2F9) | CST | Cat# 4856; RRID: AB_2181037 |
| Anti-human STAT1 pY701 (clone 4a) | BD Biosciences | Cat# 612233; RRID: AB_399555 |
| Anti-human STAT3 pY705 (clone 4) | BD Biosciences | Cat# 612357; RRID: AB_399646 |
| Anti-human STAT4 pY693 (clone 38) | BD Biosciences | Cat# 612738; RRID: AB_399957 |
| Anti-human STAT5 pY694 (clone 47) | BD Biosciences | Cat# 611965; RRID: AB_399386 |
| Anti-human STAT6 pY691 (clone 18) | BD Biosciences | Cat# 611597; RRID: AB_399013 |
| Anti-human TBK1/NAK pS172 (clone D52C2) | CST | Cat# 5483; RRID: AB_10693472 |
| Anti-human Zap70/Syk pY319/Y352 (clone 17a) | BD Biosciences | Cat# 612574; RRID: AB_399864 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to an will be fulfilled by the lead contact, Brice Gaudillière (gbrice@stanford.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
Raw data (FCS file), original data (Olink plasma proteomic data), processed data (cell frequency, phosphosignal, and chord diagram correlation matrix data), patient characteristics, and Generalized Additive Model (GAM) p values have been deposited to Dryad: https://doi.org/10.5061/dryad.9cnp5hqm.
Supplemental files are available from Mendeley Data: https://doi.org/10.17632/xss9pdtc4f.1.
Source code used for analysis can be found on github at https://github.com/julienhed/COVID-Severity.
Any additional information required to reanalyze the reported study is available upon request from the lead contact.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

Study design
This study was designed as a cross-sectional study, where samples were obtained from adults with positive test results for SARS-CoV-2 from analysis of nasopharyngeal swab specimens. Samples were obtained at any point from March to June 2020 at the Stanford Occupational Health Clinic. Testing was accomplished using Stanford Health Care clinical laboratory developed internal testing capability with a quantitative reverse-transcriptase–polymerase-chain-reaction (qRT-PCR) assay. Mechanical ventilation and therapeutics for COVID-19 (when used) were not administered until after collection of blood sample, with the exception of 2 individuals who were put on mechanical ventilation one day prior to sample collection (information in supplemental file “PatientCharacteristics”). Patients were excluded from enrollment if they were taking experimental therapeutics for COVID-19 (i.e. those medications not authorized by a regulatory agency for use in COVID-19). Healthy controls were collected prior to the detection of SARS-CoV-2 in the region (historical controls). Figure S4 shows self-reported symptom onset and diagnostic qRT-PCR timing in relation to the day of sample collection.

Data sources and clinical definitions
We obtained data from self-reported surveys and from Stanford clinical data electronic medical record system as per consented participant permission. This database contains all the clinical data available from Stanford facilities. The data obtained included patients’ demographic details, vital signs, laboratory test results, medication administration data, historical and current medication lists, historical and current diagnoses, time of COVID-19 symptom onset (self-reported), clinical notes, radiological results, biopsy results as appropriate, historical discharge disposition for previous inpatient hospitalizations, and ventilator use data. Severity diagnosis was assigned at time of SARS-CoV-2 qRT-PCR alone. Severity diagnosis was assigned according to previously defined National Institute of Health (NIH) criteria32,33 with the clinical classification of COVID-19 as follows: Asymptomatic: Positive for SARS-CoV-2 but without COVID-19 symptoms; Mild disease: Various mild symptoms (e.g. cough, fever, sore throat, loss of smell and taste, etc.) but no breathing issues (shortness of breath, dyspnea, or abnormal chest imaging) are reported; Moderate disease: Evidence of lower respiratory tract disease but oxygen saturation (SpO2) ≥ 94%; Severe disease: Requires hospitalization because of respiratory distress (SpO2 ≤ 94%, respiratory frequency <30 breaths/min, PaO2/FiO2 <300 mm Hg, or lung infiltrates >50%).

