1   Research Article

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

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6   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-1β, 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 severity[3]. 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 dysregulation[4].

Severe COVID-19 parallels the pathophysiology of sepsis[5], 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 progression[9]. 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 macrophages[10], 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 ADRS 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-1β, 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.

88   **Results**

89

90   **Study Cohort**

91

92   To better understand immune dynamics in Black/AA patients with severe COVID-19, we

93   analyzed airway and matching blood samples from a cohort of 35 individuals presenting to

94   Emory University Hospitals (severe) or the Emory Acute Respiratory Clinic (mild-acute) in

95   Atlanta, GA, USA (Fig. 1 and Extended Data Table 1), including 8 demographic-matched

96   healthy adults as controls. Of the 27 individuals who were confirmed positive by PCR from

97   nasopharyngeal swabs, 18 had an NIH severity score of "critical"

98   (https://www.covid19treatmentguidelines.nih.gov/overview/clinical-spectrum/; referred to as

99   severe herein) and were admitted to the intensive care unit (ICU) requiring mechanical ventilator

100   support, and 9 were mild (mild-acute) outpatients. All severe patients in our cohort received

101   corticosteroids (dexamethasone or equivalent). Approximately half received one or more doses

102   of the antiviral medication remdesivir, with an average ICU stay of 26 days (see Extended Data

103   Tables 1 and 2).

104

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

106

107   We first characterized major immune lineages in the airways (endotracheal aspiration, ETA) and

108   matching blood samples by Hi-D flow cytometry and observed a pronounced circulating

109   neutrophilia and lymphopenia (notably T and NK cells), which is similarly reflected in the airways

110   (Fig. 2a-e and Extended Data Fig. 1). This is in line with recent reports showing lymphopenia

111   associated with significant alterations in the myeloid compartment[15, 16]. Strikingly, in most cases

112   of severe disease, ≥85% of all pulmonary leukocytes were neutrophils (Fig. 2b). This contrasts

113   with other studies that have reported much more heterogeneity in neutrophil frequency in

114   patients' blood and lungs[17, 18]. In addition, circulating T cells and NK cells decline with increased

115   disease severity (Fig. 2d), which has recently been reported as a feature of COVID-19

116   pneumonia[19]. We also observed a notable decrease in pulmonary NK cells associated with

117   disease severity (Fig. 2b). However, there were only subtle differences in the B-cell

118   compartment (which were detected at very low levels in the airways) and myeloid-derived cells

119   (MdCs) compartment compared to other reports[8, 20, 21], highlighting the importance of

120   neutrophilia and neutrophil-to-lymphocyte ratio in our cohort. Interestingly, the patient with the

121   lowest neutrophil frequency in the airways corresponds to the youngest patient in the severe

122   disease group (Fig. 2b). However, since we only had a single patient under 35 years old in our

123   severe group, we cannot conclude any associations between neutrophilia and patient age.

124

125   Since we identified prominent neutrophilia in our cohort, we developed a Hi-D, 30-parameter

126   flow cytometry panel to interrogate inflammatory neutrophil phenotype in addition to broad

127   leukocyte characterization (see "CoV-Neutrophil" panel in Extended Data Table 3). Here, we

128   used extracellular staining of neutrophil elastase (NE) and CD63 (LAMP3) to assess primary

129   granule release[22], coupled with intracellular staining for key effectors implicated in cytokine

130   storm (IL-1β, IL-6, and IL-8). We observed that nearly all neutrophils recruited to the lung

131   developed an inflammatory profile characterized by exacerbated levels of NE and elevated

132   production of IL-1β and IL-8 with a dramatic increase in primary granule release (Fig. 2c,e).
133   Indeed, most neutrophils in the airways were positive for all 3 intracellular cytokines and
134   express CD63 on the cell surface (Fig. 2c), suggesting that neutrophils are releasing cytokines
135   upon degranulation. However, this phenotype is diminished in the circulation. A smaller
136   proportion of blood neutrophils undergo granule release and express a cytokine signature
137   limited mainly to IL-1β production and less IL-6 and IL-8 (Fig. 2e). Similarly, we find a subset of
138   neutrophils in the lung that co-express the highest levels of surface CD10, CD184 (CXCR4) and
139   NE (Fig. 2c). Interestingly, neutrophils in the lung express lower levels of FcγRII (CD32) than
140   those in the blood (Extended Data Fig. 5a). Together, these results demonstrate systemic
141   neutrophilia in severe COVID-19 patients where neutrophils accumulating in the airways
142   produce exacerbated levels of potentially damaging enzymes (e.g., NE) and inflammatory
143   cytokines (IL-1β/6/8) while undergoing pronounced primary granule release.
144
145   **Neutrophil-secreted inflammatory molecules in the airways potentiate acute respiratory**
146   **distress syndrome in severe patients**
147
148   To better understand the inflammatory signaling milieu and relate the intracellular cytokine
149   staining to protein secretion, we measured 21 total analytes in the airways and plasma using the
150   Mesoscale UPLEX platform (Extended Data Table 4). We found that IL-8 is the most abundant
151   chemokine in the airways of severe patients accompanied by high levels of IL-1β and IL-6. This
152   is in line with our Hi-D flow cytometry data showing neutrophilia and pronounced intracellular
153   staining signal for IL-8 and IL-1β (Fig. 3a-i). Strikingly, IL-8 levels were ~100-fold higher in the
154   airways of severe versus mild patients and that of IL-1β and IL-6 levels in severe patients.
155   These findings are in stark contrast to an early report[23] that showed no differences between
156   circulating and pulmonary IL-6 and IL-8 secretion, reported in the <100 pg/mL range. However,
157   more recent studies in both ETA[24] and BALF[25] samples have noted similar findings that
158   corroborate our own. These data, therefore, implicate IL-8 signaling in the prominent
159   neutrophilia observed in severe patients in our cohort.
160
161   We noticed only minor differences in circulating levels of many targets between healthy
162   individuals and those affected by severe COVID-19 disease suggesting an uncoupling of local
163   versus systemic inflammatory responses[24], particularly for IL-8 and IL-1β, but also IL-6 to a
164   lesser extent (Fig. 3a,d,g). Conversely, we observed a rise in circulating IL-1RA levels with
165   increasing disease severity, similar to other reports[26]. However, we observed an opposite trend
166   in the airways, where IL-1RA levels appear to be decreased in severe patients (Fig. 3j). This is
167   in stark contrast to IL-1β levels, which were not markedly different in the circulation but
168   significantly increased in the airways with disease severity (Fig. 3j). We also found increased
169   CCL2 (MCP-1) in the airways of severe disease, and the same trend for M-CSF and TNF was
170   observed, albeit at much lower concentrations (Fig. 3j). Interferon (IFN)-γ was detected at the
171   highest levels in the airways of severe patients with no obvious differentiation in plasma across
172   all groups. IL-10 was also increased in the airways of severe patients with notable variation at
173   low levels (Fig. 3j). In contrast to other studies[27, 28], we did not observe appreciable differences
174   in IL-18 or CXCL10 (IP-10) levels across patient groups (Extended Data Fig. 2). Additionally,

5

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

178 We further measured the concentration of neutrophil-derived MPO and its enzymatic activity
179 across patient groups. MPO is an important neutrophil effector molecule (originating from
180 primary granules like NE) implicated in respiratory illnesses such as cystic fibrosis[29], and can
181 contribute to lung tissue damage during neutrophilic pneumonitis. MPO protein concentrations
182 increased stepwise with disease severity in the plasma and respiratory supernatant (Fig. 3k).
183 However, MPO activity was mostly detected in the airways, and rose with increasing disease
184 severity (Fig. 3k).

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

189 To gain insight into the cellular states and transcriptional regulation in patients with severe
190 COVID-19 disease, we performed multi-omics scRNA-seq on cells from whole blood and ETA
191 samples of severe patients and whole blood from demographic-matched healthy control (Fig. 1).
192 We assessed immune features in data integrated from four or more patients from the same
193 cohort (i.e., healthy vs. severe; see Methods). First, major lineages from blood (Fig. 4a) and
194 ETA (Fig. 4g) were gated manually using the antibody-derived tag (ADT) data for surface
195 protein expression. Next, we used the gene expression (GEX) data to generate clusters for
196 each major lineage identified based on their surface markers, as per our previously described
197 SuPERR-seq pipeline[30] (Extended Data Fig. 3). For example, neutrophils identified by CD66b
198 and CD16 surface ADT in the blood (Fig. 4b) and ETA (Fig. 4h) were then selected and
199 clustered independently by GEX data (Fig. 4c,i) for further analyses.

201 A prior study had reported an increase in immature neutrophils in the lung concomitant to
202 increased circulating immature neutrophils from emergency hematopoiesis in severe COVID-19
203 patients[16]. We, therefore, assessed the immature neutrophil phenotype in the blood (Fig 4a-e)
204 and lung (Fig 4f-j) by scRNA-seq in our patient cohort. We readily identified a cluster of
205 neutrophils expressing *CAMP*, *LTF*, *RETN*, *OLFM4*, *DEFA3*, *CD24*, and *MMP8* (Fig. 4e and
206 Extended Data Fig. 4) in the blood of severe patients (cluster 3 in blood neutrophils) that also
207 has high expression of calprotectin (S100A8/9) and other calgranulins (Fig 4d). This is
208 consistent with immature neutrophil phenotypes described by others[7, 15] and was notably absent
209 in neutrophils from healthy blood, confirming an emergency hematopoiesis/granulopoiesis in our
210 patient cohort. However, in contrast to previous studies[16], we did not observe any signature of
211 immature neutrophils in the lungs of severe patients (Fig. 4j), suggesting that immature
212 neutrophils either are not directly recruited to the lung, or quickly differentiate upon infiltration
213 into the lung.

