Research Article

Pathogenic neutrophilia drives acute respiratory distress syndrome in severe COVID-19 patients

Running title: Pathogenic neutrophilia in severe COVID-19

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Abstract

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the ensuing COVID-19 pandemic have caused ~40 million cases and over 648,000 deaths in the United States alone. Troubling disparities in COVID-19-associated mortality emerged early, with nearly 70% of deaths confined to Black/African-American (AA) patients in some areas, yet targeted studies within this demographic are scant. Multi-omics single-cell analyses of immune profiles from airways and matching blood samples of Black/AA patients revealed low viral load, yet pronounced and persistent pulmonary neutrophilia with advanced features of cytokine release syndrome and acute respiratory distress syndrome (ARDS), including exacerbated production of IL-8, IL-1 β , IL-6, and CCL3/4 along with elevated levels of neutrophil elastase and myeloperoxidase. Circulating S100A12⁺/IFITM2⁺ mature neutrophils are recruited via the IL-8/CXCR2 axis, which emerges as a potential therapeutic target to reduce pathogenic neutrophilia and constrain ARDS in severe COVID-19.

Introduction

The coronavirus disease 2019 (COVID-19) pandemic, caused by SARS-CoV-2, is associated with high morbidity and mortality. To date, the pandemic has caused ~40 million cases and over 648,000 deaths in the United States alone, where communities of color are disproportionately burdened with disease severity and mortality^{1, 2}. A hallmark of COVID-19 pathogenesis is the vast array of clinical presentations and outcomes, ranging from asymptomatic or mild, self-limiting disease to acute respiratory distress syndrome (ARDS), multiorgan failure, and death. Indeed, such diversity in COVID-19 pathogenesis poses challenges to identify processes that dictate progression to severe disease. Early reports highlighted systemic hyperinflammatory responses that are linked to disease severity³. Cytokine release syndrome, also called a cytokine storm, has been observed in many patients and is suspected of causing the detrimental progression of COVID-19 and sustained immune dysregulation⁴.

Severe COVID-19 parallels the pathophysiology of sepsis⁵, where clinical presentation often includes granulocytosis, elevated proinflammatory cytokine production, aberrant myeloid activation, altered dendritic cell (DC) population dynamics, and lymphopenia^{6, 7}. Early single-cell analyses of bronchoalveolar lavage fluid (BALF) samples implicated dysregulated monocyte and macrophage responses as central features in poor outcomes^{4, 8}. As such, most early efforts have been focused on characterizing and constraining aberrant monocyte/macrophage responses. Concurrently, reports of neutrophilia in the peripheral blood arose, and the neutrophil to lymphocyte ratio emerged as an independent risk factor for disease progression⁹. Interestingly, a prior investigation of ARDS following sepsis identified sustained neutrophilia associated with worsened prognosis and death compared to patients who resolved neutrophilia and showed an increase in tissue-resident alveolar macrophages¹⁰, which may present another shared feature between COVID-19 and sepsis.

To date, there have been many studies of neutrophil responses in the blood of COVID-19 patients^{11, 12, 13, 14, 15}. However, an in-depth analysis of the neutrophil activity in the lungs is lacking. Expressly, the extent to which neutrophils contribute to cytokine release syndrome, tissue damage, and ultimately ARDS in severe COVID-19 cases is incompletely understood. Our systems immunology approach, combining high-dimensional (Hi-D, 30-parameter) flow cytometry and multi-omics single-cell sequencing analyses of immune profiles from the airways and matching blood samples of Black/African-American (AA) patients, revealed pronounced and sustained pulmonary neutrophilia as a hallmark of severe disease. Furthermore, mature pulmonary neutrophils produce very high levels of the neutrophil chemotactic factor IL-8 in addition to IL-1 β , IL-6, and CCL3/4 along with copious amounts of neutrophil elastase (NE) and myeloperoxidase (MPO). Altogether, our findings highlight that transcriptionally active and highly inflammatory neutrophils are sustained in the airways of severe patients and that reducing pathogenic neutrophilia may constrain ARDS in severe COVID-19 disease.

Results

Study Cohort

To better understand immune dynamics in Black/AA patients with severe COVID-19, we analyzed airway and matching blood samples from a cohort of 35 individuals presenting to Emory University Hospitals (severe) or the Emory Acute Respiratory Clinic (mild-acute) in Atlanta, GA, USA (Fig. 1 and Extended Data Table 1), including 8 demographic-matched healthy adults as controls. Of the 27 individuals who were confirmed positive by PCR from nasopharyngeal swabs, 18 had an NIH severity score of "critical" (<https://www.covid19treatmentguidelines.nih.gov/overview/clinical-spectrum/>; referred to as severe herein) and were admitted to the intensive care unit (ICU) requiring mechanical ventilator support, and 9 were mild (mild-acute) outpatients. All severe patients in our cohort received corticosteroids (dexamethasone or equivalent). Approximately half received one or more doses of the antiviral medication remdesivir, with an average ICU stay of 26 days (see Extended Data Tables 1 and 2).

Exacerbated neutrophilia in the airways and matching blood of severe COVID-19 patients

We first characterized major immune lineages in the airways (endotracheal aspiration, ETA) and matching blood samples by Hi-D flow cytometry and observed a pronounced circulating neutrophilia and lymphopenia (notably T and NK cells), which is similarly reflected in the airways (Fig. 2a-e and Extended Data Fig. 1). This is in line with recent reports showing lymphopenia associated with significant alterations in the myeloid compartment^{15, 16}. Strikingly, in most cases of severe disease, ≥85% of all pulmonary leukocytes were neutrophils (Fig. 2b). This contrasts with other studies that have reported much more heterogeneity in neutrophil frequency in patients' blood and lungs^{17, 18}. In addition, circulating T cells and NK cells decline with increased disease severity (Fig. 2d), which has recently been reported as a feature of COVID-19 pneumonia¹⁹. We also observed a notable decrease in pulmonary NK cells associated with disease severity (Fig. 2b). However, there were only subtle differences in the B-cell compartment (which were detected at very low levels in the airways) and myeloid-derived cells (MdCs) compartment compared to other reports^{8, 20, 21}, highlighting the importance of neutrophilia and neutrophil-to-lymphocyte ratio in our cohort. Interestingly, the patient with the lowest neutrophil frequency in the airways corresponds to the youngest patient in the severe disease group (Fig. 2b). However, since we only had a single patient under 35 years old in our severe group, we cannot conclude any associations between neutrophilia and patient age.

Since we identified prominent neutrophilia in our cohort, we developed a Hi-D, 30-parameter flow cytometry panel to interrogate inflammatory neutrophil phenotype in addition to broad leukocyte characterization (see "CoV-Neutrophil" panel in Extended Data Table 3). Here, we used extracellular staining of neutrophil elastase (NE) and CD63 (LAMP3) to assess primary granule release²², coupled with intracellular staining for key effectors implicated in cytokine storm (IL-1β, IL-6, and IL-8). We observed that nearly all neutrophils recruited to the lung developed an inflammatory profile characterized by exacerbated levels of NE and elevated

production of IL-1 β and IL-8 with a dramatic increase in primary granule release (Fig. 2c,e). Indeed, most neutrophils in the airways were positive for all 3 intracellular cytokines and express CD63 on the cell surface (Fig. 2c), suggesting that neutrophils are releasing cytokines upon degranulation. However, this phenotype is diminished in the circulation. A smaller proportion of blood neutrophils undergo granule release and express a cytokine signature limited mainly to IL-1 β production and less IL-6 and IL-8 (Fig. 2e). Similarly, we find a subset of neutrophils in the lung that co-express the highest levels of surface CD10, CD184 (CXCR4) and NE (Fig. 2c). Interestingly, neutrophils in the lung express lower levels of Fc γ RII (CD32) than those in the blood (Extended Data Fig. 5a). Together, these results demonstrate systemic neutrophilia in severe COVID-19 patients where neutrophils accumulating in the airways produce exacerbated levels of potentially damaging enzymes (e.g., NE) and inflammatory cytokines (IL-1 β /6/8) while undergoing pronounced primary granule release.

Neutrophil-secreted inflammatory molecules in the airways potentiate acute respiratory distress syndrome in severe patients

To better understand the inflammatory signaling milieu and relate the intracellular cytokine staining to protein secretion, we measured 21 total analytes in the airways and plasma using the Mesoscale UPLEX platform (Extended Data Table 4). We found that IL-8 is the most abundant chemokine in the airways of severe patients accompanied by high levels of IL-1 β and IL-6. This is in line with our Hi-D flow cytometry data showing neutrophilia and pronounced intracellular staining signal for IL-8 and IL-1 β (Fig. 3a-i). Strikingly, IL-8 levels were ~100-fold higher in the airways of severe versus mild patients and that of IL-1 β and IL-6 levels in severe patients. These findings are in stark contrast to an early report²³ that showed no differences between circulating and pulmonary IL-6 and IL-8 secretion, reported in the <100 pg/mL range. However, more recent studies in both ETA²⁴ and BALF²⁵ samples have noted similar findings that corroborate our own. These data, therefore, implicate IL-8 signaling in the prominent neutrophilia observed in severe patients in our cohort.

We noticed only minor differences in circulating levels of many targets between healthy individuals and those affected by severe COVID-19 disease suggesting an uncoupling of local versus systemic inflammatory responses²⁴, particularly for IL-8 and IL-1 β , but also IL-6 to a lesser extent (Fig. 3a,d,g). Conversely, we observed a rise in circulating IL-1RA levels with increasing disease severity, similar to other reports²⁶. However, we observed an opposite trend in the airways, where IL-1RA levels appear to be decreased in severe patients (Fig. 3j). This is in stark contrast to IL-1 β levels, which were not markedly different in the circulation but significantly increased in the airways with disease severity (Fig. 3j). We also found increased CCL2 (MCP-1) in the airways of severe disease, and the same trend for M-CSF and TNF was observed, albeit at much lower concentrations (Fig. 3j). Interferon (IFN)- γ was detected at the highest levels in the airways of severe patients with no obvious differentiation in plasma across all groups. IL-10 was also increased in the airways of severe patients with notable variation at low levels (Fig. 3j). In contrast to other studies^{27, 28}, we did not observe appreciable differences in IL-18 or CXCL10 (IP-10) levels across patient groups (Extended Data Fig. 2). Additionally,

CXCL12 was not detected in the airways of patients, and there were no notable differences in plasma levels across patient groups (Extended Data Fig. 2).

We further measured the concentration of neutrophil-derived MPO and its enzymatic activity across patient groups. MPO is an important neutrophil effector molecule (originating from primary granules like NE) implicated in respiratory illnesses such as cystic fibrosis²⁹, and can contribute to lung tissue damage during neutrophilic pneumonitis. MPO protein concentrations increased stepwise with disease severity in the plasma and respiratory supernatant (Fig. 3k). However, MPO activity was mostly detected in the airways, and rose with increasing disease severity (Fig. 3k).