Phlebotomy and initial blood processing
Blood was collected from 97 patients positive for SARS-CoV-2 and 40 controls via venipuncture. Anticoagulated blood was processed into peripheral blood mononuclear cells (PBMC) by density gradient centrifugation using published methods.103 PBMC were stored in 10% DMSO and frozen in liquid nitrogen until thawing and staining. Plasma was isolated from blood collected in EDTA tubes as follows: Within 4hrs of collection, tubes were centrifuged at 500 x g for 10 min at room temperature (RT), plasma was transferred into fresh conical tubes and centrifuged again (500 x g, 10 min, RT) before aliquoting into 500 μL cryovials and transferred to −80°C for long-term storage.

Study approval
We conducted this study at Stanford University Medical Center, where the samples from COVID-19 patients were collected at the Stanford Occupational Health Clinic under an IRB approved protocol (55,689; Protocol Director Dr. Nadeau). Informed consent was obtained from each patient prior to enrolling in the study or from the patient’s legally authorized representative if the patient was unable to provide consent. Healthy controls (historical controls) were consented using a separate IRB-approved protocol (8629; Protocol Director Dr. Nadeau).

METHOD DETAILS

Mass cytometry analysis of single-cell immune cell responses in PBMC

In vitro PBMC stimulation
Cryopreserved PBMC were quickly thawed, washed two times with supplemented medium, and rested for 1h at 37°C in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1% Penicillin-Streptomycin, and 1% L-Glutamine. PBMC were counted and checked for viability. 0.5-1x10⁵ cells were either stimulated with lipopolysaccharide (LPS; 1 μg/ml) and CL097 (TLR7/8 agonist; 1 μg/ml), interleukin-2 (IL-2), IL-4, IL-6 and interferon-α (IFNα; all 100 ng/mL), a cocktail of phorbol 12-myristate 13-acetate 13-acetate (PMA), ionomycin, brefeldin A and monensin (1x; PI cocktail), or left unstimulated for 15 min at 37°C. After stimulation, samples were fixed with Proteomic Stabilizer (SmartTube) and stored at −80°C until further processing for mass cytometry analysis.

Barcoding and antibody staining
The 42-marker mass cytometry antibody panel included 25 cell surface antibodies and 17 intracellular antibodies recognizing primarily phospho-specific signaling epitopes (Data S2). In brief, following in vitro stimulation, fixed PBMCs were thawed, reconstituted in cell staining medium, and arranged in a 96-well block. Subsequent steps were performed using a previously described robotics platform.104 Sets of 16 samples were barcoded with palladium metal105 and pooled into a single well. Pooled barcoded samples were treated with FC-block (Human TruStain FcX, Biolegend) for 10 min then surface antibody stained for 30 min in cell staining medium
(PBS with 0.5% BSA and 0.02% sodium azide). After surface staining, cells were permeabilized in ice-cold 100% methanol, washed, and stained for 60 min with intracellular antibodies in cell staining medium. Following intracellular staining, cells were washed and resuspended in an iridium intercalator (Fluidigm) solution containing 1.6% paraformaldehyde. Finally, samples were washed, resuspended in 1X five-element normalization beads (La, Pr, Tb, Tm, Lu) (Fluidigm), and analyzed on a freshly cleaned and tuned CyTOF instrument. The resulting mass cytometry data were bead-normalized across all runs and debarcoded as previously described.108

**Minimization of experimental batch effects**

To minimize potential batch effects, each of the unstimulated and stimulated samples from the same individual were processed, barcoded, stained, and measured simultaneously in the same tube. Samples were processed, barcoded, stained, and measured simultaneously in batches equalized to the extent possible for age, gender, healthy control, mild, moderate, and severe categories. Internal controls – i.e. aliquots of the same sample – were added during each mass cytometer run to evaluate consistent performance between runs. To control for consistent tuning parameters of the mass cytometer, batches of samples measured on separate days were normalized to metal impregnated beads mixed with samples during runs.