215 To determine which neutrophil subset in the blood can infiltrate the lung and further differentiate
216 into a pathogenic state, we explored cell-cell communication (CellChat[31]) of clustered cells from
217 blood (Fig. 4c) and lung (Fig. 4h) samples. We found significant communication through the
218 CXCL pathway between the lung neutrophil, myeloid, and non-immune populations (i.e.,

219   epithelial/stromal cells) with *CXCR2*-expressing neutrophils from the blood (blood cluster 2; Fig.
220   5a). The CXCL8 (IL-8)/CXCR2 pathway was identified as the primary recruitment axis for
221   circulating *CXCR2*+ neutrophils (Fig. 5b). These data are in line with our finding that IL-8 was
222   increased in neutrophils by flow cytometry (Fig. 2) and secreted at very high levels in the
223   airways (Fig. 3) and suggests that the CXCL8/CXCR2 signaling axis is important for neutrophil
224   recruitment to the lungs during COVID-19 pathogenesis. Virtually all lung neutrophils
225   (particularly cluster 5), non-immune cells, and monocytes show the potential to recruit
226   circulating neutrophils (Fig. 5a,d), indicating a robust and redundant mechanism of neutrophil
227   recruitment to the airways via the CXCL8/CXCR2 axis. Surprisingly, the immature neutrophils
228   (blood cluster 3) lacked *CXCR2* (Fig. 5c), suggesting that immature neutrophils are unlikely to
229   infiltrate the lung via IL-8. In contrast, a defined subset of mature neutrophils in blood
230   expressing high levels of *CXCR2*, along with interferon-induced *IFITM2* and *S100A11/12*,
231   identify blood cluster 2 as the putative neutrophil subset that can infiltrate the lung via IL-8 (Figs.
232   4d and 5c-d). It is, therefore, probable that recent lung emigrants would still express detectable
233   levels of *CXCR2*, as well as *IFITM2* and *S100A11/12* (Fig. 5c).
234
235   After identifying the blood neutrophil cluster 2 as the putative source of lung-recruited
236   neutrophils, we sought to identify their cell trajectory/differentiation once they enter the inflamed
237   airway. Cell trajectory analyses (scVelo[32]) revealed two differentiation pathways (Trajectories 1
238   and 2) for the recently infiltrated neutrophils (Fig. 5e). Indeed, cells expressing the highest levels
239   of *CXCR2* and *S100A11/12* were at the beginning of the trajectory, further supporting these as
240   the putative emigrant population from the blood (Fig. 5f). Notably, neutrophils recruited to the
241   lung and differentiated along Trajectory 2 experience transcriptional reprogramming to acquire a
242   heightened inflammatory phenotype (Fig. 5e,f). In contrast to the canonical neutrophil
243   differentiation pathway, including a short half-life, the infiltrated neutrophils in severe COVID-19
244   patients are transcriptionally active (Fig. 5e-g) in comparison to blood neutrophils (Extended
245   Data Fig. 5b) and further differentiate into a hyperinflammatory state (Fig. 5f). This is consistent
246   with more recent reports of neutrophil transmigration in other respiratory illnesses such as cystic
247   fibrosis[33], where neutrophils undergo lung/condition-specific adaptations upon recruitment from
248   the circulation[34] instead of the canonical rapid and transient effector function proceeding cell
249   death. Indeed, we observed increased expression of the interferon-stimulated gene (ISG) *IFI30*
250   along with increased expression of *CCL3* (MIP-1α) and *CCL4* (MIP-1β) (Fig. 5f), which in turn
251   can promote the recruitment of inflammatory monocytes to the lung and/or proinflammatory
252   macrophage phenotypes in the airways.
253
254   Importantly, most neutrophils increase expression of *CXCL8* (IL-8) when recruited to the
255   airways (Fig. 5d), perpetuating neutrophil recruitment to the lung. These findings further
256   corroborate our Hi-D flow cytometry, gene expression, and secreted protein data analysis
257   identifying IL-8 as the most abundant neutrophil-derived chemokine (and neutrophil chemotactic
258   factor) in the airways of severe COVID-19 patients. Furthermore, most neutrophils increase the
259   expression of *CXCR4* when recruited to the airways (Fig. 5f). Interestingly, we find that *CXCR4*
260   expression, previously attributed to immature neutrophils[16], is increased in mature lung-recruited
261   neutrophils (Fig. 5f). This is consistent with *CXCR4* expression dynamics previously detected in
262   non-COVID lung inflammation, including in cystic fibrosis[35] and malaria[36]. Taken together, the

263 progressive increase in *IFI30, CCL3/4*, along with abundant *de novo CXCL8* and *CXCR4* mRNA
264 transcripts, reveal a transcriptionally active state in neutrophils that is poised to sustain a
265 hyperinflammatory milieu in the lung of severe COVID-19 patients.
266
267 **Viral load in the respiratory tract does not correlate with disease severity**
268
269 We then investigated whether the recruited neutrophils or other cell types (i.e., myeloid,
270 lymphoid, and non-immune cells) in the airways of severe patients were infected with SARS-
271 CoV-2. By including the SARS-CoV-2 genome sequence into our human reference
272 transcriptome in the multi-omics single-cell analysis, we were able to assess viral mRNA
273 (vRNA) transcripts at a single-cell level (Fig. 7a). Notably, we did not detect vRNA in any cell
274 types in the blood or airways of severe patients (Fig. 7b,c). Further, SARS-CoV-2-specific RT-
275 qPCR revealed that viral load was decreased in the upper airways of severe patients admitted
276 to the ICU compared to mild-acute patients (Fig. 7d,e). However, we did note a gene signature
277 in neutrophils associated with response to IFN-γ (Fig. 5g and Extended Data Fig. 5b), including
278 a pronounced increase in *IFITM2* (Extended Data Fig. 5c), which has been shown to promote
279 SARS-CoV-2 infection in human lung cells[37].
280
281
282 **Pulmonary TNF and IL-1β promote neutrophil reprogramming in the lungs**
283
284 To ascertain which ligand-receptor pairs are potentially responsible for the transcriptional
285 reprogramming of neutrophils in the lung, we first performed differential gene expression (DGE)
286 analysis between blood neutrophil cluster 2 and lung neutrophils to identify genes that are
287 upregulated in lung-recruited neutrophils (Fig. 6a). Next, we used the computational method
288 NicheNet[38] to identify the potential (prioritized) ligands that could induce the upregulation of the
289 genes identified by the DGE analysis, indicating their potential role in neutrophil reprogramming
290 in the lung. *TNF*, *IL1B*, and *APOE* were the highest prioritized ligands with high ligand activity
291 whose signaling axes have the regulatory potential to drive gene expression profiles observed in
292 lung-recruited neutrophils (Fig. 6b). Importantly, NicheNet analysis revealed *TNF* as the ligand
293 predicted to increase *BCL2A1* expression in pulmonary neutrophils, as well as *CCL4* (MIP-1β)
294 and *CXCL16*. Furthermore, both *TNF* and *IL1B* are the ligands predicted to induce *NFKBIA* and
295 *CXCL8* (IL-8), and *IL1B* shows the greatest potential to induce *CCL3* (MIP-1α) expression in
296 recruited neutrophils. *APOE* is predicted to upregulate the expression of *FCER1G* in neutrophils
297 in the lung. Notably, *TNF, IL1B,* and *HMGB1* are the ligands that have the widest range of
298 regulatory potential, inducing neutrophil reprogramming by upregulating most genes that we
299 identified as differentially expressed (Fig. 6a) in lung-recruited neutrophils (Fig 6b).
300
301 Pulmonary T cells represent the primary cell population expressing *TNF* in the airways, where
302 MdCs have the highest *IL1B* and *APOE* transcripts (Fig. 6c,e). From the transcriptome data,
303 ligand-receptor pair analyses further identified putative signaling mediators for the top 15
304 prioritized ligands (Fig. 6d). *TNF* is predicted to signal through a receptor that has a *CALM1*
305 association (Fig. 6d). Intriguingly, it has been previously reported that CALM1 can bind other
306 transmembrane proteins, including ACE-2[39], and regulate their cell surface expression[40]. *IL1B* is
307 predicted to signal through the canonical IL1B/IL1R2 pathway, and *APOE* through the
308 APOE/SORL1 lipid pathway. *HMGB1* is predicted to signal through *LY96* (MD-2), which is
309 commonly associated with TLR4 (Fig. 6d)[41, 42, 43]. Accordingly, *IL1R2* and *CALM1* transcripts are

310   abundant in neutrophils from blood cluster 2, and their expression are sustained upon
311   recruitment to the airways (Fig. 6e).
312
313   Collectively, we show here that TNF- and IL-1β-mediated transcriptional reprogramming of lung
314   infiltrating neutrophils leads to induction of proinflammatory *NFKBIA*, *CCL3* (MIP-1α), and *CCL4*
315   (MIP-1β) along with *TNFAIP3/6* and *CXCL8* (Figs. 5 and 6). Of note, neutrophil-derived *CCL3*
316   and *CCL4* can attract inflammatory monocytes from the circulation to the airways. Interestingly,
317   lung-infiltrating monocytes also produce elevated levels of *CXCL8*, potentiating the recruitment
318   of circulating neutrophils via the CXCL8/CXCR2 axis (Fig. 5a,d). Hence, both neutrophils and
319   inflammatory monocytes in the airways exacerbate and potentiate pathogenic neutrophilia in the
320   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 mortality[48], 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 disproportionally 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 ARDS[11].

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-1β, 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 FcγRII (CD32) expression on pulmonary neutrophils (see Extended Data Fig. 5a). Of note, the reduction in CD32 expression may prevent IgG-mediated suppression of ISG induction[14] 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-1β 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

10

365 progression. For example, we and others[16] have shown that infiltrating neutrophils sustain local
366 production of calgranulins (S100A8/9/11/12), which can signal through and activate myeloid
367 cells via TLRs and RAGE receptors, compounding the already hyperinflammatory lung milieu.
368 Interestingly, the inflammatory monocytes that are likely recruited to the lungs via neutrophil-
369 secreted CCL3/4 also show elevated levels of neutrophil chemotactic factor *CXCL8* (IL-8),
370 which helps sustain recruitment of pathogenic neutrophils in a positive feedback loop.

372 Other immune and non-immune cell populations in the lung may contribute to COVID-19
373 pathogenesis in severe patients. Non-immune cells (e.g., stromal and epithelial cells) are known
374 targets of SARS-CoV-2 in the lung[56]. Therefore, these cells are also posited to influence
375 immune cell dynamics, especially at the outset of infection[57]. Indeed, our data indicate that non-
376 immune cells play a role in granulocyte activation (Extended Data Fig. 5d) and therefore may
377 initiate neutrophilia at the outset of infection[58]. Additionally, T cell-derived TNF and HMGB1,
378 along with myeloid-derived IL-1β, have regulatory potential to induce inflammatory gene
379 signatures observed in lung-recruited neutrophils. Strikingly, TNF is the ligand predicted to
380 increase *BCL2A1* expression in pulmonary neutrophils, which is an anti-apoptotic factor known
381 to regulate neutrophil survival[59, 60]. *TNF* signaling in recruited neutrophils also has the potential
382 to drive *CCL4* (MIP-1β) expression, along with *CXCL16*, which is another chemotactic factor
383 that can recruit neutrophils and macrophages via the CXCL16/CXCR6 pathway[61, 62]. In addition,
384 myeloid-derived *APOE* is predicted to regulate the expression of *FCER1G* in lung-recruited
385 neutrophils, which is associated with neutrophil activation during other viral respiratory
386 infections[63]. Further studies should investigate other effector functions of myeloid and lymphoid
387 subsets and how these cells interact with neutrophils to promote protection or pathology in the
388 lungs of severe COVID-19 patients.