Recruited airway neutrophils are mature, transcriptionally active, and further differentiate into a highly inflammatory state

To gain insight into the cellular states and transcriptional regulation in patients with severe COVID-19 disease, we performed multi-omics scRNA-seq on cells from whole blood and ETA samples of severe patients and whole blood from demographic-matched healthy control (Fig. 1). We assessed immune features in data integrated from four or more patients from the same cohort (i.e., healthy vs. severe; see Methods). First, major lineages from blood (Fig. 4a) and ETA (Fig. 4g) were gated manually using the antibody-derived tag (ADT) data for surface protein expression. Next, we used the gene expression (GEX) data to generate clusters for each major lineage identified based on their surface markers, as per our previously described SuPERR-seq pipeline³⁰ (Extended Data Fig. 3). For example, neutrophils identified by CD66b and CD16 surface ADT in the blood (Fig. 4b) and ETA (Fig. 4h) were then selected and clustered independently by GEX data (Fig. 4c,i) for further analyses.

A prior study had reported an increase in immature neutrophils in the lung concomitant to increased circulating immature neutrophils from emergency hematopoiesis in severe COVID-19 patients¹⁶. We, therefore, assessed the immature neutrophil phenotype in the blood (Fig 4a-e) and lung (Fig 4f-j) by scRNA-seq in our patient cohort. We readily identified a cluster of neutrophils expressing *CAMP*, *LTF*, *RETN*, *OLFM4*, *DEFA3*, *CD24*, and *MMP8* (Fig. 4e and Extended Data Fig. 4) in the blood of severe patients (cluster 3 in blood neutrophils) that also has high expression of calprotectin (S100A8/9) and other calgranulins (Fig 4d). This is consistent with immature neutrophil phenotypes described by others^{7, 15} and was notably absent in neutrophils from healthy blood, confirming an emergency hematopoiesis/granulopoiesis in our patient cohort. However, in contrast to previous studies¹⁶, we did not observe any signature of immature neutrophils in the lungs of severe patients (Fig. 4j), suggesting that immature neutrophils either are not directly recruited to the lung, or quickly differentiate upon infiltration into the lung.

To determine which neutrophil subset in the blood can infiltrate the lung and further differentiate into a pathogenic state, we explored cell-cell communication (CellChat³¹) of clustered cells from blood (Fig. 4c) and lung (Fig. 4h) samples. We found significant communication through the CXCL pathway between the lung neutrophil, myeloid, and non-immune populations (i.e.,

epithelial/stromal cells) with *CXCR2*-expressing neutrophils from the blood (blood cluster 2; Fig. 5a). The *CXCL8* (IL-8)/*CXCR2* pathway was identified as the primary recruitment axis for circulating *CXCR2*⁺ neutrophils (Fig. 5b). These data are in line with our finding that IL-8 was increased in neutrophils by flow cytometry (Fig. 2) and secreted at very high levels in the airways (Fig. 3) and suggests that the *CXCL8*/*CXCR2* signaling axis is important for neutrophil recruitment to the lungs during COVID-19 pathogenesis. Virtually all lung neutrophils (particularly cluster 5), non-immune cells, and monocytes show the potential to recruit circulating neutrophils (Fig. 5a,d), indicating a robust and redundant mechanism of neutrophil recruitment to the airways via the *CXCL8*/*CXCR2* axis. Surprisingly, the immature neutrophils (blood cluster 3) lacked *CXCR2* (Fig. 5c), suggesting that immature neutrophils are unlikely to infiltrate the lung via IL-8. In contrast, a defined subset of mature neutrophils in blood expressing high levels of *CXCR2*, along with interferon-induced *IFITM2* and *S100A11/12*, identify blood cluster 2 as the putative neutrophil subset that can infiltrate the lung via IL-8 (Figs. 4d and 5c-d). It is, therefore, probable that recent lung emigrants would still express detectable levels of *CXCR2*, as well as *IFITM2* and *S100A11/12* (Fig. 5c).

After identifying the blood neutrophil cluster 2 as the putative source of lung-recruited neutrophils, we sought to identify their cell trajectory/differentiation once they enter the inflamed airway. Cell trajectory analyses (scVelo³²) revealed two differentiation pathways (Trajectories 1 and 2) for the recently infiltrated neutrophils (Fig. 5e). Indeed, cells expressing the highest levels of *CXCR2* and *S100A11/12* were at the beginning of the trajectory, further supporting these as the putative emigrant population from the blood (Fig. 5f). Notably, neutrophils recruited to the lung and differentiated along Trajectory 2 experience transcriptional reprogramming to acquire a heightened inflammatory phenotype (Fig. 5e,f). In contrast to the canonical neutrophil differentiation pathway, including a short half-life, the infiltrated neutrophils in severe COVID-19 patients are transcriptionally active (Fig. 5e-g) in comparison to blood neutrophils (Extended Data Fig. 5b) and further differentiate into a hyperinflammatory state (Fig. 5f). This is consistent with more recent reports of neutrophil transmigration in other respiratory illnesses such as cystic fibrosis³³, where neutrophils undergo lung/condition-specific adaptations upon recruitment from the circulation³⁴ instead of the canonical rapid and transient effector function proceeding cell death. Indeed, we observed increased expression of the interferon-stimulated gene (ISG) *IFI30* along with increased expression of *CCL3* (MIP-1 α) and *CCL4* (MIP-1 β) (Fig. 5f), which in turn can promote the recruitment of inflammatory monocytes to the lung and/or proinflammatory macrophage phenotypes in the airways.

Importantly, most neutrophils increase expression of *CXCL8* (IL-8) when recruited to the airways (Fig. 5d), perpetuating neutrophil recruitment to the lung. These findings further corroborate our Hi-D flow cytometry, gene expression, and secreted protein data analysis identifying IL-8 as the most abundant neutrophil-derived chemokine (and neutrophil chemotactic factor) in the airways of severe COVID-19 patients. Furthermore, most neutrophils increase the expression of *CXCR4* when recruited to the airways (Fig. 5f). Interestingly, we find that *CXCR4* expression, previously attributed to immature neutrophils¹⁶, is increased in mature lung-recruited neutrophils (Fig. 5f). This is consistent with *CXCR4* expression dynamics previously detected in non-COVID lung inflammation, including in cystic fibrosis³⁵ and malaria³⁶. Taken together, the

progressive increase in *IFI30*, *CCL3/4*, along with abundant *de novo* *CXCL8* and *CXCR4* mRNA transcripts, reveal a transcriptionally active state in neutrophils that is poised to sustain a hyperinflammatory milieu in the lung of severe COVID-19 patients.

Viral load in the respiratory tract does not correlate with disease severity

We then investigated whether the recruited neutrophils or other cell types (i.e., myeloid, lymphoid, and non-immune cells) in the airways of severe patients were infected with SARS-CoV-2. By including the SARS-CoV-2 genome sequence into our human reference transcriptome in the multi-omics single-cell analysis, we were able to assess viral mRNA (vRNA) transcripts at a single-cell level (Fig. 7a). Notably, we did not detect vRNA in any cell types in the blood or airways of severe patients (Fig. 7b,c). Further, SARS-CoV-2-specific RT-qPCR revealed that viral load was decreased in the upper airways of severe patients admitted to the ICU compared to mild-acute patients (Fig. 7d,e). However, we did note a gene signature in neutrophils associated with response to IFN- γ (Fig. 5g and Extended Data Fig. 5b), including a pronounced increase in *IFITM2* (Extended Data Fig. 5c), which has been shown to promote SARS-CoV-2 infection in human lung cells³⁷.

Pulmonary TNF and IL-1 β promote neutrophil reprogramming in the lungs

To ascertain which ligand-receptor pairs are potentially responsible for the transcriptional reprogramming of neutrophils in the lung, we first performed differential gene expression (DGE) analysis between blood neutrophil cluster 2 and lung neutrophils to identify genes that are upregulated in lung-recruited neutrophils (Fig. 6a). Next, we used the computational method NicheNet³⁸ to identify the potential (prioritized) ligands that could induce the upregulation of the genes identified by the DGE analysis, indicating their potential role in neutrophil reprogramming in the lung. *TNF*, *IL1B*, and *APOE* were the highest prioritized ligands with high ligand activity whose signaling axes have the regulatory potential to drive gene expression profiles observed in lung-recruited neutrophils (Fig. 6b). Importantly, NicheNet analysis revealed *TNF* as the ligand predicted to increase *BCL2A1* expression in pulmonary neutrophils, as well as *CCL4* (MIP-1 β) and *CXCL16*. Furthermore, both *TNF* and *IL1B* are the ligands predicted to induce *NFKBIA* and *CXCL8* (IL-8), and *IL1B* shows the greatest potential to induce *CCL3* (MIP-1 α) expression in recruited neutrophils. *APOE* is predicted to upregulate the expression of *FCER1G* in neutrophils in the lung. Notably, *TNF*, *IL1B*, and *HMGB1* are the ligands that have the widest range of regulatory potential, inducing neutrophil reprogramming by upregulating most genes that we identified as differentially expressed (Fig. 6a) in lung-recruited neutrophils (Fig 6b).

Pulmonary T cells represent the primary cell population expressing *TNF* in the airways, where MdCs have the highest *IL1B* and *APOE* transcripts (Fig. 6c,e). From the transcriptome data, ligand-receptor pair analyses further identified putative signaling mediators for the top 15 prioritized ligands (Fig. 6d). *TNF* is predicted to signal through a receptor that has a *CALM1* association (Fig. 6d). Intriguingly, it has been previously reported that *CALM1* can bind other transmembrane proteins, including ACE-2³⁹, and regulate their cell surface expression⁴⁰. *IL1B* is predicted to signal through the canonical IL1B/IL1R2 pathway, and *APOE* through the APOE/SORL1 lipid pathway. *HMGB1* is predicted to signal through *LY96* (MD-2), which is commonly associated with TLR4 (Fig. 6d)^{41, 42, 43}. Accordingly, *IL1R2* and *CALM1* transcripts are

abundant in neutrophils from blood cluster 2, and their expression are sustained upon recruitment to the airways (Fig. 6e).

Collectively, we show here that TNF- and IL-1 β -mediated transcriptional reprogramming of lung infiltrating neutrophils leads to induction of proinflammatory *NFKBIA*, *CCL3* (MIP-1 α), and *CCL4* (MIP-1 β) along with *TNFAIP3/6* and *CXCL8* (Figs. 5 and 6). Of note, neutrophil-derived *CCL3* and *CCL4* can attract inflammatory monocytes from the circulation to the airways. Interestingly, lung-infiltrating monocytes also produce elevated levels of *CXCL8*, potentiating the recruitment of circulating neutrophils via the *CXCL8/CXCR2* axis (Fig. 5a,d). Hence, both neutrophils and inflammatory monocytes in the airways exacerbate and potentiate pathogenic neutrophilia in the lungs of severe patients in a positive feedback loop.