**Cell frequency, endogenous intracellular signaling, and intracellular signaling responses**

Mass cytometry data was examined using CellEngine (Primity Bio) to define cell populations using manual gates and quantify differential expression of signaling markers in response to stimulation. The gating strategy and representative example of phosphosignaling responses can be found in Figure S1 and S2.

Cell frequencies were expressed as a percentage of gated singlet mononuclear cells (DNA⁺ CD235a⁻ CD61⁺ CD66⁺), except for granulocyte frequency which was expressed as a percentage of singlet leukocytes (DNA⁺ CD235a⁻ CD61⁻). Signal intensity was quantified per single cell for each phospho-signaling protein (pSTAT1, pSTAT3, pSTAT4, pSTAT5, pSTAT6, pMAPKAPK2, pCREB, pPLCy2, pS6, PERK1/2, pP38, pZAP70/Syk, pTBK1, p4EBP1, and total IκBα) and for a set of markers that relate to immune subset specific functionality (HLA-DR, FoxP3, CD38, IgM, and Ki67) using an arcsinh transformed value (arcsinh(x/5)) from the median signal intensity. Endogenous intracellular signaling activity was derived from the analysis of unstimulated cells, while intracellular signaling responses to stimulation were reported as the arcsinh transformed ratio over the endogenous signaling, i.e., the difference in arcsinh transformed signal intensity between the stimulated and unstimulated condition. For cell subsets in a given sample that had an event count below 20 events, that cell subset and related phospho-signal were excluded from downstream analysis. A combination of five stimulations x 44 immune cell subsets x 20 functional proteins (i.e. 15 phospho-signaling + 5 functionality-specific proteins) resulted in a total of 4400 single features obtained per sample. A penalization matrix, based on mechanistic immunological knowledge, was applied to the immune cell response data,35,107 resulting in a final total of 2662 features for each sample that was used for further analysis. These included 44 innate and adaptive immune cell subset frequency features, 789 endogenous signaling features, 299 LPS/CL097 stimulation response features, 752 IFNa/IL-2/IL-4/IL-6 stimulation response features, and 778 PI stimulation response features.

**Plasma protein profiling using olink multiplex panel**

Plasma protein levels were quantified using Olink multiplex proximity extension assay (PEA) panels (Olink Proteomics; www.olink.com) according to the manufacturer’s instructions and as described before.108 The basis of PEA is a dual-recognition immunoassay, where two matched antibodies labeled with unique DNA oligonucleotides simultaneously bind to a target protein in solution. This brings the two antibodies into proximity, allowing their DNA oligonucleotides to hybridize, serving as a template for a DNA polymerase-dependent extension step. This double-stranded DNA which is unique for a specific antigen will get amplified using P5/P7 Illumina adapters along with sample indexing, which is quantitatively proportional to the initial concentration of target protein. These amplified targets will finally get quantified by Next Generation Sequencing using Illumina Nova Seq 6000 (Illumina Corporation, San Diego, California). In this study, we have used the Explore 1536 panel which measures 1,472 proteins using 3 μl plasma samples, which were treated with 1% Triton X-100 and incubated at room temperature for 2 h to inactivate the virus.

The raw expression values obtained with the Olink assay are provided in the arbitrary unit Normalized Protein Expression (NPX), where high NPX values represent high protein concentration. Values were log2-transformed to account for heteroskedasticity. Proteins close to the limit of detection are flagged in the raw data.

**Benchmarking of models**

In addition to the LASSO model for which we built the stack generalization model (see “Multivariate analysis and stacked generalization”), we also compared the performance of the LASSO regression with different regression strategies, including: ordinary least square regression, random forest regressor, elastic-net regression, and support vector machine regression. For all these methods, we report (1) the RMSE (root-mean-square-error) obtained through the leave-one-out cross validation (LOOCV) strategy and (2) the RMSE on the validation set (Table S3).