390 Interestingly, *CXCR4* is upregulated in recruited neutrophils in severe patients (Figs. 4d,i and
391 5f), which may promote neutrophil survival and retention during pneumonitis and/or further
392 influence inflammatory neutrophil phenotype. Accordingly, CXCR4 was shown to promote
393 transcriptional reprogramming of neutrophils in pulmonary tissues[64]. Although we do not find
394 detectable levels of the canonical CXCR4 ligand (i.e., CXCL12) in the airways, we did observe
395 transcripts for *HMGB1* (Fig. 6), which is an alternative ligand for CXCR4. Since the IL-8/CXCR2
396 pathway is most notably increased and likely the primary neutrophil recruitment axis to the
397 airways, we speculate that CXCR4 signaling instead may promote neutrophil survival and
398 retention at the site of inflammation, which has been previously reported[65]. Additionally, CXCR4
399 signaling can stimulate de novo *CXCL8*/IL-8 production[66, 67], and has been shown to promote
400 neutrophil extracellular trap release during malaria disease progression[68]. Alternatively, CXCR4
401 is also associated with neutrophil aging and senescence[69, 70, 71]. As such, elevated CXCR4 may
402 be associated with prolonged neutrophil survival in COVID-19 pathogenesis. In a prior study[72],
403 we noted a similar pattern of CXCR4 expression on T cells from severe COVID-19 patients,
404 where a progressive decrease of surface CXCR4 is associated with recovery. In contrast,
405 patients that succumbed to the disease, showed a time-dependent escalation in CXCR4$^+$
406 circulating T cells concomitant to increased CXCR4$^+$ T cells in the lungs[72], implicating CXCR4
407 on the dysregulated lung-homing inflammatory T cells in COVID-19. Further investigation into
408 the potential role of CXCR4 in cell survival, retention, or recruitment may unravel novel

11

409 therapeutic targets to modulate inflammation and treat severe COVID-19. Notably, therapeutic
410 intervention with a CXCR4 antagonist during malaria-associated ARDS has shown significant
411 benefit in animal models[36].
412
413 Modulating inflammation through corticosteroids (particularly dexamethasone) has shown
414 clinical efficacy and is now the standard-of-care for patients progressing to severe COVID-19[73].
415 However, the pleiotropic effects of glucocorticoids and their propensity to cause neutrophilia are
416 well documented in asthma and chronic obstructive pulmonary disease (COPD)[74]. Although a
417 well-established mechanism of action for dexamethasone is via transcriptional repression of
418 proinflammatory cytokines, we observed very high *CXCL8* and *IL1B* transcripts concomitant
419 with elevated pulmonary IL-8 and IL-1β protein with prominent signatures by intracellular
420 staining—particularly in pulmonary neutrophils. In contrast, IL-6 levels were lower than that of
421 IL-8 and IL-1β in our patient cohort, and overly abundant neutrophils were not producing as
422 much IL-6 comparatively, which may explain, in part, why anti-IL-6/IL-6R studies failed to meet
423 primary endpoints[75]. Here, we provide evidence of an uncoupled cytokine profile in airway fluids
424 versus plasma where the lung microenvironment exhibits features of cytokine-induced ARDS
425 driven largely by a proinflammatory, neutrophilic feed-forward loop. Beyond COVID-19, it has
426 been shown that resolution of neutrophilia in ARDS has substantial prognostic benefit[10]. Taken
427 together, the CXCL8 (IL-8)/CXCR2 signaling axis emerges as a key potential target for next-
428 generation immunomodulatory therapy to reduce pathogenic neutrophilia and constrain severe
429 disease in patients in addition to corticosteroids.
430
431 We also contend that recognizing the lung pathology in severe COVID-19 to be a neutrophilic
432 and hyperinflammatory disease is paramount to achieve better outcomes in next-generation
433 therapies. Although the lung pathology in COVID-19 is initiated by a viral infection, severe
434 patients in the ICU no longer show signs of uncontrolled viral replication. In fact, not only did we
435 not detect viral transcripts by scRNA-seq within the cells in the airways, but we also noted
436 decreased viral burden in severe patients in the ICU versus mild-acute patients seen in the
437 outpatient clinic (Fig. 7). This is further supported by our previous study where we performed
438 plaque assays on the respiratory secretions from severe patients and revealed significantly
439 diminished, if any, viral plaques from the endotracheal aspirates[76]. This may explain, in part,
440 why antiviral drugs such as remdesivir are not able to prevent death when administered to
441 severe patients in ICU[77].
442
443 It is important to consider potentially confounding factors and limitations in our study. Though a
444 major strength of our study is the uniformity of sample collection in a single disease state, our
445 airway samples are limited to ETA, which contrasts with bronchoalveolar lavage fluid (BALF)
446 used in other studies[7, 17]. The ETA procedure can sample material from the medial airways—
447 i.e., an intermediate between the distal/lower airways (e.g., BALF) and the proximal/upper
448 airways (e.g., sputum or oro-/naso-pharyngeal samples). Although the relative abundance of
449 immune cells may vary across the upper and lower airways, previous studies observe a
450 correlation between paired ETA and BALF samples. In addition, these studies show that ETA
451 samples are not inherently neutrophilic[78], though neutrophilia/granulocytosis may be a shared
452 feature of non-COVID-19 and COVID-19 ARDS[10, 79]. In any event, our study is one of the only

453   known to date to employ integrated multi-omics single-cell investigation of immunity in
454   exclusively Black/AA subjects, which are disproportionately burdened with severe disease and
455   worse outcomes. Importantly, other studies of COVID-19 immune responses have noted similar
456   features of disease severity[7, 15, 16], which suggests that the findings in our target demographic
457   will be broadly applicable to other groups. Finally, our study was limited in scope to 35 total
458   subjects and single collection time points, limiting our abilities to interrogate correlations with
459   clinical outcomes, warranting further longitudinal studies in larger patient cohorts.
460

461   In conclusion, we present evidence that neutrophils are poised to be the leukocyte population
462   most responsible for the dysregulated hyperinflammatory response that drives ARDS in severe
463   COVID-19 patients. Neutrophil frequency and inflammatory profiles reveal that they are not only
464   the most abundant leukocyte population in the medial airways, but also major producers of
465   hallmark effector molecules associated with disease severity, including IL-8, IL-1β, and IL-6,
466   along with potent proteases such as NE and MPO, which are inherently inflammatory and
467   contribute to lung damage/pathology. Furthermore, we provide evidence for a neutrophil
468   feedforward loop where IL-8, produced by virtually all pulmonary neutrophils (and some myeloid
469   and stromal cells), is the primary chemokine recruiting circulating neutrophils and promoting
470   neutrophilia in the inflamed airways.
471

472   Collectively, our findings implicate neutrophilia in the immunopathophysiology of severe COVID-
473   19 disease where perpetual, transcriptionally active, and highly inflammatory pulmonary
474   neutrophils drive ARDS despite low viral burden. Thus, therapeutic intervention targeting
475   neutrophil recruitment/retention and/or survival/reprogramming at the site of inflammation has
476   the potential to constrain ARDS in severe patients, particularly those most vulnerable to
477   succumb to COVID-19 disease.

478 **Methods**

479

480 **Ethics and biosafety.** A total of 35 individuals were recruited for this study (Extended Data

481 Tables 1 and 2). The 18 severe COVID-19+ patients were recruited from the Intensive Care

482 Units of Emory University, Emory St. Joseph's, Emory Decatur, and Emory Midtown Hospitals.

483 We also recruited 9 mild COVID-19-infected outpatients in the Emory Acute Respiratory Clinic

484 and 8 healthy adults from the Emory University Hospital. All studies were approved by the

485 Emory Institutional Review Board (IRB) under protocol numbers IRB00058507, IRB00057983,

486 and IRB00058271. Informed consent was obtained from the patients when they had decision-

487 making ability or from a legally authorized representative (LAR) if the patient was unable to

488 provide consent. Blood and sputum or endotracheal aspirate (ETA) were obtained. Control

489 blood samples were obtained from healthy adults matched by age and race. Study inclusion

490 criteria included a confirmed COVID-19 diagnosis by PCR amplification of SARS-CoV-2 viral

491 RNA obtained from nasopharyngeal or oropharyngeal swabs, age of 18 years or greater, and

492 willingness to provide informed consent. Individuals with a confirmed history of COVID-19

493 diagnosis were excluded from the healthy donor group. All work with infectious virus and

494 respiratory samples from COVID-19 patients was conducted inside a biosafety cabinet within

495 the Emory Health and Safety Office (EHSO) and the United States Department of Agriculture

496 (USDA)-approved BSL3 containment facility in the Health Sciences Research Building at Emory

497 University following protocols approved by the Institutional Biosafety Committee (IBC) and

498 Biosafety Officer (see ref[76]).

499

500 **Patient sample collection and processing.** Primary leukocytes from the airways of COVID-19

501 patients requiring mechanical ventilator support were collected bedside via endotracheal

502 aspiration (ETA) and whole blood collected by standard venipuncture. Plasma from whole blood

503 was isolated via centrifugation at 400 x g for 10 min at 4˚C. To remove platelets, the isolated

504 plasma was centrifuged at 4,000 x g for 10 min at 4˚C. Untouched circulating leukocytes were

505 isolated using the EasySep™ RBC Depletion Reagent (StemCell Technologies). ETA (from

506 severe patients) or non-induced sputum (from mild patients) was mixed 1:1 with a 50mM EDTA

507 solution (final concentration 25 mM EDTA) in custom RPMI-1640 media deficient in biotin, L-

508 glutamine, phenol red, riboflavin, and sodium bicarbonate (defRPMI-1640), with 3% newborn

509 calf serum (NBCS) and mechanically dissociated using a syringe to liberate leukocytes from

510 mucins and other respiratory secretions. Supernatants were collected for further analysis, and

511 then cells underwent an additional mechanical dissociation step using 1-3 mL of a 10 mM EDTA

512 in defRPMI-1640 + 3% NBCS and a P1000 pipettor. Cells were then washed with 10 mL

513 defRPMI-1640 + 3% NBCS, passed through a 70 µm nylon strainer, and pelleted through a 2

514 mL 100% NBCS layer prior to counting and downstream processing.

515

516 **High-dimensional (Hi-D) 30-parameter flow cytometry.** Cells (up to 10^7 total) were

517 resuspended in defRPMI-1640 with 3% newborn calf serum and Benzonase™ (FACS buffer) in

518 5 mL FACS tubes and pre-incubated with GolgiStop™ (BD Biosciences) for ~60 min at 4˚C.