Discussion

Previous studies have reported increased neutrophilia in severe COVID-19, particularly in the circulation^{14, 44, 45, 46}. In contrast, reports on exacerbated airway neutrophilia and the implication of lung neutrophils as the main cell type driving ARDS in severe patients have yielded inconclusive results^{7, 8, 17, 47}. Here, we present the first comprehensive study of the lung immune response to SARS-CoV-2 in Black/AA individuals and unequivocally identify a robust and sustained airway neutrophilia associated with disease severity. The COVID-19 pandemic further highlighted some of the socio-economic and behavioral inequalities that may have contributed to troubling disparities in COVID-19-associated morbidity and mortality⁴⁸, with almost 70% of deaths being Black/AA patients in some areas^{1, 2}. Although the socio-economic and behavioral differences indeed contribute to health disparities among demographics, a systematic investigation to determine the immunological features that characterize disease severity within Black/AA patients is lacking. Our systems biology approach addresses this knowledge gap and reveals new therapeutic targets to inhibit neutrophil migration, retention, and/or survival in the lung as potential effective interventions for individuals with severe disease that have been disproportionately affected by COVID-19.

Although previous studies on severe COVID-19 in humans have not been conclusive with regards to the role of airway neutrophilia in ARDS, studies in SARS-CoV-2-infected rhesus macaques and mice, where conditions and sample collection are more controlled, have identified exacerbated neutrophilia as a key immunological feature associated with disease severity^{49, 50, 51}. Hence, taken together with previous studies on neutrophils^{8, 14, 17, 45, 46, 47} along with animal models^{49, 50, 51}, our current findings provide compelling evidence that targeting exacerbated airway neutrophilia may constrain ARDS and prevent further lung damage in most patients requiring mechanical ventilator support. Although our studies represent one of the few targeted investigations in Black/AA patients, we believe our results have broader implications and may apply to most patients suffering from neutrophilic ARDS¹¹.

The heightened numbers of neutrophils in the lung are likely to induce and sustain inflammatory signatures by an autocrine/paracrine feedback loop among neutrophils, and paracrine signaling to other cell types that can potentiate disease severity. Notably, NE is a potent serine protease that we show is released from degranulating neutrophils in the lungs (see Fig. 2), and has potential to stimulate production of TNF, IL-1 β , and IL-8^{52, 53}, and also abrogate protective effector functions of T cells and MdCs in the lung by cleaving cell surface receptors such as TLRs and Fc-receptors^{54, 55}. Indeed, we demonstrate NE staining on the surface of T cells and MdCs in the lung (see Extended Data Fig. 5e) in addition to neutrophils, which may explain, in part, the reduction of Fc γ RII (CD32) expression on pulmonary neutrophils (see Extended Data Fig. 5a). Of note, the reduction in CD32 expression may prevent IgG-mediated suppression of ISG induction¹⁴ in pulmonary neutrophils. Indeed, we demonstrate a pronounced signature for the ISG *IFI30* (see Fig. 5f). Similarly, we show by intracellular staining that pulmonary neutrophils are producing exacerbated levels of IL-1 β protein that is likely released upon degranulation along with IL-8 and IL-6 (see Figs. 2 and 3). In addition to neutrophils, the myeloid lineage is also abundant in the airways and as such they are poised to influence disease

progression. For example, we and others¹⁶ have shown that infiltrating neutrophils sustain local production of calgranulins (S100A8/9/11/12), which can signal through and activate myeloid cells via TLRs and RAGE receptors, compounding the already hyperinflammatory lung milieu. Interestingly, the inflammatory monocytes that are likely recruited to the lungs via neutrophil-secreted CCL3/4 also show elevated levels of neutrophil chemotactic factor CXCL8 (IL-8), which helps sustain recruitment of pathogenic neutrophils in a positive feedback loop.

Other immune and non-immune cell populations in the lung may contribute to COVID-19 pathogenesis in severe patients. Non-immune cells (e.g., stromal and epithelial cells) are known targets of SARS-CoV-2 in the lung⁵⁶. Therefore, these cells are also posited to influence immune cell dynamics, especially at the outset of infection⁵⁷. Indeed, our data indicate that non-immune cells play a role in granulocyte activation (Extended Data Fig. 5d) and therefore may initiate neutrophilia at the outset of infection⁵⁸. Additionally, T cell-derived TNF and HMGB1, along with myeloid-derived IL-1 β , have regulatory potential to induce inflammatory gene signatures observed in lung-recruited neutrophils. Strikingly, TNF is the ligand predicted to increase *BCL2A1* expression in pulmonary neutrophils, which is an anti-apoptotic factor known to regulate neutrophil survival^{59, 60}. *TNF* signaling in recruited neutrophils also has the potential to drive *CCL4* (MIP-1 β) expression, along with *CXCL16*, which is another chemotactic factor that can recruit neutrophils and macrophages via the CXCL16/CXCR6 pathway^{61, 62}. In addition, myeloid-derived *APOE* is predicted to regulate the expression of *FCER1G* in lung-recruited neutrophils, which is associated with neutrophil activation during other viral respiratory infections⁶³. Further studies should investigate other effector functions of myeloid and lymphoid subsets and how these cells interact with neutrophils to promote protection or pathology in the lungs of severe COVID-19 patients.

Interestingly, *CXCR4* is upregulated in recruited neutrophils in severe patients (Figs. 4d,i and 5f), which may promote neutrophil survival and retention during pneumonitis and/or further influence inflammatory neutrophil phenotype. Accordingly, *CXCR4* was shown to promote transcriptional reprogramming of neutrophils in pulmonary tissues⁶⁴. Although we do not find detectable levels of the canonical *CXCR4* ligand (i.e., *CXCL12*) in the airways, we did observe transcripts for *HMGB1* (Fig. 6), which is an alternative ligand for *CXCR4*. Since the IL-8/CXCR2 pathway is most notably increased and likely the primary neutrophil recruitment axis to the airways, we speculate that *CXCR4* signaling instead may promote neutrophil survival and retention at the site of inflammation, which has been previously reported⁶⁵. Additionally, *CXCR4* signaling can stimulate de novo *CXCL8*/IL-8 production^{66, 67}, and has been shown to promote neutrophil extracellular trap release during malaria disease progression⁶⁸. Alternatively, *CXCR4* is also associated with neutrophil aging and senescence^{69, 70, 71}. As such, elevated *CXCR4* may be associated with prolonged neutrophil survival in COVID-19 pathogenesis. In a prior study⁷², we noted a similar pattern of *CXCR4* expression on T cells from severe COVID-19 patients, where a progressive decrease of surface *CXCR4* is associated with recovery. In contrast, patients that succumbed to the disease, showed a time-dependent escalation in *CXCR4*⁺ circulating T cells concomitant to increased *CXCR4*⁺ T cells in the lungs⁷², implicating *CXCR4* on the dysregulated lung-homing inflammatory T cells in COVID-19. Further investigation into the potential role of *CXCR4* in cell survival, retention, or recruitment may unravel novel

therapeutic targets to modulate inflammation and treat severe COVID-19. Notably, therapeutic intervention with a CXCR4 antagonist during malaria-associated ARDS has shown significant benefit in animal models³⁶.

Modulating inflammation through corticosteroids (particularly dexamethasone) has shown clinical efficacy and is now the standard-of-care for patients progressing to severe COVID-19⁷³. However, the pleiotropic effects of glucocorticoids and their propensity to cause neutrophilia are well documented in asthma and chronic obstructive pulmonary disease (COPD)⁷⁴. Although a well-established mechanism of action for dexamethasone is via transcriptional repression of proinflammatory cytokines, we observed very high *CXCL8* and *IL1B* transcripts concomitant with elevated pulmonary IL-8 and IL-1 β protein with prominent signatures by intracellular staining—particularly in pulmonary neutrophils. In contrast, IL-6 levels were lower than that of IL-8 and IL-1 β in our patient cohort, and overly abundant neutrophils were not producing as much IL-6 comparatively, which may explain, in part, why anti-IL-6/IL-6R studies failed to meet primary endpoints⁷⁵. Here, we provide evidence of an uncoupled cytokine profile in airway fluids versus plasma where the lung microenvironment exhibits features of cytokine-induced ARDS driven largely by a proinflammatory, neutrophilic feed-forward loop. Beyond COVID-19, it has been shown that resolution of neutrophilia in ARDS has substantial prognostic benefit¹⁰. Taken together, the *CXCL8* (IL-8)/CXCR2 signaling axis emerges as a key potential target for next-generation immunomodulatory therapy to reduce pathogenic neutrophilia and constrain severe disease in patients in addition to corticosteroids.

We also contend that recognizing the lung pathology in severe COVID-19 to be a neutrophilic and hyperinflammatory disease is paramount to achieve better outcomes in next-generation therapies. Although the lung pathology in COVID-19 is initiated by a viral infection, severe patients in the ICU no longer show signs of uncontrolled viral replication. In fact, not only did we not detect viral transcripts by scRNA-seq within the cells in the airways, but we also noted decreased viral burden in severe patients in the ICU versus mild-acute patients seen in the outpatient clinic (Fig. 7). This is further supported by our previous study where we performed plaque assays on the respiratory secretions from severe patients and revealed significantly diminished, if any, viral plaques from the endotracheal aspirates⁷⁶. This may explain, in part, why antiviral drugs such as remdesivir are not able to prevent death when administered to severe patients in ICU⁷⁷.

It is important to consider potentially confounding factors and limitations in our study. Though a major strength of our study is the uniformity of sample collection in a single disease state, our airway samples are limited to ETA, which contrasts with bronchoalveolar lavage fluid (BALF) used in other studies^{7, 17}. The ETA procedure can sample material from the medial airways—i.e., an intermediate between the distal/lower airways (e.g., BALF) and the proximal/upper airways (e.g., sputum or oro-/naso-pharyngeal samples). Although the relative abundance of immune cells may vary across the upper and lower airways, previous studies observe a correlation between paired ETA and BALF samples. In addition, these studies show that ETA samples are not inherently neutrophilic⁷⁸, though neutrophilia/granulocytosis may be a shared feature of non-COVID-19 and COVID-19 ARDS^{10, 79}. In any event, our study is one of the only

known to date to employ integrated multi-omics single-cell investigation of immunity in exclusively Black/AA subjects, which are disproportionately burdened with severe disease and worse outcomes. Importantly, other studies of COVID-19 immune responses have noted similar features of disease severity^{7, 15, 16}, which suggests that the findings in our target demographic will be broadly applicable to other groups. Finally, our study was limited in scope to 35 total subjects and single collection time points, limiting our abilities to interrogate correlations with clinical outcomes, warranting further longitudinal studies in larger patient cohorts.

In conclusion, we present evidence that neutrophils are poised to be the leukocyte population most responsible for the dysregulated hyperinflammatory response that drives ARDS in severe COVID-19 patients. Neutrophil frequency and inflammatory profiles reveal that they are not only the most abundant leukocyte population in the medial airways, but also major producers of hallmark effector molecules associated with disease severity, including IL-8, IL-1 β , and IL-6, along with potent proteases such as NE and MPO, which are inherently inflammatory and contribute to lung damage/pathology. Furthermore, we provide evidence for a neutrophil feedforward loop where IL-8, produced by virtually all pulmonary neutrophils (and some myeloid and stromal cells), is the primary chemokine recruiting circulating neutrophils and promoting neutrophilia in the inflamed airways.