**MGH dataset validation**

We used a recent proteomic study106 of patients enrolled at the Mass General Hospital (MGH, Boston, MA) as an independent validation cohort of the severity model trained on the plasma proteomic data. For this analysis, we used our previous proteomic model fitted on our training cohort to predict disease severity and compared to the WHO scale established for those samples at day 0 to reproduce the hypothesis of our model. We report the r and p value of the Spearman correlation test between the predictions of our model and the WHO scale labels (Related to Figure S6 and Data S1). As the MGH dataset was missing 43 protein expressions...
compared to the assay that we used, we imputed the median value of the training cohort to samples of the MGH dataset before predicting.

QUANTIFICATION AND STATISTICAL ANALYSIS

Univariate analysis
We used the R environment (http://www.r-project.org/) for statistical analysis. We chose to apply a ranked regression analysis for each feature relative to the severity of the patient at the time of sampling using a spearman correlation test. Healthy controls were labeled with the numerical value 1, mild cases with 2, moderate = 3, and severe = 4. For each statistic, we report both p values associated with the spearman correlation coefficient, and the r correlation coefficient.

Multi-omic factor analysis
Multi-omic factor analysis (MOFA) was applied simultaneously across plasma (Olink) and the single-cell proteomics data (cell frequency, endogenous signaling, IFNa/IL-2/IL-4/IL-6 signaling response, LPS/CL097 signaling response, and PI signaling response). MOFA infers a set of factors that capture both biological and technical sources of variability that are shared across different datasets. MOFA models were constructed using the six data layers which were supplied as a list of matrices. We followed the developers’ directions for model selection and downstream analysis.32 Since MOFA is not guaranteed to find a global optimum, 10 model trials were performed using different random initializations. For each trial, the number of factors was calculated by requiring at least 2% variance explained for any single dataset. The model with the highest evidence lower bound was selected out of these 10 trials (model 6, Figure S3A). The factors calculated by model six of our 10 trials were extracted for downstream analysis. MOFA enables variance decomposition of the calculated factors and uses a coefficient of determination (R^2) to quantify the fraction of variance explained by each factor for each dataset, which we examined first to determine how each dataset contributes to each factor (Figure S3B). Next, we re-gressed the 17 factors on COVID-19 severity, which was dummy encoded as follows: Control = 1, Mild = 2, Moderate = 3, Severe = 4. Regression estimates, 95% confidence intervals, and p values were examined (Figure S3C). Factors 3, 8, and 10 were significantly associated with COVID-19 severity. To assess the robustness of factors across model trials, we calculated Pearson correlation coefficients between every pair of factors across all trials. Factors 3 and 10 were consistently discovered in all model instances (data not shown). Finally, we visualized our samples across factors 3 and 10 using a bivariate scatterplot (Figure 1D).

Covariate analysis
Correlations between the covariates “age”, “gender”, “obesity”, “Hispanic ethnicity”, and “days between self-reported symptom onset and sample collection” with our outcome “severity” was assessed with Pearson correlation and are reported as a correlation heatmap (Figure S5). Gender was encoded as ‘Male’ = 0, ‘Female’ = 1. Obesity was encoded as ‘not obese’ = 0, ‘obese’ = 1. Hispanic ethnicity was encoded as ‘not Hispanic’ = 0, ‘Hispanic’ = 1.

Multivariate analysis and stacked generalization
For the multivariate analysis, a LASSO model was trained independently on each omics dataset independently using the caret and glmnet packages. For a matrix X of all biological features from a given -omic dataset of N samples, and a vector of disease severity Y, the LASSO algorithm (29) calculates coefficients b to minimize the error term L(β) = 1/2(Y − XB)^2 + λ||B||_1. The L1 regularization is used to increase model sparsity for the sake of biological interpretation and model robustness. Once a LASSO model is trained for each omics modality (individual model performance Table S2), the multi-omics analysis can be carried out by performing stacked generalization35 on the new representation of the data by using the outputs of the previous layer of models as predictors with the constraint that their participation should be a non-negative coefficient. Specifically, a LASSO model is first constructed on each omics modality, then all estimations of disease severity are used as predictors for a second-layer constrained regression model. Intrinsically, this is equivalent to a weighted average of the individual models with the coefficients of the LASSO model as desired weights.