519 Human TruStain FcX™ was then added, followed by a 10 min incubation at RT. The 24-color

520 extracellular staining master mix (Extended Data Table 2) was prepared 2X in BD Horizon™

521 Brilliant Stain Buffer to prevent staining artifacts from BD Horizon Brilliant dye interactions and

522 added 1:1 to cells, then incubated for 30 min at 4˚C. Following staining (and total 1 h exposure
523 to GolgiStop™), cells were washed with ~4 mL FACS buffer. Next, the cells were resuspended
524 in 200 μL of BD Cytofix/Cytoperm™ fixation/permeabilization solution and incubated at 4˚C for
525 30 min followed by a wash with ~4 mL BD Perm/Wash™ Buffer. The 4-color intracellular
526 staining (Extended Data Table 3) was prepared in BD Perm/Wash™ Buffer, and cells were
527 stained for 30 min at 4˚C. Cells were washed with ~4 mL BD Perm/Wash™ Buffer, then
528 resuspended for a final 20 min incubation in 4% PFA and transported out of the BSL3
529 containment facility. Cells were washed in ~4 mL FACS buffer, then resuspended in 200-1000
530 μL FACS buffer for acquisition using BD FACSDiva™ Software on the Emory Pediatric/Winship
531 Flow Cytometry Core BD FACSymphony™ A5. To distinguish auto-fluorescent cells from cells
532 expressing low levels of a particular surface marker, we established upper thresholds for auto-
533 fluorescence by staining samples with fluorescence-minus-one (FMO) control stain sets in
534 which a reagent for a channel of interest is omitted. Data were analyzed with FlowJo™ v10.8
535 (FlowJo LLC).
536
537 **Cell-surface antibody-derived tag (ADT) staining, single-cell encapsulation, and library**
538 **generation.** Leukocytes from whole blood and ETA samples were incubated with oligo-
539 conjugated Ig-A/D/G/M for 10 min at 4˚C followed by the addition of Human TruStain FcX™
540 (BioLegend) and 10 min incubation at RT. Cells were then surface stained with oligo-conjugated
541 monoclonal antibody panel (total 89 antibodies; Extended Data Table 5) for 30 min at 4˚C,
542 followed by two washes in def-RPMI-1640/0.04% BSA. Cells were resuspended at a
543 concentration of 1200-1500 cells/μL in def-RPMI-1640/0.04% BSA and passed through a 20 or
544 40 μm cell strainer before loading onto a Chromium Controller (10X Genomics, Pleasanton,
545 CA). Cells were loaded to target encapsulation of 10,000 cells. Gene expression (GEX) and
546 antibody-derived tag (ADT) libraries were generated using the Chromium Single Cell 5′ Library
547 & Gel Bead Kit v1.1 with feature barcoding following the manufacturer's instructions. GEX
548 libraries were pooled and sequenced at a depth of approximately 540,000,000 reads per sample
549 in a single S4 flow cell and ADT libraries at a depth of approximately 79,000,000 reads per
550 sample in a single lane of an S4 flow cell on a NovaSeq™ 6000 (Illumina, San Diego, CA;
551 Extended Data Table 6)
552
553 **Multi-omics single-cell RNA sequencing (scRNA-seq) analysis.** Single-cell 5′ unique
554 molecular identifier (UMI) counting and barcode de-multiplexing were performed using
555 CellRanger Software (v.5.0.0). To detect SARS-CoV-2 viral RNA reads, we built a custom
556 reference genome from human GRCh38 and SARS-CoV-2 references (severe acute respiratory
557 syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome, GenBank MN908947.3).
558 Splicing-aware aligner STAR[80] was implemented to align FASTQ inputs to the reference
559 genome, and the resulting files are automatically filtered by CellRanger to include only cell
560 barcodes representing real cells. This determination is based on the distribution of UMI counts.
561 ADT reads were aligned to a feature reference file containing the antibody-specific barcode
562 sequences. To recover neutrophils, we applied our SuPERR-seq pipeline as previously
563 described[30]. Briefly, we recovered neutrophils from CellRanger unfiltered count matrices by
564 plotting surface CD16 ADT and CD66b ADT using the "FeatureScatter" function in Seurat v4.0[81]
565 (R version 4.0.2). The double-positive cell barcodes were then extracted and further evaluated

566   by GEX to confirm viable neutrophil identity. A threshold for mitochondrial content per barcode
567   was determined for each sample independently and applied as a cutoff to remove dead or dying
568   cells (Extended Data Table 7). Most samples show high cell viability with a minimal proportion of
569   dead cells.
570
571   The UMI counts of the GEX data were log-normalized by the "NormalizeData" function in Seurat
572   before downstream analysis, following the optimal workflow we previously described for sample
573   normalization and data integration[82]. Center log-ratio (CLR) transform in Seurat was performed
574   on ADT UMIs when recovering neutrophils from the unfiltered matrices. For surface protein
575   visualization to classify major lineages using our SuPERR-seq workflow[30], ADT UMIs were
576   normalized using the R package Denoised and Scaled by Background[83] (DSB) to remove
577   ambient UMI counts (i.e., background) prior to manual sequential gating by surface expression
578   (Extended Data Fig. 4) in SeqGeq v1.7 (FlowJo, LLC). DSB uses empty droplets to calculate
579   background expression, which was manually selected according to the distribution of total ADT
580   per cell in the raw count matrices (Extended Data Table 8). To minimize the influence from non-
581   informative empty droplets, we removed cell barcodes with less than 100 total ADT UMIs before
582   plotting the ADT distribution.
583
584   Before integrating the multiple datasets, we first classified major lineages in individual samples
585   based on a combination of gene transcript and surface protein markers (SuPERR-seq
586   workflow[30]) as in Fig. 4 for samples where the ADT library was of sufficient quality to allow
587   manual gating (Extended Data Fig. 4). Cell barcodes within each major lineage that co-
588   expressed markers exclusive to other major lineages were considered cell doublets and
589   removed (Extended Data Fig. 4). In addition, we removed cell barcodes with extremely high
590   total ADT UMIs, which we considered to be aggregated cells. To efficiently integrate replicate
591   samples, we concatenated major lineages derived from the same tissue in different donors. To
592   minimize batch effects and optimize data integration, we followed the data normalization and
593   merging strategies described previously[82]. Briefly, samples were first treated individually, and
594   log-normalized count matrices were scaled/Z-transformed, and the "vst" method of the Seurat
595   function "FindVariableFeatures" was utilized to select the top 1000 highly variable genes
596   (HVGs) of each sample. HVGs shared between replicate samples were used to perform
597   principal component analysis (PCA). To visualize the data, we performed UMAP reduction of
598   the first 30 PCs, and cell clustering was generated using the Leiden community detection
599   algorithm at a resolution of 0.8. UMAP visualizations for the integrated Blood and integrated
600   ETA were generated using Seurat v4 data integration workflow.
601
602   **Receptor-ligand interaction analyses**. Clustered cells from lung and blood samples from each
603   patient were investigated for evidence of intercellular communication using CellChat[31].
604   Clustered cell populations from the lung samples were combined with the blood neutrophils to
605   determine which lung cell populations could recruit circulating neutrophils. We utilized
606   NicheNet[38] to determine which ligand-receptor pairs could be responsible for the different
607   transcriptional states of the neutrophil populations in the lung and blood samples. We focused
608   on certain neutrophil clusters as the receiver populations, considering the remaining neutrophils
609   and other lung cell populations as the senders and thus potential interactors. The target set of

610   genes was determined using Seurat::FindMarkers(min.pct = 0.1), keeping only those genes with
611   an adjusted p-value lower than 0.05 and average log2-fold change of more than 0.2. To address
612   gene expression changes due to lung infiltration, we ran the algorithm using blood neutrophil
613   cluster 2 as the most likely candidate for lung infiltration. The genes considered were those
614   differentially expressed between blood cluster 2 and the lung neutrophils and genes
615   differentially expressed between blood cluster 2 and those cells that progress along "Trajectory
616   2" in the lung (see Fig.5).
617
618   **Cell trajectory analyses.** The Python toolkit scVelo[32] inferred the trajectories using biological
619   data of the ETA neutrophils. Input data for scVelo analysis was intron, exon, and spanning
620   count matrices estimated using the dropEST tool[84], then filtered with previously identified
621   neutrophil cell barcodes in R studio. Intron, exon, and spanning matrices were compared to
622   identify missing rows (genes) and were added to each matrix to equalize dimensions. The exon
623   matrix contained the spliced matrix, and the sum of the intron and spanning matrices constituted
624   the unspliced matrix. Spliced and unspliced matrices were imported with anndata library, and
625   pandas library was used to import gene names and cell barcodes. Raw count matrices were
626   added to anndata object layers as 'spliced' and 'unspliced.' Then, gene names and cell
627   barcodes were attached to variables and observations of anndata object, respectively. Anndata
628   object was transposed, followed by the regular scVelo analysis. The default parameters of
629   plotting velocity streams include vkey='velocity', colorbar=True, alpha=0.3, sort_order=True, and
630   legend_loc='on data'.
631
632   **Pathway and process enrichment analyses**. Differential gene expression (DGE) analyses
633   were performed in Seurat v4 and imported for gene annotation and further analysis using
634   Metascape[85]. DGE between neutrophils versus the total ETA were used to generate Fig. 5g and
635   non-immune cells versus total ETA were used for Extended Data Fig. 5d.
636
637   **Mesoscale U-PLEX assays**. U-PLEX Biomarker Group 1 Human Multiplex Assays (Meso
638   Scale Discovery) were used to evaluate levels of 21 analytes following the manufacturer's
639   protocol (Extended Data Table 4) in plasma and UVC-inactivated respiratory supernatants (see
640   ref[76]). Samples were diluted 1:5 for all assays except for IL-8, MCP-1, and IL-1RA, which were
641   above the upper limit of detection for the assay and were diluted 1:200 to acquire measurement
642   within the assay range. Final values were obtained by multiplying measurements by their
643   respective dilution factor.
644
645   **Myeloperoxidase (MPO) content and activity**. The abundance and activity of MPO were
646   quantified as previously described[29]. MPO activity and protein concentration were measured
647   sequentially following the immunocapture. On average, across six 96-well plate assays, lower
648   limits of quantification were 4.0 ng/mL (activity) and 0.84 ng/mL (protein). Samples above the
649   lower limit of detection but below the lower limit of quantification were imputed as half of the
650   latter, and those detected above the highest standard of 50 ng/mL (i.e., above the upper limit of
651   detection) at all dilutions were imputed as twice the standard concentration (Extended Data
652   Table 4).
653

654 **SARS-CoV-2 quantitative reverse transcription PCR (RT-qPCR).** Viral (v)RNA was
655 extracted from the respiratory secretions of COVID-19 patients using the *Quick*-RNA™ Viral Kit
656 (Zymo Research) following the manufacturer's protocol and complementary (c)DNA synthesized
657 using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems™) per the
658 manufacturer's instructions, then diluted 1:5 in nuclease-free water. 10 µL diluted cDNA was
659 used with the NEB Luna® Universal Probe qPCR Master Mix (New England BioLabs® Inc.)
660 following the manufacturer's protocol and performed in 384-well plates using a QuantStudio™ 5
661 Real-Time PCR System (Applied Biosystems™). Primer/probe pairs were:
662 AGAAGATTGGTTAGATGATGATAGT (forward), TTCCATCTCTAATTGAGGTTGAACC
663 (reverse), and /56-FAM/TCCTCACTGCCGTCTTGTTGACCA/3IABkFQ/ (probe), which were
664 designed from sequences previously described[86] (Integrated DNA Technologies; IDT). To
665 generate a standard curve for the quantification of SARS-CoV-2 genome copies a gBlock from
666 IDT with the following sequence was used as a standard:
667 AATTAAGAACACGTCACCGCAAGAAGAAGATTGGTTAGATGATGATAGTCAACAAACTGTT
668 GGTCAACAAGACGGCAGTGAGGACAATCAGACAACTACTATTCAAACAATTGTTGAGGTTC
669 AACCTCAATTAGAGATGGAACTTACAGTTTCAGTGTTCAATTAA.
670
671 **Statistical analyses**. Statistical analyses were performed using GraphPad Prism9. Data were
672 analyzed for distribution (normal (Gaussian) vs. lognormal) independently using the D'Agostino
673 and Pearson test for normality in the untransformed and Log10-transformed data. In cases
674 where the sample size (N) was too small for D'Agostino and Pearson normality test, the
675 Shapiro-Wilk test was used to assess distribution. When data passed both distribution tests, the
676 likelihood of each distribution (normal vs. lognormal) was computed, and QQ-plots were
677 generated. When Log10 transformed data had a higher likelihood of a normal distribution
678 (passing normal distribution test) and/or failed lognormal distribution test, paired t-tests were
679 performed to compare matching blood and respiratory supernatant samples within a single
680 group. If the data had unequal variance (as determined by an F-test), a ratio paired t-test was
681 performed. All instances where lognormal distribution was likely non-parametric Wilcoxon
682 matched-pairs sign ranked tests were performed. For comparisons across the three patient
683 groups (i.e., healthy, mild-acute, severe), ordinary one-way ANOVA (if equal variance) or
684 Brown-Forsythe and Welch ANOVA (if unequal variance) tests were performed for data with a
685 normal distribution. Alternatively, data with a lognormal distribution were analyzed with a
686 Kruskal-Wallis test.
687
688 **Data availability.** Single-cell sequencing datasets presented here are available through NCBI
689 GEO, accession number XXX.