Collectively, our findings implicate neutrophilia in the immunopathophysiology of severe COVID-19 disease where perpetual, transcriptionally active, and highly inflammatory pulmonary neutrophils drive ARDS despite low viral burden. Thus, therapeutic intervention targeting neutrophil recruitment/retention and/or survival/reprogramming at the site of inflammation has the potential to constrain ARDS in severe patients, particularly those most vulnerable to succumb to COVID-19 disease.

Methods

Ethics and biosafety. A total of 35 individuals were recruited for this study (Extended Data Tables 1 and 2). The 18 severe COVID-19+ patients were recruited from the Intensive Care Units of Emory University, Emory St. Joseph's, Emory Decatur, and Emory Midtown Hospitals. We also recruited 9 mild COVID-19-infected outpatients in the Emory Acute Respiratory Clinic and 8 healthy adults from the Emory University Hospital. All studies were approved by the Emory Institutional Review Board (IRB) under protocol numbers IRB00058507, IRB00057983, and IRB00058271. Informed consent was obtained from the patients when they had decision-making ability or from a legally authorized representative (LAR) if the patient was unable to provide consent. Blood and sputum or endotracheal aspirate (ETA) were obtained. Control blood samples were obtained from healthy adults matched by age and race. Study inclusion criteria included a confirmed COVID-19 diagnosis by PCR amplification of SARS-CoV-2 viral RNA obtained from nasopharyngeal or oropharyngeal swabs, age of 18 years or greater, and willingness to provide informed consent. Individuals with a confirmed history of COVID-19 diagnosis were excluded from the healthy donor group. All work with infectious virus and respiratory samples from COVID-19 patients was conducted inside a biosafety cabinet within the Emory Health and Safety Office (EHSO) and the United States Department of Agriculture (USDA)-approved BSL3 containment facility in the Health Sciences Research Building at Emory University following protocols approved by the Institutional Biosafety Committee (IBC) and Biosafety Officer (see ref⁷⁶).

Patient sample collection and processing. Primary leukocytes from the airways of COVID-19 patients requiring mechanical ventilator support were collected bedside via endotracheal aspiration (ETA) and whole blood collected by standard venipuncture. Plasma from whole blood was isolated via centrifugation at 400 x g for 10 min at 4°C. To remove platelets, the isolated plasma was centrifuged at 4,000 x g for 10 min at 4°C. Untouched circulating leukocytes were isolated using the EasySep™ RBC Depletion Reagent (StemCell Technologies). ETA (from severe patients) or non-induced sputum (from mild patients) was mixed 1:1 with a 50mM EDTA solution (final concentration 25 mM EDTA) in custom RPMI-1640 media deficient in biotin, L-glutamine, phenol red, riboflavin, and sodium bicarbonate (defRPMI-1640), with 3% newborn calf serum (NBCS) and mechanically dissociated using a syringe to liberate leukocytes from mucins and other respiratory secretions. Supernatants were collected for further analysis, and then cells underwent an additional mechanical dissociation step using 1-3 mL of a 10 mM EDTA in defRPMI-1640 + 3% NBCS and a P1000 pipettor. Cells were then washed with 10 mL defRPMI-1640 + 3% NBCS, passed through a 70 µm nylon strainer, and pelleted through a 2 mL 100% NBCS layer prior to counting and downstream processing.

High-dimensional (Hi-D) 30-parameter flow cytometry. Cells (up to 10⁷ total) were resuspended in defRPMI-1640 with 3% newborn calf serum and Benzonase™ (FACS buffer) in 5 mL FACS tubes and pre-incubated with GolgiStop™ (BD Biosciences) for ~60 min at 4°C. Human TruStain FcX™ was then added, followed by a 10 min incubation at RT. The 24-color extracellular staining master mix (Extended Data Table 2) was prepared 2X in BD Horizon™ Brilliant Stain Buffer to prevent staining artifacts from BD Horizon Brilliant dye interactions and

added 1:1 to cells, then incubated for 30 min at 4°C. Following staining (and total 1 h exposure to GolgiStop™), cells were washed with ~4 mL FACS buffer. Next, the cells were resuspended in 200 µL of BD Cytfix/Cytoperm™ fixation/permeabilization solution and incubated at 4°C for 30 min followed by a wash with ~4 mL BD Perm/Wash™ Buffer. The 4-color intracellular staining (Extended Data Table 3) was prepared in BD Perm/Wash™ Buffer, and cells were stained for 30 min at 4°C. Cells were washed with ~4 mL BD Perm/Wash™ Buffer, then resuspended for a final 20 min incubation in 4% PFA and transported out of the BSL3 containment facility. Cells were washed in ~4 mL FACS buffer, then resuspended in 200-1000 µL FACS buffer for acquisition using BD FACSDiva™ Software on the Emory Pediatric/Winship Flow Cytometry Core BD FACSymphony™ A5. To distinguish auto-fluorescent cells from cells expressing low levels of a particular surface marker, we established upper thresholds for auto-fluorescence by staining samples with fluorescence-minus-one (FMO) control stain sets in which a reagent for a channel of interest is omitted. Data were analyzed with FlowJo™ v10.8 (FlowJo LLC).

Cell-surface antibody-derived tag (ADT) staining, single-cell encapsulation, and library generation. Leukocytes from whole blood and ETA samples were incubated with oligo-conjugated Ig-A/D/G/M for 10 min at 4°C followed by the addition of Human TruStain FcX™ (BioLegend) and 10 min incubation at RT. Cells were then surface stained with oligo-conjugated monoclonal antibody panel (total 89 antibodies; Extended Data Table 5) for 30 min at 4°C, followed by two washes in def-RPMI-1640/0.04% BSA. Cells were resuspended at a concentration of 1200-1500 cells/µL in def-RPMI-1640/0.04% BSA and passed through a 20 or 40 µm cell strainer before loading onto a Chromium Controller (10X Genomics, Pleasanton, CA). Cells were loaded to target encapsulation of 10,000 cells. Gene expression (GEX) and antibody-derived tag (ADT) libraries were generated using the Chromium Single Cell 5' Library & Gel Bead Kit v1.1 with feature barcoding following the manufacturer's instructions. GEX libraries were pooled and sequenced at a depth of approximately 540,000,000 reads per sample in a single S4 flow cell and ADT libraries at a depth of approximately 79,000,000 reads per sample in a single lane of an S4 flow cell on a NovaSeq™ 6000 (Illumina, San Diego, CA; Extended Data Table 6)

Multi-omics single-cell RNA sequencing (scRNA-seq) analysis. Single-cell 5' unique molecular identifier (UMI) counting and barcode de-multiplexing were performed using CellRanger Software (v.5.0.0). To detect SARS-CoV-2 viral RNA reads, we built a custom reference genome from human GRCh38 and SARS-CoV-2 references (severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome, GenBank MN908947.3). Splicing-aware aligner STAR⁸⁰ was implemented to align FASTQ inputs to the reference genome, and the resulting files are automatically filtered by CellRanger to include only cell barcodes representing real cells. This determination is based on the distribution of UMI counts. ADT reads were aligned to a feature reference file containing the antibody-specific barcode sequences. To recover neutrophils, we applied our SuPERR-seq pipeline as previously described³⁰. Briefly, we recovered neutrophils from CellRanger unfiltered count matrices by plotting surface CD16 ADT and CD66b ADT using the "FeatureScatter" function in Seurat v4.0⁸¹ (R version 4.0.2). The double-positive cell barcodes were then extracted and further evaluated

by GEX to confirm viable neutrophil identity. A threshold for mitochondrial content per barcode was determined for each sample independently and applied as a cutoff to remove dead or dying cells (Extended Data Table 7). Most samples show high cell viability with a minimal proportion of dead cells.

The UMI counts of the GEX data were log-normalized by the "NormalizeData" function in Seurat before downstream analysis, following the optimal workflow we previously described for sample normalization and data integration⁸². Center log-ratio (CLR) transform in Seurat was performed on ADT UMIs when recovering neutrophils from the unfiltered matrices. For surface protein visualization to classify major lineages using our SuPERR-seq workflow³⁰, ADT UMIs were normalized using the R package Denoised and Scaled by Background⁸³ (DSB) to remove ambient UMI counts (i.e., background) prior to manual sequential gating by surface expression (Extended Data Fig. 4) in SeqGeq v1.7 (FlowJo, LLC). DSB uses empty droplets to calculate background expression, which was manually selected according to the distribution of total ADT per cell in the raw count matrices (Extended Data Table 8). To minimize the influence from non-informative empty droplets, we removed cell barcodes with less than 100 total ADT UMIs before plotting the ADT distribution.

Before integrating the multiple datasets, we first classified major lineages in individual samples based on a combination of gene transcript and surface protein markers (SuPERR-seq workflow³⁰) as in Fig. 4 for samples where the ADT library was of sufficient quality to allow manual gating (Extended Data Fig. 4). Cell barcodes within each major lineage that co-expressed markers exclusive to other major lineages were considered cell doublets and removed (Extended Data Fig. 4). In addition, we removed cell barcodes with extremely high total ADT UMIs, which we considered to be aggregated cells. To efficiently integrate replicate samples, we concatenated major lineages derived from the same tissue in different donors. To minimize batch effects and optimize data integration, we followed the data normalization and merging strategies described previously⁸². Briefly, samples were first treated individually, and log-normalized count matrices were scaled/Z-transformed, and the "vst" method of the Seurat function "FindVariableFeatures" was utilized to select the top 1000 highly variable genes (HVGs) of each sample. HVGs shared between replicate samples were used to perform principal component analysis (PCA). To visualize the data, we performed UMAP reduction of the first 30 PCs, and cell clustering was generated using the Leiden community detection algorithm at a resolution of 0.8. UMAP visualizations for the integrated Blood and integrated ETA were generated using Seurat v4 data integration workflow.

Receptor-ligand interaction analyses. Clustered cells from lung and blood samples from each patient were investigated for evidence of intercellular communication using CellChat³¹. Clustered cell populations from the lung samples were combined with the blood neutrophils to determine which lung cell populations could recruit circulating neutrophils. We utilized NicheNet³⁸ to determine which ligand-receptor pairs could be responsible for the different transcriptional states of the neutrophil populations in the lung and blood samples. We focused on certain neutrophil clusters as the receiver populations, considering the remaining neutrophils and other lung cell populations as the senders and thus potential interactors. The target set of

genes was determined using Seurat::FindMarkers(min.pct = 0.1), keeping only those genes with an adjusted p-value lower than 0.05 and average log2-fold change of more than 0.2. To address gene expression changes due to lung infiltration, we ran the algorithm using blood neutrophil cluster 2 as the most likely candidate for lung infiltration. The genes considered were those differentially expressed between blood cluster 2 and the lung neutrophils and genes differentially expressed between blood cluster 2 and those cells that progress along "Trajectory 2" in the lung (see Fig.5).