Leave-one-out cross-validation
An underlying assumption of the LASSO algorithm is statistical independence between all observations. At each iteration of this algorithm, one sample is kept for independent validation. This is an extreme case of the k-fold cross validation called leave-one-out cross validation (LOOCV).108 The model is trained on all the samples except the one blinded sample and the reported results are exclusively based on the blinded subject. We repeat this method for every sample, hence building N successive models. For stacked generalization, a cross-validation was implemented in a two-step approach where the first layer selects the best values of λ for each individual omic and reports the intermediate prediction. Then, the second layer optimizes the constrained regression of all predictors for the stacked generalization step.

The results of the training cohort were validated with an independent cohort that is totally blinded during the design and fitting of the models. This independent validation is used only for performance evaluation throughout the results and used to assess the performance as a second step after the cross-validation.
Multi-class receiver operating characteristics (AUC)
To characterize our predictions’ separability in a multi-class setting, we used a combined metric of the area under the receiver operating curve (AUC) in both the training and validation model. This metric for multi-class analysis uses every combination of labels in one-to-one comparisons. Every subset of predictions on a pair of classes is considered independently, and the AUC is calculated for each specific pair. Once all the subset AUCs are computed, the generalizable AUC combines them, taking their mean across all comparisons, to give the multi-class model’s overall performance.110

Bootstrap analysis for feature selection
For each omics dataset, we performed a bootstrap analysis where we repeat a bootstrapping procedure on the dataset and train a cross-validated model. At each iteration, we keep the non-zero coefficients selected by the LASSO procedure on the bootstrapped dataset and we repeat the procedure 1,000 times.45,46 We report the frequency of selection of the features as well as their median coefficient in all the bootstrapped models. This method selected 44 frequency, 599 endogenous, 536 PI-response, 492 IFNa/IL-2/IL-4/IL-6, and 783 proteome features that were informative in at least one iteration of the bootstrap procedure (see Data S1). To assess the relative importance of each feature to the model, we ranked features in each data layer based on their frequency of selection.

Correlation network
The features are visualized using a correlation graph structure to identify correlated feature populations. We used a tSNE111 layout for the visualization of all the features calculated from the matrix including all the samples available. On this graph, each biological feature is denoted by a node whose size is dependent on the $-\log_{10}$ of p value of correlation with disease severity (Spearman). The correlation network between the features is represented by an edge where the width of the edges is proportional to the Spearman p value of the correlation between a pair of nodes on a log10 scale.

Confounder analysis
A post-hoc linear regression analysis was used as a statistical method to exclude the likelihood that certain clinical or demographic variables confounded the predictive accuracy of the severity model. We considered the following confounders: “days between symptoms onset and sample collection”, “age”, “gender”, “Hispanic ethnicity”, and “obesity”. This analysis fits a regression using the values of the cross-validated SG model and adds the confounders together to regress them against the severity predictor. The resulting coefficients of the regression are computed and reported in Table S4. Their significance is reported using the F-statistic from which we derive the p value for the model coefficient and the confounders.

Plasma proteomic pathway identifier
To obtain pathway information of selected proteome features, we utilized Reactome (www.reactome.org), a web-based resource for identifying biological pathways, in which we used the list of the 10% bootstrap-selected proteins as an input. Reactome provided a list of pathways, identified by a Reactome gene set identifier, which we assessed for the proteins part of specific pathways.

Longitudinal modeling
Correlation analysis of bootstrap selected features (10%) with “time since symptom onset” were calculated with a generalized additive model (GAM) using the R package “mgcv”.102 For each feature, a factor-smooth interaction GAM was constructed with Severity and “time since symptom onset” as explanatory variables. Three knots were used to represent the smooth term. P-values of smooth terms for Mild, Moderate, and Severe groups were corrected for false discovery per data layer with Benjamini-Hochberg,112 and are reported in Data S1C.
Model formula (R notation) $y \sim \text{Severity} + s(DOS, \text{by = Severity, k = 3})$. 