690 **Author Contributions**

691

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708 Draft, Visualization, Supervision, Project Administration**,** Funding Acquisition**.** All authors
709 discussed the results and read and approved the final manuscript.

710

711 **Competing interests**

712

713 FEL is the founder of MicroB-plex, Inc., serves on the SAB of Be Bio Pharma, receives grants
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716

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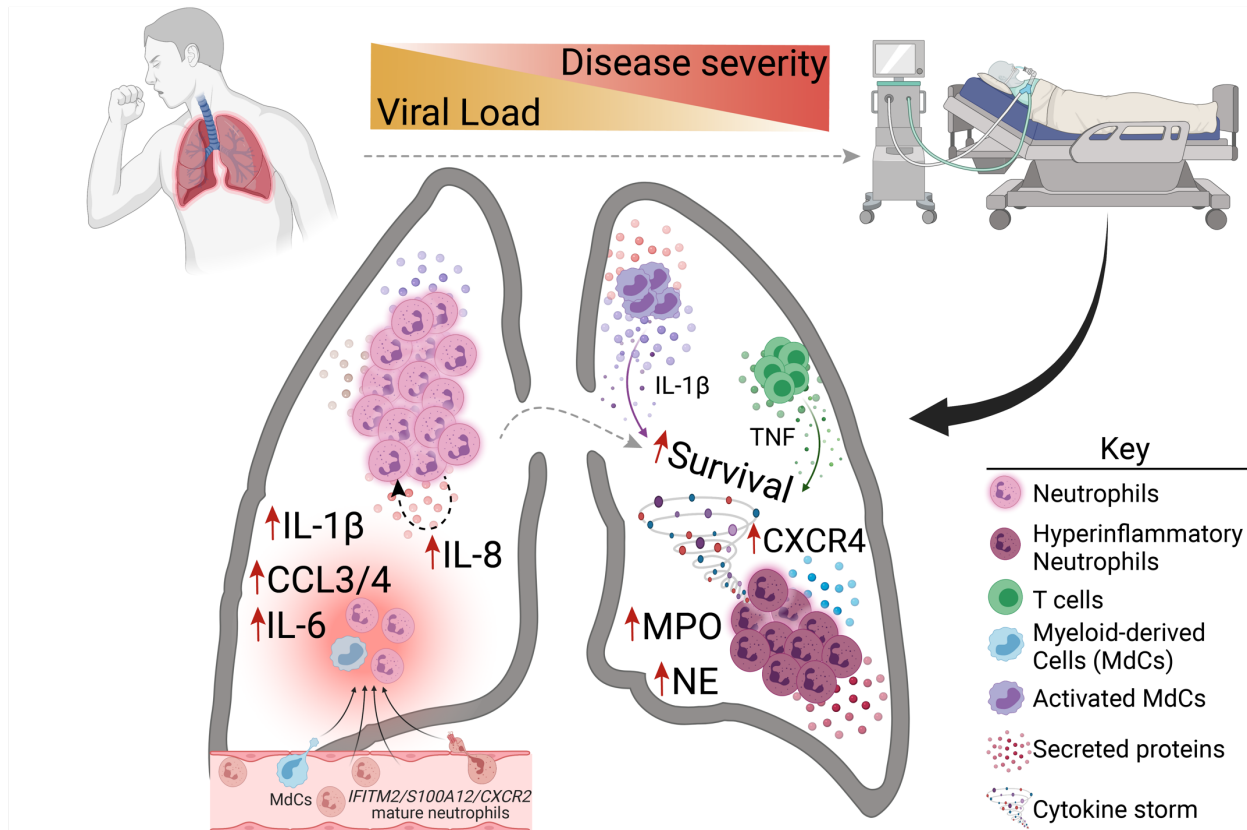
Graphical Abstract

The lung pathology due to severe COVID-19 is marked by a perpetual pathogenic neutrophilia, leading to acute respiratory distress syndrome (ARDS) even in the absence of viral burden. Circulating mature neutrophils are recruited to the airways via IL-8 (CXCL8)/CXCR2 chemotaxis. Recently migrated neutrophils further differentiate into a transcriptionally active and hyperinflammatory state, with an exacerbated expression of IL-8 (*CXCL8*), IL-1β (*IL1B*), *CCL3*, *CCL4*, neutrophil elastase (NE), and myeloperoxidase (MPO) activity. Airway neutrophils and recruited inflammatory monocytes further increase their production of IL-8 (*CXCL8*), perpetuating lung neutrophilia in a feedforward loop. MdCs and T cells produce IL-1β and TNF, driving neutrophils reprogramming and survival.
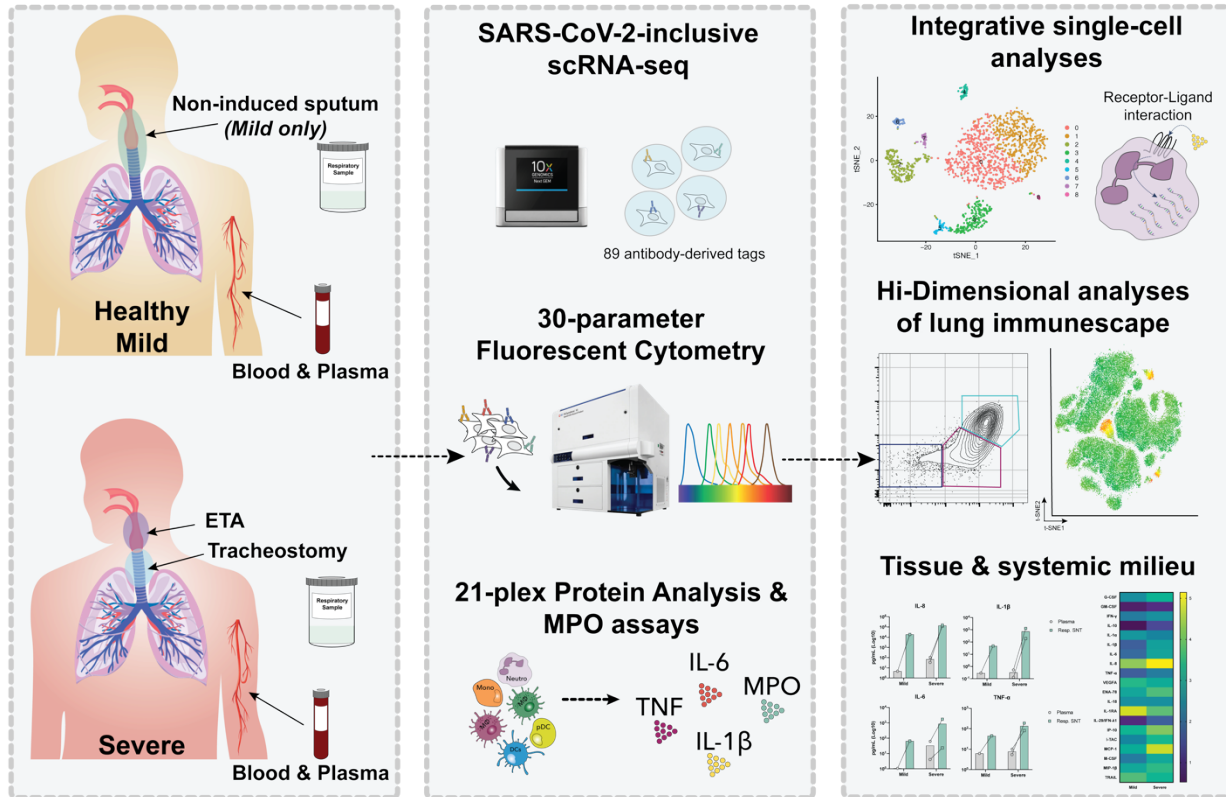
Figure 1. *Experimental design for the systems immunology approach (integrated multi-omics single-cell assays) to study COVID-19 patient samples*. Respiratory samples (sputum or endotracheal aspirates) and matching blood from all subjects were collected for analysis by 21-plex Mesoscale analysis, high-dimensional (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.

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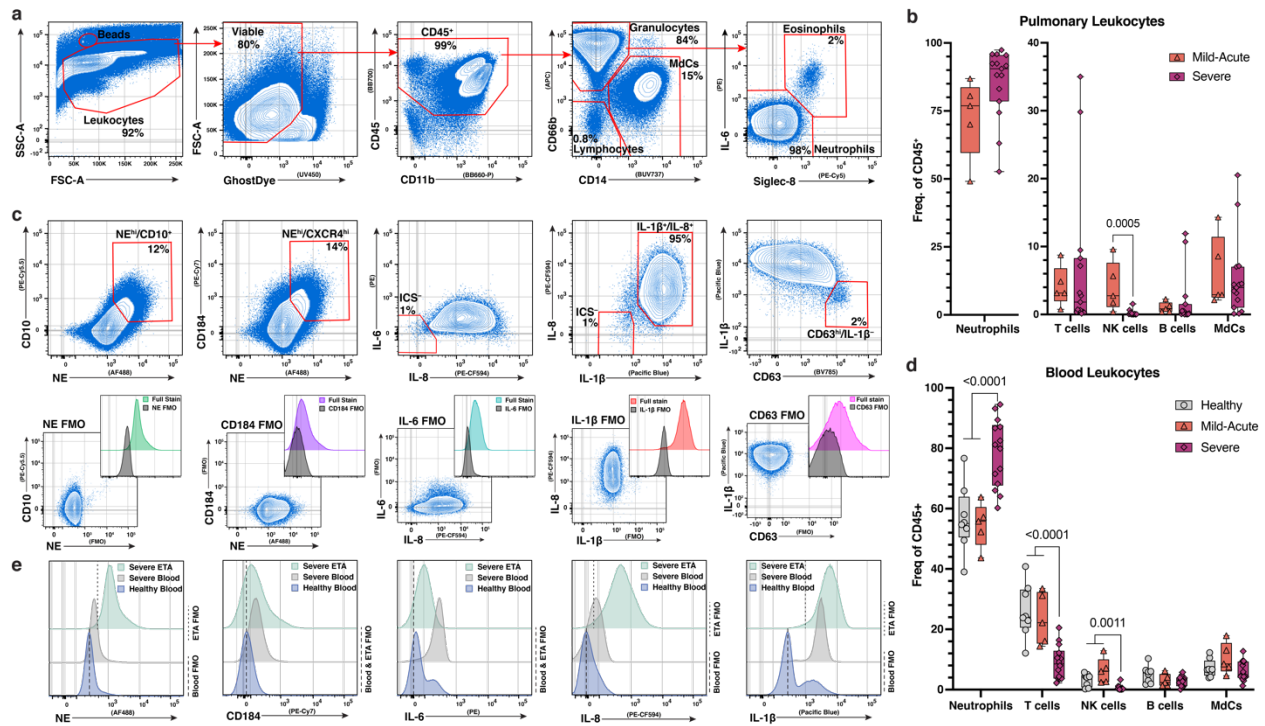
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1052 **Figure 2. *Exacerbated neutrophilia in the airways and matching blood of severe COVID-**
1053 *19 patients.*** (a) Representative gating strategy for all samples (see Extended Data Fig. 1 for full
1054 gating strategy). (b) Box plots show distributions of leukocytes isolated from endotracheal
1055 aspirates (ETA). (c) Representative plots demonstrating inflammatory profile of pulmonary
1056 neutrophils including neutrophil elastase (NE), CD184 (CXCR4), and intracellular staining of IL-
1057 6, IL-8, and IL-1β including the full stain and fluorescence minus one (FMO) controls (d) Box
1058 plots show distributions of leukocytes isolated from whole blood from severe patients. (e)
1059 Representative histograms showing median fluorescence intensity (MFI) of key markers across
1060 healthy blood (blue), severe blood (gray), and severe ETA (green) samples. MdCs = myeloid-
1061 derived cells. For comparisons across the three patient groups (i.e., healthy, mild-acute,
1062 severe), ordinary one-way ANOVA (if equal variance) or Brown-Forsythe and Welch ANOVA (if
1063 unequal variance) tests were performed for data with a normal distribution. Data with a
1064 lognormal distribution were analyzed with a Kruskal-Wallis test.