Cell trajectory analyses. The Python toolkit scVelo³² inferred the trajectories using biological data of the ETA neutrophils. Input data for scVelo analysis was intron, exon, and spanning count matrices estimated using the dropEST tool⁸⁴, then filtered with previously identified neutrophil cell barcodes in R studio. Intron, exon, and spanning matrices were compared to identify missing rows (genes) and were added to each matrix to equalize dimensions. The exon matrix contained the spliced matrix, and the sum of the intron and spanning matrices constituted the unspliced matrix. Spliced and unspliced matrices were imported with anndata library, and pandas library was used to import gene names and cell barcodes. Raw count matrices were added to anndata object layers as 'spliced' and 'unspliced.' Then, gene names and cell barcodes were attached to variables and observations of anndata object, respectively. Anndata object was transposed, followed by the regular scVelo analysis. The default parameters of plotting velocity streams include vkey='velocity', colorbar=True, alpha=0.3, sort_order=True, and legend_loc='on data'.

Pathway and process enrichment analyses. Differential gene expression (DGE) analyses were performed in Seurat v4 and imported for gene annotation and further analysis using Metascape⁸⁵. DGE between neutrophils versus the total ETA were used to generate Fig. 5g and non-immune cells versus total ETA were used for Extended Data Fig. 5d.

Mesoscale U-PLEX assays. U-PLEX Biomarker Group 1 Human Multiplex Assays (Meso Scale Discovery) were used to evaluate levels of 21 analytes following the manufacturer's protocol (Extended Data Table 4) in plasma and UVC-inactivated respiratory supernatants (see ref⁷⁶). Samples were diluted 1:5 for all assays except for IL-8, MCP-1, and IL-1RA, which were above the upper limit of detection for the assay and were diluted 1:200 to acquire measurement within the assay range. Final values were obtained by multiplying measurements by their respective dilution factor.

Myeloperoxidase (MPO) content and activity. The abundance and activity of MPO were quantified as previously described²⁹. MPO activity and protein concentration were measured sequentially following the immunocapture. On average, across six 96-well plate assays, lower limits of quantification were 4.0 ng/mL (activity) and 0.84 ng/mL (protein). Samples above the lower limit of detection but below the lower limit of quantification were imputed as half of the latter, and those detected above the highest standard of 50 ng/mL (i.e., above the upper limit of detection) at all dilutions were imputed as twice the standard concentration (Extended Data Table 4).

SARS-CoV-2 quantitative reverse transcription PCR (RT-qPCR). Viral (v)RNA was extracted from the respiratory secretions of COVID-19 patients using the *Quick-RNA*[™] Viral Kit (Zymo Research) following the manufacturer's protocol and complementary (c)DNA synthesized using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems[™]) per the manufacturer's instructions, then diluted 1:5 in nuclease-free water. 10 µL diluted cDNA was used with the NEB Luna[®] Universal Probe qPCR Master Mix (New England BioLabs[®] Inc.) following the manufacturer's protocol and performed in 384-well plates using a QuantStudio[™] 5 Real-Time PCR System (Applied Biosystems[™]). Primer/probe pairs were: AGAAGATTGGTTAGATGATGATAGT (forward), TTCCATCTCTAATTGAGGTTGAACC (reverse), and /56-FAM/TCCTCACTGCCGTCTTGTGACCA/3IABkFQ/ (probe), which were designed from sequences previously described⁸⁶ (Integrated DNA Technologies; IDT). To generate a standard curve for the quantification of SARS-CoV-2 genome copies a gBlock from IDT with the following sequence was used as a standard:
AATTAAGAACACGTCACCGCAAGAAGAAGATTGGTTAGATGATGATAGTCAACAACTGTT
GGTCAACAAGACGGCAGTGAGGACAATCAGACAACTACTATTCAAACAATTGTTGAGGTTC
AACCTCAATTAGAGATGGAAGTTACAGTTTCAGTGTTC AATTAA.

Statistical analyses. Statistical analyses were performed using GraphPad Prism9. Data were analyzed for distribution (normal (Gaussian) vs. lognormal) independently using the D'Agostino and Pearson test for normality in the untransformed and Log10-transformed data. In cases where the sample size (*N*) was too small for D'Agostino and Pearson normality test, the Shapiro-Wilk test was used to assess distribution. When data passed both distribution tests, the likelihood of each distribution (normal vs. lognormal) was computed, and QQ-plots were generated. When Log10 transformed data had a higher likelihood of a normal distribution (passing normal distribution test) and/or failed lognormal distribution test, paired t-tests were performed to compare matching blood and respiratory supernatant samples within a single group. If the data had unequal variance (as determined by an F-test), a ratio paired t-test was performed. All instances where lognormal distribution was likely non-parametric Wilcoxon matched-pairs sign ranked tests were performed. For comparisons across the three patient groups (i.e., healthy, mild-acute, severe), ordinary one-way ANOVA (if equal variance) or Brown-Forsythe and Welch ANOVA (if unequal variance) tests were performed for data with a normal distribution. Alternatively, data with a lognormal distribution were analyzed with a Kruskal-Wallis test.

Data availability. Single-cell sequencing datasets presented here are available through NCBI GEO, accession number XXX.

Author Contributions

Devon J. Eddins: Conceptualization, Methodology, Investigation, Formal Analysis, Data Curation, Writing—Original Draft, Writing—Review & Editing, Visualization. **Junkai Yang:** Methodology, Formal Analysis, Data Curation, Writing—Review & Editing, Visualization. **Astrid Kisters:** Methodology, Investigation, Data Curation, Writing—Review & Editing. **Vincent D. Giacalone:** Methodology, Formal Analysis, Investigation, Data Curation, Writing—Review & Editing. **Ximo Pechuan:** Methodology, Formal Analysis, Data Curation, Visualization, Writing—Review & Editing. **Joshua D. Chandler:** Methodology, Formal Analysis, Investigation, Data Curation, Writing—Review & Editing. **Jinyoung Eum:** Formal Analysis, Visualization. **Benjamin R. Babcock:** Investigation, Data curation. **Brian S. Dobosh:** Investigation, Data Curation, Formal Analysis. **Mindy R. Hernández:** Resources. **Fathma Abdulkhader:** Investigation. **Genoah L. Collins:** Investigation. **Richard P. Ramonell:** Resources, Data Curation, Writing—Review & Editing. **Christine Moussion:** Methodology, Formal Analysis. **Darya Y. Orlova:** Methodology, Data Curation. **Ignacio Sanz:** Resources. **F. Eun-Hyung Lee:** Methodology, Data Curation, Resources, Writing—Review & Editing. **Rabindra M. Tirouvanziam:** Methodology, Formal Analysis, Data Curation, Resources, Writing—Review & Editing. **Eliver E.B. Ghosn:** Conceptualization, Methodology, Formal Analysis, Resources, Data Curation, Writing—Original Draft, Visualization, Supervision, Project Administration, Funding Acquisition. All authors discussed the results and read and approved the final manuscript.

Competing interests

FEL is the founder of MicroB-plex, Inc., serves on the SAB of Be Bio Pharma, receives grants from BMGF and Genentech, and receives royalties from BLI, inc. All other authors have no competing interest to declare.

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Figure 1. Experimental design for the systems immunology approach (integrated multi-omics single-cell assays) to study COVID-19 patient samples. Respiratory samples (sputum or endotracheal aspirates) and matching blood from all subjects were collected for analysis by 21-plex Mesoscale analysis, high-dimensional (Hi-D) 30-parameter flow cytometry, and multi-omics scRNA-seq. Cells from endotracheal aspirates (ETA) and blood of severe COVID-19 patients along with blood from healthy individuals were surface-stained with a panel of 89 oligo-conjugated monoclonal antibodies before single-cell encapsulation, and analyses were performed with a custom human reference genome that included the SARS-CoV-2 genome to simultaneously detect viral mRNA transcripts. Integrative multi-omics analyses were performed on the resulting data sets.

Figure 2. Exacerbated neutrophilia in the airways and matching blood of severe COVID-19 patients. (a) Representative gating strategy for all samples (see Extended Data Fig. 1 for full gating strategy). (b) Box plots show distributions of leukocytes isolated from endotracheal aspirates (ETA). (c) Representative plots demonstrating inflammatory profile of pulmonary neutrophils including neutrophil elastase (NE), CD184 (CXCR4), and intracellular staining of IL-6, IL-8, and IL-1β including the full stain and fluorescence minus one (FMO) controls (d) Box plots show distributions of leukocytes isolated from whole blood from severe patients. (e) Representative histograms showing median fluorescence intensity (MFI) of key markers across healthy blood (blue), severe blood (gray), and severe ETA (green) samples. MdCs = myeloid-derived cells. For comparisons across the three patient groups (i.e., healthy, mild-acute, severe), ordinary one-way ANOVA (if equal variance) or Brown-Forsythe and Welch ANOVA (if unequal variance) tests were performed for data with a normal distribution. Data with a lognormal distribution were analyzed with a Kruskal-Wallis test.

Figure 3. Cytokine release syndrome is dominated by IL-8 and IL-1 β with pronounced myeloperoxidase content and activity in the lung microenvironment. (a, d, g, j) Concentration (pg/mL) of 15 analytes interrogated by Mesoscale analyses in plasma (gray circles) and respiratory supernatant (Resp. SNT; green squares) from healthy control (HC), mild-acute (MA), and severe COVID-19 patients. (b, e, h) Representative flow cytometric intracellular staining for IL-8 (CXCL8), IL-1 β , and IL-6, including the full stain and fluorescence minus one (FMO) controls in both blood and ETA neutrophils (CD66b⁺). (c, f, i) UMAP visualizations of CXCL8 (IL-8), IL1B, and IL6, which were also measured by intracellular flow cytometry staining and Mesoscale in both blood and ETA. (k) Concentration (ng/mL) of myeloperoxidase (MPO) protein and MPO activity in plasma vs. Resp. SNT. In (a, d, g, j, k), black dotted line = median lower limit of detection (LLOD) for assays (see Extended Data Table 3). In (b, e, h) red dashed line indicates the median fluorescence intensity (MFI) of neutrophils in the FMO control (value listed in the plot).

Figure 4. Multi-omic single-cell RNA-seq reveals emergency granulopoiesis in the circulation and abundant heterogeneous populations of mature neutrophils in the airways with distinct inflammatory states. UMAP visualization of scRNA-seq of total integrated blood (a) and endotracheal aspirate (ETA) (f) cells. Neutrophils were identified based on cell-surface markers (b and g), and total neutrophils were subclustered for further analysis (c and h). Dot plots of the intersection of the top differentially expressed genes in neutrophil clusters (d and i) sorted by average log-fold change for blood (d) and lung (i) neutrophils, respectively. UMAP visualization of signature genes of immature neutrophils (highlighted in d and i) in blood from severe patients compared to healthy individuals (e) and lungs of severe patients (j).