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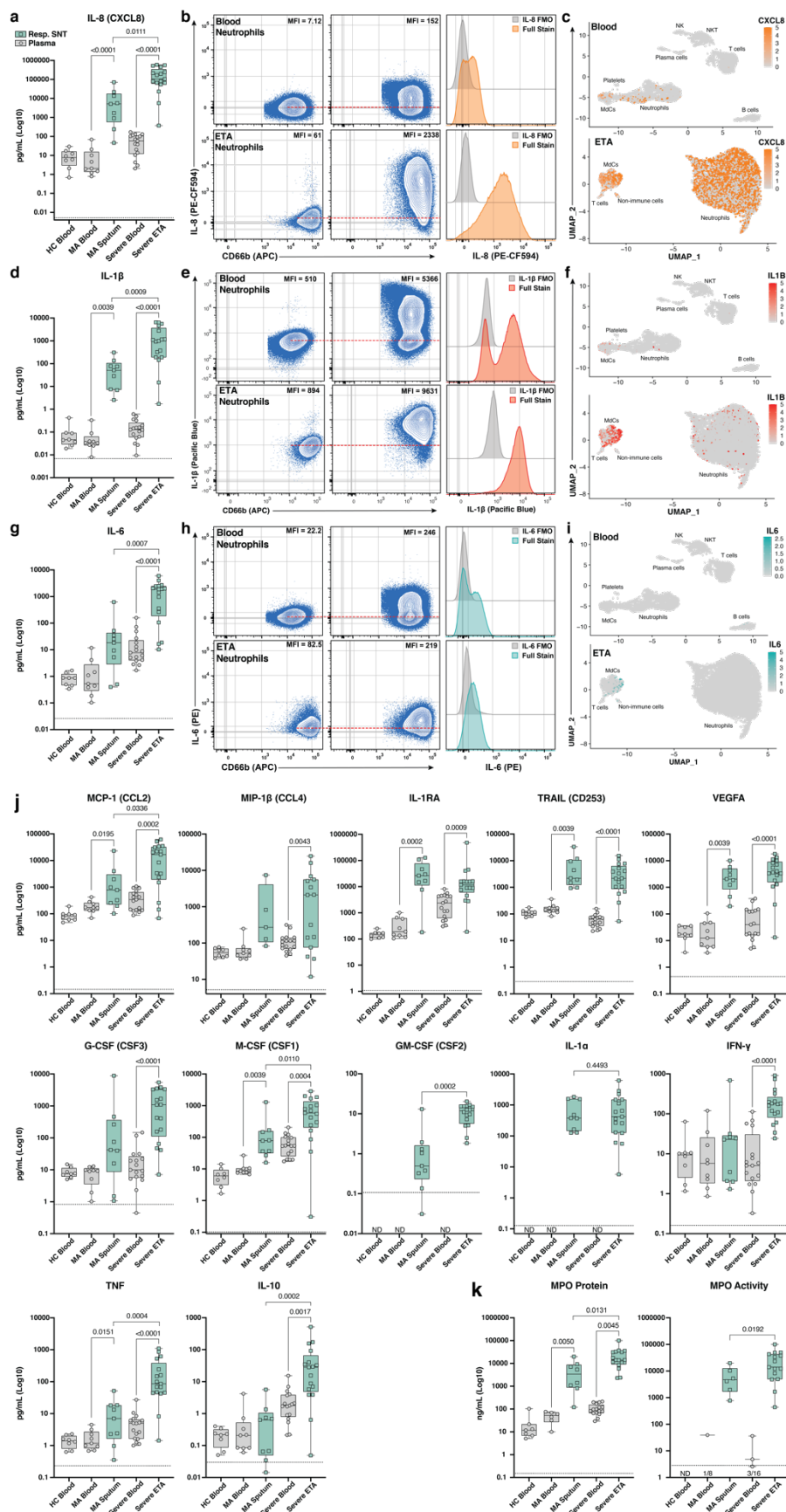
1067 **Figure 3**. *Cytokine release syndrome is dominated by IL-8 and IL-1β with pronounced*
1068 *myeloperoxidase content and activity in the lung microenvironment*. (a, d, g, j)
1069 Concentration (pg/mL) of 15 analytes interrogated by Mesoscale analyses in plasma (gray
1070 circles) and respiratory supernatant (Resp. SNT; green squares) from healthy control (HC),
1071 mild-acute (MA), and severe COVID-19 patients. (b, e, h) Representative flow cytometric
1072 intracellular staining for IL-8 (CXCL8), IL-1β, and IL-6, including the full stain and fluorescence
1073 minus one (FMO) controls in both blood and ETA neutrophils (CD66b⁺). (c, f, i) UMAP
1074 visualizations of *CXCL8* (IL-8), *IL1B*, and *IL6*, which were also measured by intracellular flow
1075 cytometry staining and Mesoscale in both blood and ETA. (k) Concentration (ng/mL) of
1076 myeloperoxidase (MPO) protein and MPO activity in plasma vs. Resp. SNT. In (a, d, g, j, k),
1077 black dotted line = median lower limit of detection (LLOD) for assays (see Extended Data Table
1078 3). In (b, e, h) red dashed line indicates the median fluorescence intensity (MFI) of neutrophils in
1079 the FMO control (value listed in the plot).
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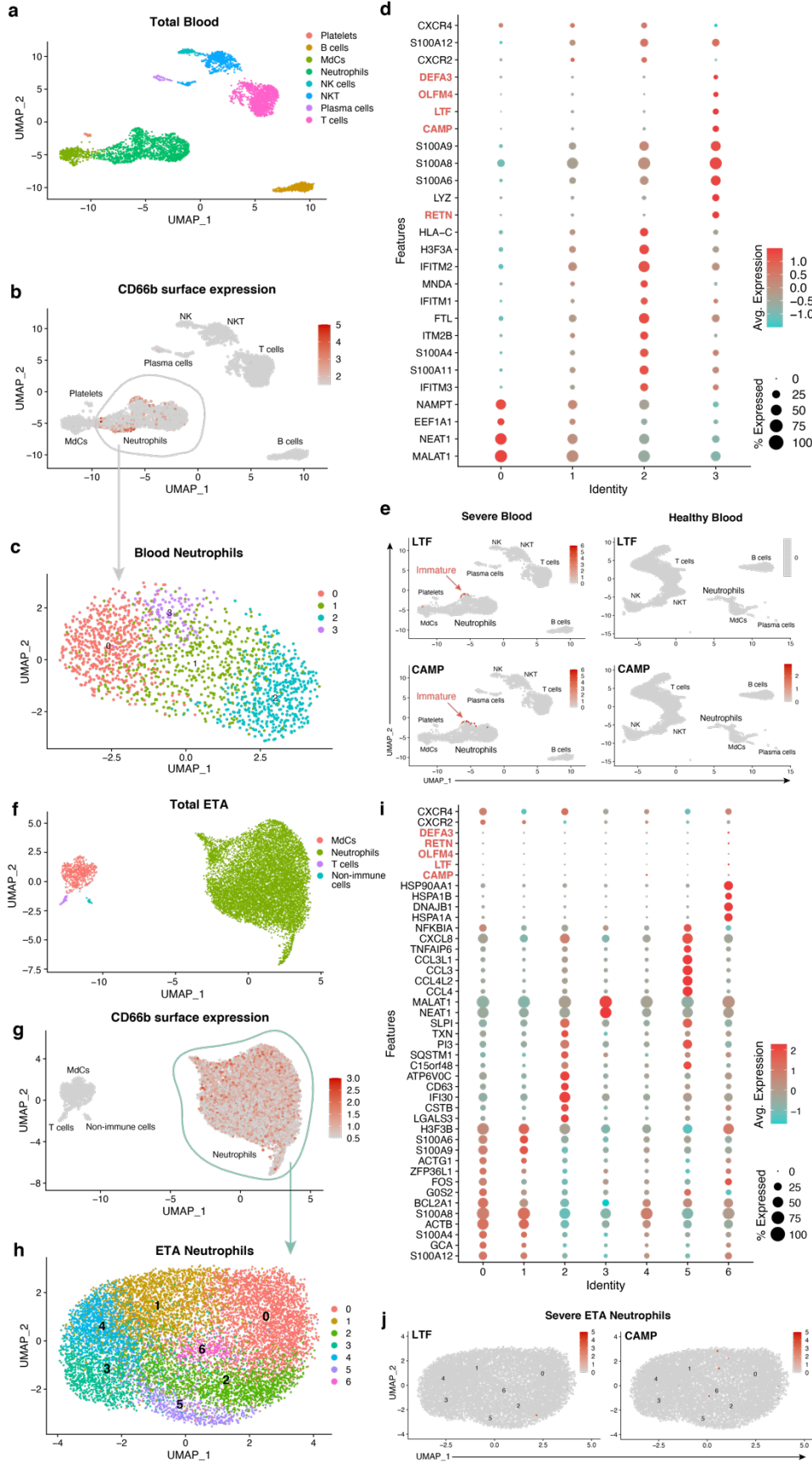
1082 **Figure 4.** *Multi-omic single-cell RNA-seq reveals emergency granulopoiesis in the*
1083 *circulation and abundant heterogeneous populations of mature neutrophils in the*
1084 *airways with distinct inflammatory states.* UMAP visualization of scRNA-seq of total
1085 integrated blood (a) and endotracheal aspirate (ETA) (f) cells. Neutrophils were identified based
1086 on cell-surface markers (b and g), and total neutrophils were subclustered for further analysis (c
1087 and h). Dot plots of the intersection of the top differentially expressed genes in neutrophil
1088 clusters (d and i) sorted by average log-fold change for blood (d) and lung (i) neutrophils,
1089 respectively. UMAP visualization of signature genes of immature neutrophils (highlighted in d
1090 and i) in blood from severe patients compared to healthy individuals (e) and lungs of severe
1091 patients (j).
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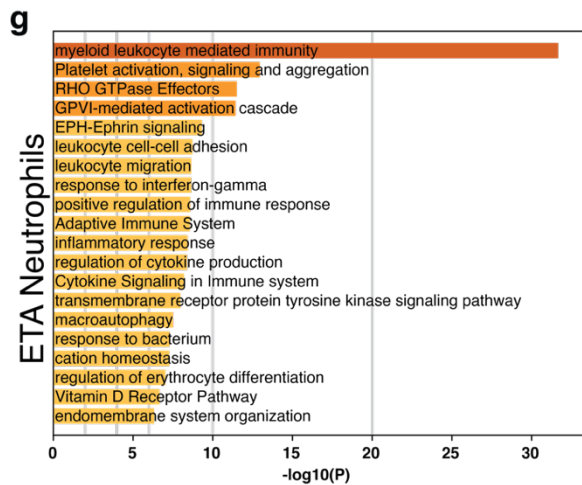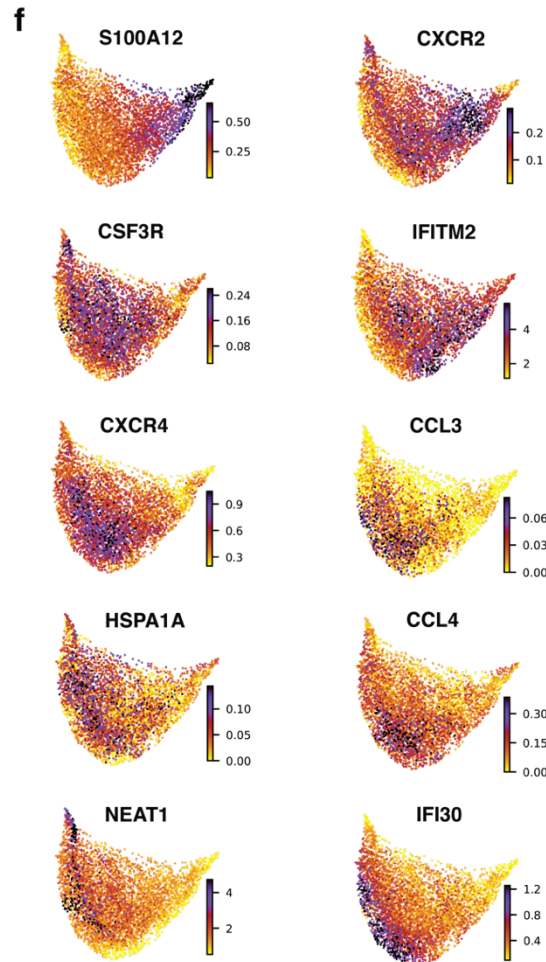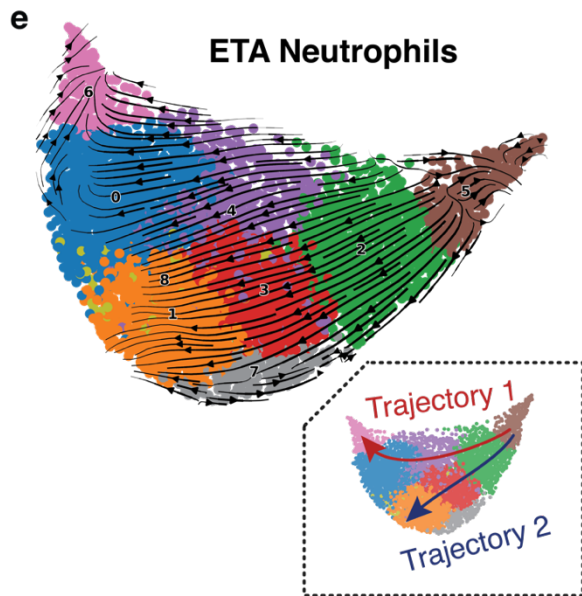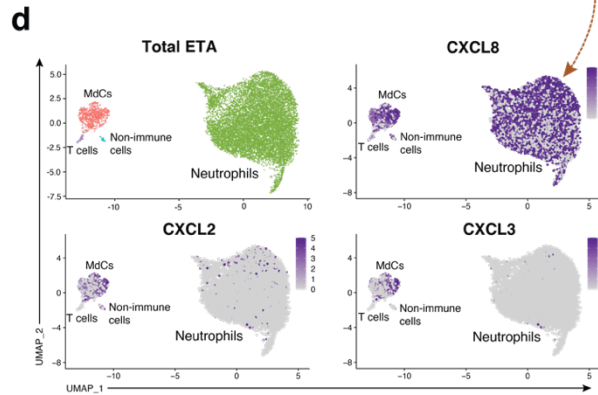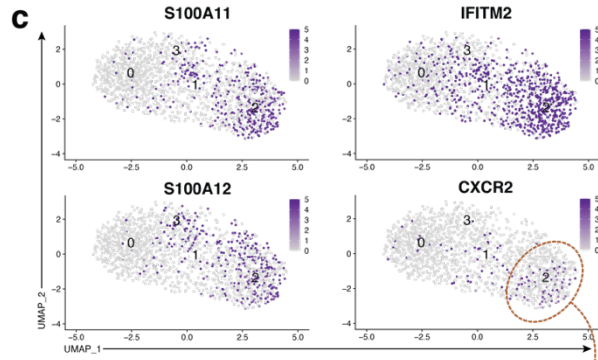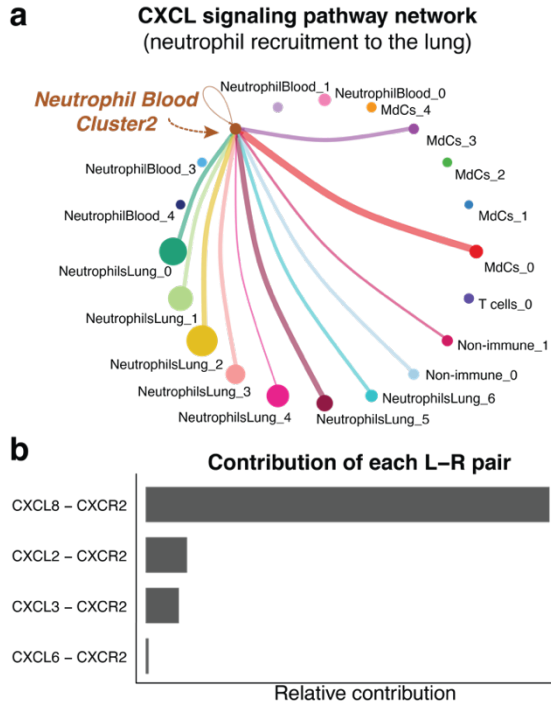
1094 **Figure 5**. *Mature neutrophils are continuously recruited from circulation and progress*
1095 *toward a hyperinflammatory state*. Cell receptor-ligand pair analyses (CellChat[31]) from
1096 scRNA-seq data identify significant CXCL signaling pathway network enrichment between lung
1097 cells and blood neutrophils (a and b). Blood neutrophil cluster 2 represents the main subset
1098 potentially recruited to the lung (a). Recruitment of blood cluster 2 (a and c) is largely
1099 orchestrated by the CXCL8 (IL-8)/CXCR2 axis, and to a lesser extent, by the CXCL2 and
1100 CXCL3 (b and d). *S100A11/12, IFITM2,* and *CXCR2* mark a cluster of mature neutrophils in the
1101 blood (c) that likely represents the neutrophil subset recruited to the lung (d). Cell trajectory
1102 analysis (scVelo[32]) identifies two potential pathways (Trajectories 1 and 2) for recently migrated
1103 neutrophils (e), that begin with a gene signature consistent with neutrophil blood cluster 2 (c and
1104 f). Neutrophils recruited to the lung acquire a hyperinflammatory profile along Trajectory 2 (e
1105 and f), characterized by high expression of interferon-stimulated gene (ISG) *IFI30* along with
1106 macrophage inflammatory proteins *CCL3* (MIP-1α) and *CCL4* (MIP-1β), whereas *CSF3R* and
1107 *CXCR4* are increased in cells along both trajectories (f). Neutrophils along Trajectory 1 may
1108 reflect cells progressing to apoptosis expressing higher levels of *HSPA1A* (HSP70) followed by
1109 *NEAT1* (f). Pathway and process enrichment analyses performed in Metascape[85] reveals that
1110 myeloid-mediated immunity and platelet activation, signaling, and aggregation are significantly
1111 enriched in neutrophils in the lungs of severe patients (g). MdCs: Myeloid-derived Cells.
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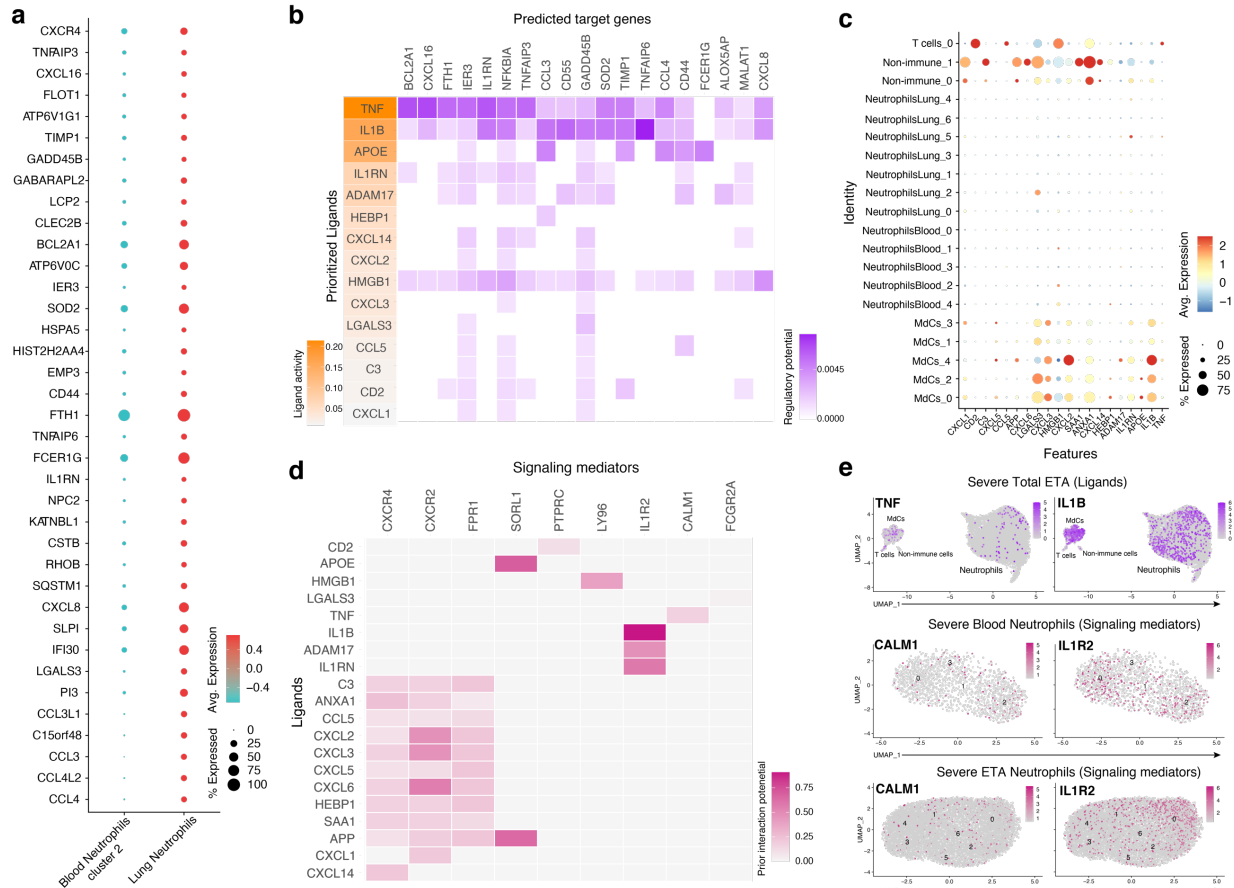
Figure 6. *TNF and IL-1β drive inflammatory program in neutrophils recruited to the lung.*
(a) Dot plots showing 37 of the 67 differentially expressed genes in lung neutrophils versus blood neutrophil cluster 2 sorted by average log-fold change used as input for NicheNet[38] analyses (b) NicheNet[38] 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.

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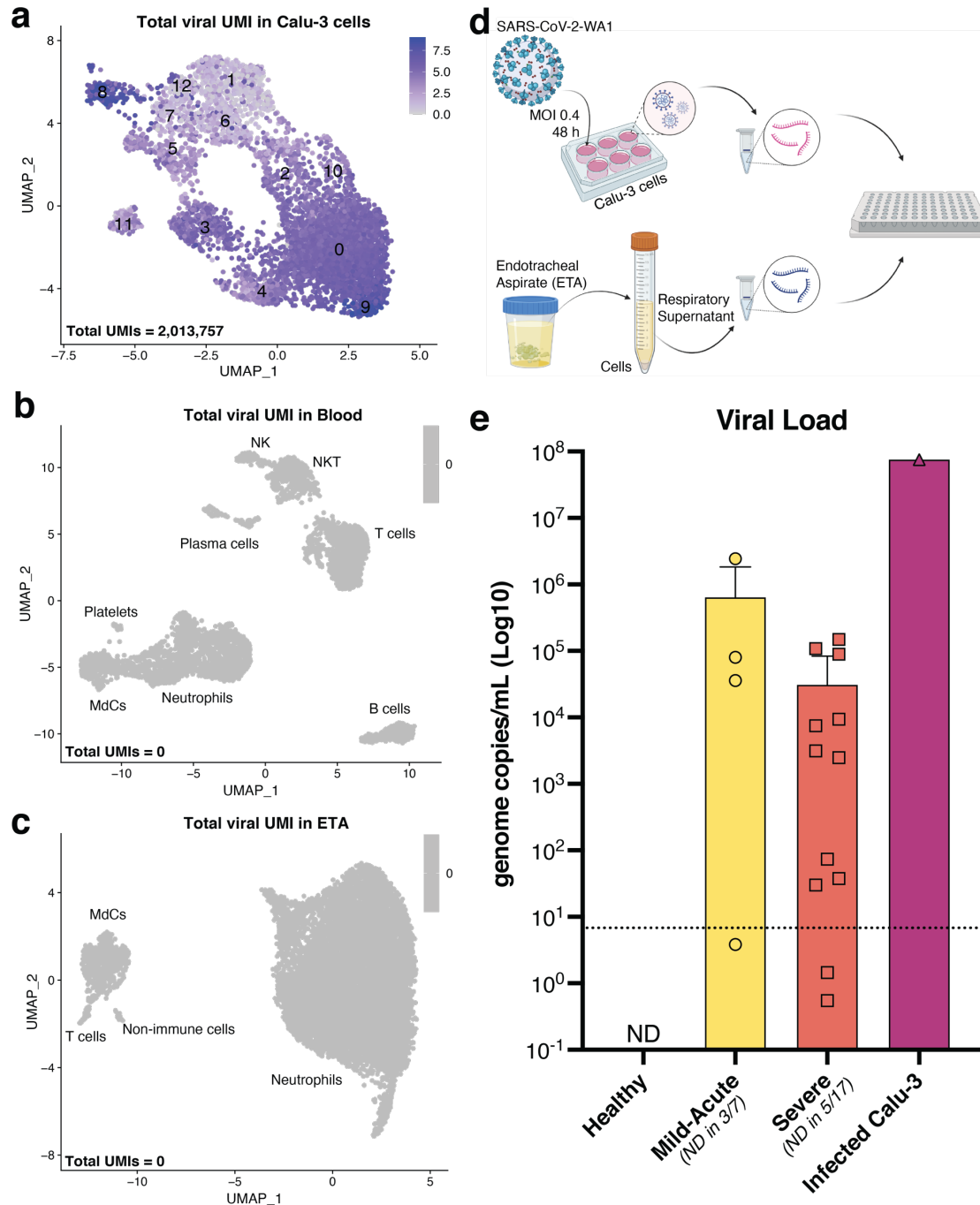
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Figure 7. *Viral transcripts (scRNA-seq) and viral load in the airways of severe patients.*
1129 Calu-3 cells infected with SARS-CoV-2 USA-WA1/2020 (MOI 0.4) were encapsulated for
1130 scRNA-seq assays (a) following the same protocol used for patient cells from blood (b) and ETA
1131 (c). Millions of viral transcripts (or unique molecular identifiers; UMIs) were detected in about
1132 5,000 Calu-3 cells (a), but not in the cells from severe patients (total UMI = 0; b and c). RT-
1133 qPCR (d) results of culture supernatant from Calu-3 infected cells and respiratory supernatants
1134 from mild-acute and severe patients (e) show a lower viral load in severe patients than in mild-
1135 acute patients and in the Calu-3-infected culture supernatant. ND = not detected; horizontal
1136 dotted line = lower limit of detection (LLOD).
1137