Figure 5. Mature neutrophils are continuously recruited from circulation and progress toward a hyperinflammatory state. Cell receptor-ligand pair analyses (CellChat³¹) from scRNA-seq data identify significant CXCL signaling pathway network enrichment between lung cells and blood neutrophils (a and b). Blood neutrophil cluster 2 represents the main subset potentially recruited to the lung (a). Recruitment of blood cluster 2 (a and c) is largely orchestrated by the CXCL8 (IL-8)/CXCR2 axis, and to a lesser extent, by the CXCL2 and CXCL3 (b and d). *S100A11/12*, *IFITM2*, and *CXCR2* mark a cluster of mature neutrophils in the blood (c) that likely represents the neutrophil subset recruited to the lung (d). Cell trajectory analysis (scVelo³²) identifies two potential pathways (Trajectories 1 and 2) for recently migrated neutrophils (e), that begin with a gene signature consistent with neutrophil blood cluster 2 (c and f). Neutrophils recruited to the lung acquire a hyperinflammatory profile along Trajectory 2 (e and f), characterized by high expression of interferon-stimulated gene (ISG) *IFI30* along with macrophage inflammatory proteins *CCL3* (MIP-1 α) and *CCL4* (MIP-1 β), whereas *CSF3R* and *CXCR4* are increased in cells along both trajectories (f). Neutrophils along Trajectory 1 may reflect cells progressing to apoptosis expressing higher levels of *HSPA1A* (HSP70) followed by *NEAT1* (f). Pathway and process enrichment analyses performed in Metascape⁸⁵ reveals that myeloid-mediated immunity and platelet activation, signaling, and aggregation are significantly enriched in neutrophils in the lungs of severe patients (g). MdCs: Myeloid-derived Cells.

Figure 6. *TNF* and *IL-1β* drive inflammatory program in neutrophils recruited to the lung.

(a) Dot plots showing 37 of the 67 differentially expressed genes in lung neutrophils versus blood neutrophil cluster 2 sorted by average log-fold change used as input for NicheNet³⁸ analyses (b) NicheNet³⁸ analyses identified the highest prioritized ligands (top 15) ordered by ligand activity (y-axis) that best predict the pulmonary neutrophil gene signature (x-axis). The predicted target genes represent the pulmonary neutrophil gene signature identified by differential gene expression analysis between blood neutrophils from cluster 2 and ETA neutrophils (see Extended Data Fig. 5d) (b) Dot plots of the intersection of the top 15 expressed prioritized ligands from all cells in the ETA samples. (c) Ligand-receptor matrix of putative signaling mediators for the top 15 prioritized ligands identified in (a). (d) UMAP visualizations of *TNF* and *IL1B* transcripts in the total ETA along with expression of predicted signaling mediators in blood (middle) and ETA (bottom) neutrophils from severe patients.

Figure 7. Viral transcripts (scRNA-seq) and viral load in the airways of severe patients. Calu-3 cells infected with SARS-CoV-2 USA-WA1/2020 (MOI 0.4) were encapsulated for scRNA-seq assays (a) following the same protocol used for patient cells from blood (b) and ETA (c). Millions of viral transcripts (or unique molecular identifiers; UMIs) were detected in about 5,000 Calu-3 cells (a), but not in the cells from severe patients (total UMI = 0; b and c). RT-qPCR (d) results of culture supernatant from Calu-3 infected cells and respiratory supernatants from mild-acute and severe patients (e) show a lower viral load in severe patients than in mild-acute patients and in the Calu-3-infected culture supernatant. ND = not detected; horizontal dotted line = lower limit of detection (LLOD).

1138 Supplemental Material:

#	Demographic	PID	Status	Sex	Age Group	Dexamethasone or equivalent	Remdesivir (No. of doses)	Date PCR positive Dx	Date intubated	Date sample collected	Length ICU stay (days)	Clinical Outcome
1	AA	PHA0001	Severe	Male	35-54	Yes	0	6/9/2020	6/8/2020	6/17/2020	32	Recovered
2	AA	PHA0004	Severe	Male	35-54	Yes	0	7/11/2020	7/18/2020	8/5/2020	37	Recovered
3	AA	PHA0006	Severe	Male	35-54	Yes	0	8/11/2020	8/16/2020	8/20/2020	8	Recovered
4	AA	PHA0018	Severe	Male	35-54	Yes	2	10/14/2020	9/29/2020	10/8/2020	13	Recovered
5	AA	PHA0041	Severe	Male	35-54	Yes	2	2/6/2021	2/6/2021	2/10/2021	14	Recovered
6	AA	PHA0003	Severe	Male	55+	Yes	2	6/23/2020	6/25/2020	6/30/2020	14	Succumbed
7	A	PHA0008	Severe	Male	55+	Yes	2	8/29/2020	8/30/2020	9/3/2020	19	Recovered
8	AA	PHA0024	Severe	Male	55+	Yes	2	12/27/2020	12/31/2020	1/6/2021	9	Recovered
9	AA	PHA0025	Severe	Male	55+	Yes	0	12/20/2020	12/24/2020	1/8/2021	26	Recovered
10	AA	PHA0031	Severe	Male	55+	Yes	1	1/23/2021	1/24/2021	1/27/2021	11	Succumbed
11	AA	PHA0032	Severe	Male	55+	Yes	1	1/9/2021	1/14/2020	1/27/2021	40	Recovered
12	AA	PHA0035	Severe	Male	55+	Yes	0	1/18/2021	1/20/2021	2/2/2021	53	Recovered
13	AA	PHA0036	Severe	Female	18-34	Yes	2	1/10/2021	12/31/2020	2/2/2021	42	Recovered
14	AA	PHA0002	Severe	Female	55+	Yes	0	6/20/2020	6/20/2020	6/24/2020	25	Recovered
15	AA	PHA0005	Severe	Female	55+	Yes	0	7/16/2020	7/18/2020	8/12/2020	33	Recovered
16	AA	PHA0007	Severe	Female	55+	Yes	0	8/9/2020	8/10/2020	9/2/2020	60	Recovered
17	AA	PHA0010	Severe	Female	55+	Yes	2	9/17/2020	9/21/2020	9/24/2020	18	Recovered
18	AA	PHA0026	Severe	Female	55+	Yes	1	12/27/2020	12/28/2020	1/8/2021	16	Succumbed
19	A	PHA0040	Mild-Acute	Male	35-54	Yes	0	1/6/2021	N/A	2/8/2021	N/A	Recovered
20	AA	PHA0043	Mild-Acute	Male	35-54	Yes	0	Unclear	N/A	2/18/2021	N/A	Recovered
21	AA	PHA0023	Mild-Acute	Male	55+	Yes	0	11/6/2020	N/A	11/11/2020	N/A	Recovered
22	AA	PHA0047	Mild-Acute	Male	55+	Yes	0	2/8/2021	N/A	2/19/2021	N/A	Recovered
23	AA	PHA0012	Mild-Acute	Female	35-54	Yes	0	9/18/2020	N/A	9/29/2020	N/A	Recovered
24	AA	PHA0013	Mild-Acute	Female	35-54	Yes	0	9/21/2020	N/A	9/29/2020	N/A	Recovered
25	AA	PHA0044	Mild-Acute	Female	35-54	Yes	0	2/16/2021	N/A	2/19/2021	N/A	Recovered
26	AA	PHA0046	Mild-Acute	Female	35-54	Yes	0	2/12/2021	N/A	2/19/2021	N/A	Recovered
27	AA	PHA0045	Mild-Acute	Female	55+	Yes	0	2/12/2021	N/A	2/19/2021	N/A	Recovered
28	AA	PHA1009	Healthy	Female	18-34	Yes	0	N/A	N/A	10/23/2020	N/A	N/A
29	AA	PHA1005	Healthy	Female	35-54	Yes	0	N/A	N/A	10/23/2020	N/A	N/A
30	AA	PHA1006	Healthy	Female	35-54	Yes	0	N/A	N/A	10/23/2020	N/A	N/A
31	AA	PHA1007	Healthy	Female	35-54	Yes	0	N/A	N/A	10/23/2020	N/A	N/A
32	AA	PHA1008	Healthy	Female	35-54	Yes	0	N/A	N/A	10/23/2020	N/A	N/A
33	AA	PHA1010	Healthy	Female	35-54	Yes	0	N/A	N/A	10/23/2020	N/A	N/A
34	AA	PHA1011	Healthy	Female	35-54	Yes	0	N/A	N/A	2/10/2021	N/A	N/A
35	AA	PHA1012	Healthy	Female	55+	Yes	0	N/A	N/A	2/17/2021	N/A	N/A

1139 Extended Data Table 1. *Demographic and clinical data from the 35 Black/African*

1140 *American individuals enrolled in our studies.* Table describing demography, status, sex, and
1141 age of study participants along with select details of clinical course including administration of
1142 Dexamethasone and Remdesivir, date first intubated (severe, ICU patients only), date of PCR
1143 positive diagnosis (Dx), date clinical specimens were collected, and total length ICU stay in
1144 days, and clinical outcome (whether recovered or succumbed to disease).
1145
1146

Characteristics	All subjects (N = 35)		
	Healthy (N = 8)	Mild-Acute (N=9)	Severe (N=18)
Age			
A. Mean age $\pm$ SD	46 $\pm$ 11	51 $\pm$ 12	56 $\pm$ 14
B. Age range	31 – 70	35 – 68	22 – 81
Gender			
A. Female	8 (100%)	5 (56%)	6 (33%)
B. Male	0 (0%)	4 (44%)	12 (67%)
Site of endotracheal aspiration			
A. Endotracheal tube	—	—	9 (50%)
B. Tracheostomy	—	—	9 (50%)
Medical History			
A. Smoking History			
i) Never	7 (87.5%)	7 (78%)	12 (67%)
ii) Former	1 (12.5%)	2 (22%)	2 (11%)
iii) Current	0 (0%)	0 (0%)	4 (22%)
B. Hypertension (HTN)	3 (37.5%)	3 (33%)	11 (61%)
C. Obesity	2 (25%)	1 (11%)	4 (22%)
D. Asthma	2 (25%)	2 (22%)	2 (11%)
E. COPD	0 (0%)	0 (0%)	2 (11%)
F. Diabetes mellitus (DM)			
i) Type I DM	0 (0%)	0 (0%)	0 (0%)
ii) Type II DM	1 (12.5%)	0 (0%)	8 (44%)
G. Deep vein thrombosis (DVT)	0 (0%)	0 (0%)	1 (5%)
H. Hypothyroidism	0 (0%)	0 (0%)	1 (5%)
I. Congestive Heart Failure (CHF)	1 (12.5%)	0 (0%)	5 (28%)
J. Gastroesophageal reflux disease (GERD)	1 (12.5%)	1 (11%)	1 (5%)
K. Dyslipidemia	0 (0%)	1 (11%)	1 (5%)
L. Obstructive sleep apnea (OSA)	0 (0%)	0 (0%)	2 (11%)
M. Coronary artery disease (CAD)	0 (0%)	0 (0%)	1 (5%)
N. Chronic kidney disease	0 (0%)	1 (11%)	1 (5%)
O. Cancer	0 (0%)	0 (0%)	0 (0%)
P. Autoimmune hemolytic anemia	0 (0%)	0 (0%)	1 (5%)
Q. Thrombocytopenia	0 (0%)	0 (0%)	1 (5%)
R. Arthritis/Rheumatoid arthritis (RA)	1 (12.5%)	0 (0%)	1 (5%)
Complications			
A. Acute respiratory distress syndrome (ARDS)	—	—	18 (100%)
B. Hypoxemic respiratory failure	—	—	13 (72%)
C. Extracorporeal membrane oxygenation (ECMO)	—	—	2 (11%)
D. Shock	—	—	15 (83%)
E. Nosocomial pneumonia	—	—	6 (33%)
F. Coagulopathy	—	—	3 (17%)
G. Seizures	—	—	3 (17%)
H. Cardiac arrhythmia	—	—	3 (17%)
I. Renal failure	—	—	8 (44%)
J. Renal replacement therapy	—	—	6 (33%)

1148 **Extended Data Table 2. *Clinical characteristics of the 35 Black/African American***
 1149 ***individuals enrolled in our studies.*** Table describing clinical characteristics of 35 patients
 1150 across the 3 cohorts (healthy, mild-acute, and severe) including age, gender, site of
 1151 endotracheal aspiration (ETA), medical history, along with complications for the severe patients
 1152 admitted to the ICU.
 1153

1154

a CoV-Neutrophil

Laser	Detector	Antigen	Fluorophore	Clone	Isotype	Vendor	Cat. No.	Titration
	B 488/10	Side Scatter (SSC)	—	—	—	—	—	—
1	B 515/20	Neutrophil elastase (NE)	AF488	950318	Mouse IgG1, κ	R&D Systems	IC91671G	1:25
2	B 610/20	CD45-RA	BB630-P	HI-100	Mouse IgG2a, κ	BD Biosciences	624294	1:160
3	488 nm B 670/30	CD11b	BB660-P	ICRF44	Mouse IgG1, κ	BD Biosciences	624295	1:50
4	B 710/50	CD45	BB700	HI-30	Mouse IgG1, κ	BD Biosciences	746090	1:640
5	B 750/30	CD4	BB755-P	RPA-T4	Mouse IgG1, κ	BD Biosciences	624391	1:100
6	B 780/60	HLA-DR (MHC II)	BB790-P	G46-6	Mouse IgG2a, κ	BD Biosciences	624296	1:50
7	V 431/28	CD163	BV421	GHI/6I	Mouse IgG1, κ	BioLegend	333612	1:25
8	V 470/15	IL-1β	Pacific Blue	H1b-98	Mouse IgG1, κ	BioLegend	511710	1:20
9	V 586/15	CD56	BV570	HCD56	Mouse IgG1, κ	BioLegend	318330	1:100
10	V 610/20	CD86 *	BV605	FUN-1	Mouse IgG1, κ	BD Biosciences	562999	1:50
11	405 nm V 670/30	TNF	BV650	MAb11	Mouse IgG1, κ	BD Biosciences	563418	1:80
12	V 710/50	CD19	BV711	H1B19	Mouse IgG1, κ	BioLegend	302246	1:20
13	V 740/35	CD16 (FcγRIII)	BV750	3G8	Mouse IgG1, κ	BD Biosciences	747461	1:320
14	V 780/60	CD63 (LAMP-3)	BV785	H5C6	Mouse IgG1, κ	BioLegend	353044	1:80
15	YG 586/15	IL-6	PE	MQ2-13A5	Rat IgG1, κ	BD Biosciences	554545	1:20
16	YG 610/20	IL-8 (CXCL8)	PE-CF594	G265-8	Mouse IgG2b, κ	BD Biosciences	563531	1:20
17	561 nm YG 670/30	Siglec-8 *	PE-Cy5	7C9	Mouse IgG1, κ	BioLegend	347114	1:100
18	YG 710/50	CD10	PE-Cy5.5	HI10a	Mouse IgG1, κ	Homemade	—	1:40
19	YG 780/60	CD184 *	PE-Cy7	12G5	Mouse IgG2a, κ	BioLegend	306514	1:40
20	UV 379/28	CD64 (FcγRI)	BUV395	10.1	Mouse IgG1, κ	BD Biosciences	740300	1:80
21	UV 450/50	GhostDye (Viability)	UV450	—	—	Tonbo Biosciences	13-0868-T500	1:100
22	355 nm UV 586/15	CD172a/b (SIRPα/β1)	BUV563	SE5A5	Mouse IgG1, κ	BD Biosciences	748435	1:10
23	UV 670/30	CD123 (IL-3Rα)	BUV661	9F5	Mouse IgG1, κ	BD Biosciences	741628	1:40
24	UV 740/35	CD14	BUV737	M5E2	Mouse IgG2a, κ	BD Biosciences	612763	1:50
25	UV 820/60	CD3ε	BUV805	SK1	Mouse IgG1, κ	BD Biosciences	612889	1:40
26	R 670/30	CD66b	APC	QA17A51	Mouse IgG1, κ	BioLegend	396906	1:200
27	640 nm R 710/50	CD11c	AF700	Bu15	Mouse IgG1, κ	BioLegend	337220	1:200
28	R 780/60	CD32 (FcγRII)	APC-Fire750	FUN-2	Mouse IgG2b, κ	BioLegend	303220	1:400

* = alternate reagents used in panel iterations (see c below); -P denotes prototype reagents

b CoV-Global Distributions

Laser	Detector	Antigen	Fluorophore	Clone	Isotype	Vendor	Cat. No.	Titration
	B 488/10	Side Scatter (SSC)	—	—	—	—	—	—
1	488 nm B 515/20	CD45	AF488	HI-30	Mouse IgG1, κ	BioLegend	304017	1:160
2	V 431/28	CD56	BV421	HCD56	Mouse IgG1, κ	BioLegend	318328	1:40
3	V 610/20	CD8α	BV605	SK1	Mouse IgG1, κ	BioLegend	344742	1:20
4	405 nm V 670/30	HLA-DR (MHC II)	BV650	L243	Mouse IgG2a, κ	BioLegend	307650	1:50
5	V 710/50	CD4	BV711	RPA-T4	Mouse IgG1, κ	BioLegend	300558	1:40
6	V 740/35	CD16 (FcγRIII)	BV750	3G8	Mouse IgG1, κ	BD Biosciences	747461	1:320
7	V 780/60	CD19	BV785	H1B19	Mouse IgG1, κ	BioLegend	302240	1:40
8	YG 586/15	CD123 (IL-3Rα)	PE	6H6	Mouse IgG1, κ	BioLegend	306006	1:400
9	YG 670/30	Siglec-8	PE-Cy5	7C9	Mouse IgG1, κ	BioLegend	347114	1:100
10	561 nm YG 710/50	CD14	PE-Fire700	63D3	Mouse IgG1, κ	BioLegend	367158	1:100
11	YG 780/60	CD11c	PE-Cy7	Bu-15	Mouse IgG1, κ	BioLegend	337216	1:160
12	355 nm UV 450/50	GhostDye (Viability)	UV450	—	—	Tonbo Biosciences	13-0868-T500	1:100
13	R 670/30	CD11b	APC	ICRF44	Mouse IgG1, κ	BioLegend	301350	1:400
14	640 nm R 710/50	CD3ε	AF700	HIT3a	Mouse IgG2a, κ	BioLegend	300324	1:320
15	R 780/60	CD66b	APC-Fire750	QA17A51	Mouse IgG1, κ	BioLegend	396908	1:80

c Alternate Reagents

Laser	Detector	Antigen	Fluorophore	Clone	Isotype	Vendor	Cat. No.	Titration
1	405 nm V670/30	CD169 (Siglec-1)	BV605	7-239	Mouse IgG1, κ	BioLegend	346010	1:40
2	V670/30	CD182 (CXCR2)	BV605	6C6	Mouse IgG1, λ	BD Biosciences	744197	1:40
3	561 nm YG 670/30	CD86	PE-Cy5	IT2.2	Mouse IgG1, κ	BioLegend	305408	1:20
4	YG 780/60	CD206 (MMR)	PE-Cy7	15-2	Mouse IgG2a, κ	BioLegend	321124	1:10

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Extended Data Table 3. *High-dimensional 30-parameter, including intracellular cytokine staining, and 17-parameter flow cytometry panels used for airway and blood cells.* Flow cytometer configuration and cytometry reagents used in the final panels to interrogate (a) neutrophil phenotype and (b) global immune cell distributions and blood and ETA samples. Channels marked with an asterisk (*) denote alternate reagents (c) used in earlier panel iterations for some samples in this study. Monoclonal antibody (mAb) master mixes were prepared in BD Horizon™ Brilliant Stain Buffer and samples stained as described in the methods section. Titrations of all reagents were determined in house for each lot independently prior to use. AF: AlexaFluor, APC: Allophycocyanin, BB: Brilliant Blue, BUV: Brilliant Ultraviolet, BV: Brilliant Violet, FITC: Fluorescein isothiocyanate, PE: Phycoerythrin. Fluorophores marked with -P denote prototype reagents and are custom conjugations from BD Biosciences.

a	Analyte	Median LLOD (pg/mL)	LLOD Range (pg/mL)
1	G-CSF (CSF3)	0.84 ± 0.88	0.15—2.12
2	GM-CSF (CSF2)	0.11 ± 0.13	0.01—0.31
3	IFN-γ	1.31 ± 1.12	0.16—2.79
4	IL-10	0.03 ± 0.02	0.01—0.06
5	IL-1α	0.13 ± 0.07	0.04—0.19
6	IL-1β	0.07 ± 0.05	0.01—0.12
7	IL-6	0.31 ± 0.39	0.03—0.89
8	IL-8 (CXCL8)	0.03 ± 0.03	0.02—0.08
9	TNF	0.24 ± 0.13	0.07—0.35
10	VEGFA	0.43 ± 0.44	0.06—1.06
11	ENA-78 (CXCL5)	0.15 ± 0.07	0.08—0.23
12	IL-18	1.02 ± 1.31	0.14—2.92
13	IL-1RA	1.08 ± 0.23	0.83—1.28
14	IL-29 (IFNλ1)	0.28 ± 0.11	0.13—0.38
15	IP-10 (CXCL10)	0.70 ± 0.99	0.18—2.19
16	I-TAC (CXCL11)	1.82 ± 0.23	1.59—2.13
17	MCP-1 (CCL2)	0.14 ± 0.05	0.09—0.21
18	M-CSF (CSF1)	0.10 ± 0.04	0.06—0.14
19	MIP-1β (CCL4)	5.17 ± 2.40	2.78—8.51
20	TRAIL (CD253)	0.28 ± 0.11	0.18—0.44
21	SDF1α (CXCL12)	30.13 ± 26.73	10.60—60.60

b	Assay	Median LLOD (ng/mL)	LLOD Range (ng/mL)	Median LLOQ (ng/mL)	LLOQ Range (ng/mL)
1	Total MPO	0.15 ± 0.15	0.03—0.42	0.30 ± 0.30	0.05—0.84
2	MPO Activity	2.86 ± 1.98	0.38—6.03	5.72 ± 3.96	0.76—12.07

Extended Data Table 4. Mesoscale U-PLEX biomarker group 1 human and myeloperoxidase (MPO) assays. Median ± SD and range of lower limits of detection (LLOD) for (a) Mesoscale UPLEX analytes and (b) MPO assays. Lower limits of quantification (LLOQ; MPO assays) and LODs (determined by standard curve) were run for each assay plate independently and the median LLOD plotted as horizontal dotted lines for each assay (see Fig. 3 and Extended Data Fig. 2).

Antigen	Clone	Vendor	Cat. No.	Antigen	Clone	Vendor	Cat. No.
1 CD10	HI10a	Biolegend	312233	46 CD324	67A4	Biolegend	324127
2 CD101 (BB27)	BB27	Biolegend	331017	47 CD326 (EpCAM)	9C4	Biolegend	324247
3 CD103 (Integrin α E)	Ber-ACT8	Biolegend	350233	48 CD335 (NKp46)	9.00E+02	Biolegend	331941
4 CD107a (LAMP-1)	H4A3	Biolegend	328649	49 CD370 (CLEC9A)	8F9	Biolegend	353809
5 CD117 (c-kit)	104D2	Biolegend	313243	50 CD38	HIT2	Biolegend	303543
6 CD11b	ICRF44	Biolegend	301359	51 CD4	RPA-T4	Biolegend	300567
7 CD11c	S-HCL-3	Biolegend	371521	52 CD40	5C3	Biolegend	334348
8 CD123 (IL-3R α)	6H6	Biolegend	306045	53 CD41	HIP8	Biolegend	303739
9 CD127 (IL-7R α)	A019D5	Biolegend	351356	54 CD44	BJ18	Biolegend	338827
10 CD133	S16016B	Biolegend	394007	55 CD45	2D1	Biolegend	368545
11 CD137	4B4-1	Biolegend	309839	56 CD45R	RA3-6B2	Biolegend	103273
12 CD138 (Syndecan-1)	MI15	Biolegend	356539	57 CD45RA	HI100	Biolegend	304163
13 CD14	M5E2	Biolegend	301859	58 CD45RO	UCHL1	Biolegend	304259
14 CD15 (SSEA-1)	W6D3	Biolegend	323053	59 CD49a	TS2/7	Biolegend	328319
15 CD16 (Fc γ RIII)	3G8	Biolegend	302065	60 CD5	UCHT2	Biolegend	300637
16 CD161	HP-3G10	Biolegend	339947	61 CD56 (NCAM)	QA17A16	Biolegend	392425
17 CD163	GHI/61	Biolegend	333637	62 CD62L	DREG-56	Biolegend	304851
18 CD169	7-239	Biolegend	346021	63 CD64 (Fc γ RI)	10.1	Biolegend	305045
19 CD183 (CXCR3)	G025H7	Biolegend	353747	64 CD66b	6/40c	Biolegend	392909
20 CD184 (CXCR4)	12G5	Biolegend	306533	65 CD69	FN50	Biolegend	310951
21 CD185 (CXCR5)	J252D4	Biolegend	356939	66 CD80	2D10	Biolegend	305243
22 CD19	HIB19	Biolegend	302265	67 CD86	IT2.2	Biolegend	305447
23 CD196 (CCR6)	G034E3	Biolegend	353440	68 CD8 α	RPA-T8	Biolegend	301071
24 CD197 (CCR7)	G043H7	Biolegend	353251	69 CD95 (Fas)	DX2	Biolegend	305651
25 CD1c	L161	Biolegend	331547	70 CX3CR1	K0124E1	Biolegend	355705
26 CD1d	51.1	Biolegend	350319	71 HLA-DR	L243	Biolegend	307663
27 CD20	2H7	Biolegend	302363	72 IgD	IA6-2	Biolegend	348245
28 CD206 (MMR)	15-2	Biolegend	321147	73 IgG	M1310G05	Biolegend	410727
29 CD209 (DC-SIGN)	9E9A8	Biolegend	330121	74 IgM	MHM-88	Biolegend	314547
30 CD21	Bu32	Biolegend	354923	75 Ig light chain κ	MHK-49	Biolegend	316533
31 CD23	EBVCS-5	Biolegend	338525	76 Ig light chain λ	MHL-38	Biolegend	316629
32 CD24	ML5	Biolegend	311143	77 Siglec-8	7C9	Biolegend	347115
33 CD25	BC96	Biolegend	302649	78 TCR V α 7.2	3C10	Biolegend	351735
34 CD269 (BCMA)	19F2	Biolegend	357523	79 TCR γ/δ	B1	Biolegend	331231
35 CD27	O323	Biolegend	302853	80 TIGIT (VSTM3)	A15153G	Biolegend	372729
36 CD274 (PD-L1)	29E.2A3	Biolegend	329751	81 XCR1	S15046E	Biolegend	372617
37 CD275 (ICOSL)	2D3	Biolegend	309419	Custom oligo-conjugated in-house			
38 CD279 (PD-1)	EH12.2H7	Biolegend	329963	82 ACE2	535919	Sino Biological	MAB9332-100
39 CD28	CD28.2	Biolegend	302963	83 CD160	BY55	Biolegend	341202
40 CD29 (Integrin β 1)	TS2/16	Biolegend	303029	84 CD16b	2D2G5B9	Sino Biological	11046-MM01
41 CD3	UCHT1	Biolegend	300479	85 CD178	NOK-1	Biolegend	306402
42 CD307d (FcRL4)	413D12	Biolegend	340213	86 CD34	581	Biolegend	343602
43 CD307e (FcRL5)	509f6	Biolegend	340309	87 CD43	CD43-10G7	Biolegend	343202
44 CD309 (VEGFR2)	7D4-6	Biolegend	359921	88 CD9	HI9a	Biolegend	312102
45 CD32 (Fc γ RII)	FUN-2	Biolegend	303225	89 IgA	IS11-8E10	Mytenyi	130-093-073

Extended Data Table 5. Panel of oligo-conjugated antibodies used in multi-omics scRNA-seq assays to measure surface protein markers on cells from airways and blood. A total of 89 oligo-conjugated antibody-derived tags (ADT) were used to evaluate surface protein expression of target antigens via scRNA-seq. Reagents no. 82-89 were not commercially available in the TotalSeq-C™ format (BioLegend), so custom oligo-conjugated reagents were generated in-house using purified monoclonal antibodies and commercially available 5' Feature Barcode Antibody Conjugation - Lightning-Link® kits (abcam). Titrations of all reagents were determined in-house for each lot independently prior to use. Cells were surface stained prior to encapsulation and surface marker expression of major lineage markers were used to help cluster/validate gene expression data as described in the methods.

		PID	Total reads	Mean reads/cell
ETA	GEX	PHA0007	593,835,060	350,345
		PHA0008	555,825,992	2,241,234
		PHA0010	587,153,474	872,442
		PHA0018	541,561,148	459,729
	ADT	PHA0007	85,070,531	2,101
		PHA0008	78,647,311	7,514
		PHA0010	46,042,783	2,989
		PHA0018	73,265,461	5,399
Blood	GEX	PHA0007	518,672,044	273,129
		PHA0008	540,400,013	641,044
		PHA0010	592,394,112	543,980
		PHA0018	556,699,985	1,484,533
		PHA1005	541,306,254	158,787
		PHA1006	501,896,927	200,198
		PHA1007	512,999,099	172,902
		PHA1008	506,988,059	160,898
		PHA1009	527,201,752	130,463
		PHA1010	480,796,663	332,042
	ADT	PHA0007	74,597,277	2,115
		PHA0008	80,175,737	1,536
		PHA0010	62,927,249	1,067
		PHA0018	79,818,668	1,395
		PHA1005	77,435,258	1,145
		PHA1006	88,850,369	1,334
		PHA1007	88,329,209	1,169
		PHA1008	93,579,790	1,351
		PHA1009	91,162,676	1,281
		PHA1010	89,597,131	1,251

Extended Data Table 6. Sequencing depth of independent samples. All gene expression (GEX) libraries were pooled and run in a single S4 flow cell on a NovaSeq™ 6000. All antibody-derived tag (ADT) libraries were pooled and sequenced in a single lane of a S4 flow cell on a NovaSeq™ 6000. Total reads and mean number of reads per cell for GEX and ADT are reported independently for each sample, with an average read depth of 540,000,000 reads per sample (GEX) and 79,000,000 reads per sample (ADT).

a

PID	Blood (%)	ETA (%)
PHA0007	30	30
PHA0008	20	25
PHA0010	10	25
PHA0018	30	25
PHA1005	30	
PHA1006	30	
PHA1007	30	
PHA1008	20	
PHA1009	20	
PHA1010	25	

b

PID	Total ADT UMI	
	Blood	ETA
PHA0007	125–500	125–1250
PHA0008	125–1000	125–1250
PHA0010	125–1500	125–750
PHA0018	125–1500	125–1000
PHA1005	125–1250	
PHA1006	125–1250	
PHA1007	125–1500	
PHA1008	125–1500	
PHA1009	125–1500	
PHA1010	125–1500	

Extended Data Table 7. Mitochondrial gene thresholds and total ADT UMIs of individual samples. (a) Threshold for mitochondrial gene distribution (percentage) for each sample used to exclude potential dead cells in scRNA-seq data. Threshold was evaluated and set for each sample independently. (b) The distribution of total ADT UMIs was determined and recorded per each sample independently.

Extended Data Figure 1. Representative gating strategy for Hi-D FACS data.

Representative gating of blood (a) and ETA (b) samples. Extracellular stain FMOs for markers used to interrogate neutrophil phenotype in blood (c) and ETA (d).

Extended Data Figure 2. Additional cytokine assessment in blood and lungs.

Concentration (pg/mL) of remaining 6 analytes interrogated by Mesoscale analyses in plasma (gray circles) and respiratory supernatant (Res. SNT; green squares) from healthy control (HC), mild-acute (MA), and severe COVID-19 patients. Dotted line = assay limit of detection (LOD).

1222 **Extended Data Figure 4. *Gene signature for immature neutrophils is lacking in healthy***
 1223 ***donors and lungs of COVID-19 patients.*** UMAP visualizations of genes that identify immature
 1224 neutrophils in the blood healthy individuals (a) and severe COVID-19 patients (b), along with
 1225 cells from the lungs of severe COVID-19 patients (c).
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Extended Data Figure 5. Cellular signatures during SARS-CoV-2 pathogenesis. (a) Pulmonary neutrophils have a reduction in CD32 (FcγRII) expression as compared to circulating

1231 neutrophils. (b) Neutrophils undergo transcriptional reprogramming and increase de novo
 1232 transcription (total RNA) upon migrating to the lung. (c) Neutrophils in the blood and lung
 1233 increase expression of *IFITM2* during SARS-CoV-2 pathogenesis. (d) Metascape⁸⁵ pathway and
 1234 process enrichment analyses reveal non-immune cells have an increased gene signature for
 1235 granulocyte activation in the airways of severe COVID-19 patients. (e) Extracellular neutrophil
 1236 elastase is also detected on the surface of pulmonary T cells and myeloid-derived cells (MdCs)
 1237 in severe COVID-19 patients.
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