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

1140 **Extended Data Table 1.** *Demographic and clinical data from the 35 Black/African*
1141 *American individuals enrolled in our studies.* Table describing demography, status, sex, and
1142 age of study participants along with select details of clinical course including administration of
1143 Dexamethasone and Remdesivir, date first intubated (severe, ICU patients only), date of PCR
1144 positive diagnosis (Dx), date clinical specimens were collected, and total length ICU stay in
1145 days, and clinical outcome (whether recovered or succumbed to disease).
1146

Characteristics	All subjects (N = 35)		
	Healthy (N = 8)	**Mild-Acute** (N=9)	**Severe** (N=18)
Age			
A. Mean age ± SD	46 ± 11	51 ± 12	56 ± 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. Gastroesophegeal 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. Athritis/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. Extracorpeal membrane oxygenation (ECMO)	—	—	2 (11%)
D. Shock	—	—	15 (83%)
E. Nosocomial pneumonia	—	—	6 (33%)
F. Coagulopathy	—	—	3 (17%)
G. Seizures	—	—	3 (17%)
H. Cardiac arrythmia	—	—	3 (17%)
I. Renal failure	—	—	8 (44%)
J. Renal replacement therapy	—	—	6 (33%)

1147

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	405 nm	V 610/20	CD86 *	BV605	FUN-1	Mouse IgG1, κ	BD Biosciences	562999	1:50
11		V 670/30	TNF	BV650	MAb11	Mouse IgG1, κ	BD Biosciences	563418	1:80
12		V 710/50	CD19	BV711	HIB19	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
	488 nm	B 488/10	Side Scatter (SSC)	—	—	—	—	—	—
1		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	HIB19	Mouse IgG1, κ	BioLegend	302240	1:40
8		YG 586/15	CD123 (IL-3Rα)	PE	6H6	Mouse IgG1, κ	BioLegend	306006	1:400
9	561 nm	YG 670/30	Siglec-8	PE-Cy5	7C9	Mouse IgG1, κ	BioLegend	347114	1:100
10		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

1155

1156   **Extended Data Table 3.** *High-dimensional 30-parameter, including intracellular cytokine*
1157   *staining, and 17-parameter flow cytometry panels used for airway and blood cells.* Flow
1158   cytometer configuration and cytometry reagents used in the final panels to interrogate (a)
1159   neutrophil phenotype and (b) global immune cell distributions and blood and ETA samples.
1160   Channels marked with an asterisk (*) denote alternate reagents (c) used in earlier panel
1161   iterations for some samples in this study. Monoclonal antibody (mAb) master mixes were
1162   prepared in BD Horizon™ Brilliant Stain Buffer and samples stained as described in the
1163   methods section. Titrations of all reagents were determined in house for each lot independently
1164   prior to use. AF: AlexaFluor, APC: Allophycocyanin, BB: Brilliant Blue, BUV: Brilliant Ultraviolet,
1165   BV: Brilliant Violet, FITC: Fluorescein isothiocyanate, PE: Phycoerythrin. Fluorophores marked
1166   with -P denote prototype reagents and are custom conjugations from BD Biosciences.
1167

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

1168

1169 **Extended Data Table 4.** *Mesoscale U-PLEX biomarker group 1 human and*
1170 *myeloperoxidase (MPO) assays.* Median ± SD and range of lower limits of detection (LLOD)
1171 for (a) Mesoscale UPLEX analytes and (b) MPO assays. Lower limits of quantification (LLOQ;
1172 MPO assays) and LODs (determined by standard curve) were run for each assay plate
1173 independently and the median LLOD plotted as horizontal dotted lines for each assay (see Fig.
1174 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 αE)	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-3Rα)	6H6	Biolegend	306045	53	CD41	HIP8	Biolegend	303739
9	CD127 (IL-7Rα)	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 (FcγRIII)	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 (FcγRI)	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	CD8α	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 Vα7.2	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 β1)	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 (FcγRII)	FUN-2	Biolegend	303225	89	IgA	IS11-8E10	Myltenyi	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

1187
1188 **Extended Data Table 6.** *Sequencing depth of independent samples.* All gene expression
1189 (GEX) libraries were pooled and run in a single S4 flow cell on a NovaSeq™ 6000. All antibody-
1190 derived tag (ADT) libraries were pooled and sequenced in a single lane of a S4 flow cell on a
1191 NovaSeq™ 6000. Total reads and mean number of reads per cell for GEX and ADT are
1192 reported independently for each sample, with an average read depth of 540,000,000 reads per
1193 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

	Total ADT UMI	
PID	**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	

1194

1195 **Extended Data Table 7. *Mitochondrial gene thresholds and total ADT UMIs of individual**
1196 *samples.* (a)Threshold for mitochondrial gene distribution (percentage) for each sample used to
1197 exclude potential dead cells in scRNA-seq data. Threshold was evaluated and set for each
1198 sample independently. (b) The distribution of total ADT UMIs was determined and recorded per
1199 each sample independently.
1200
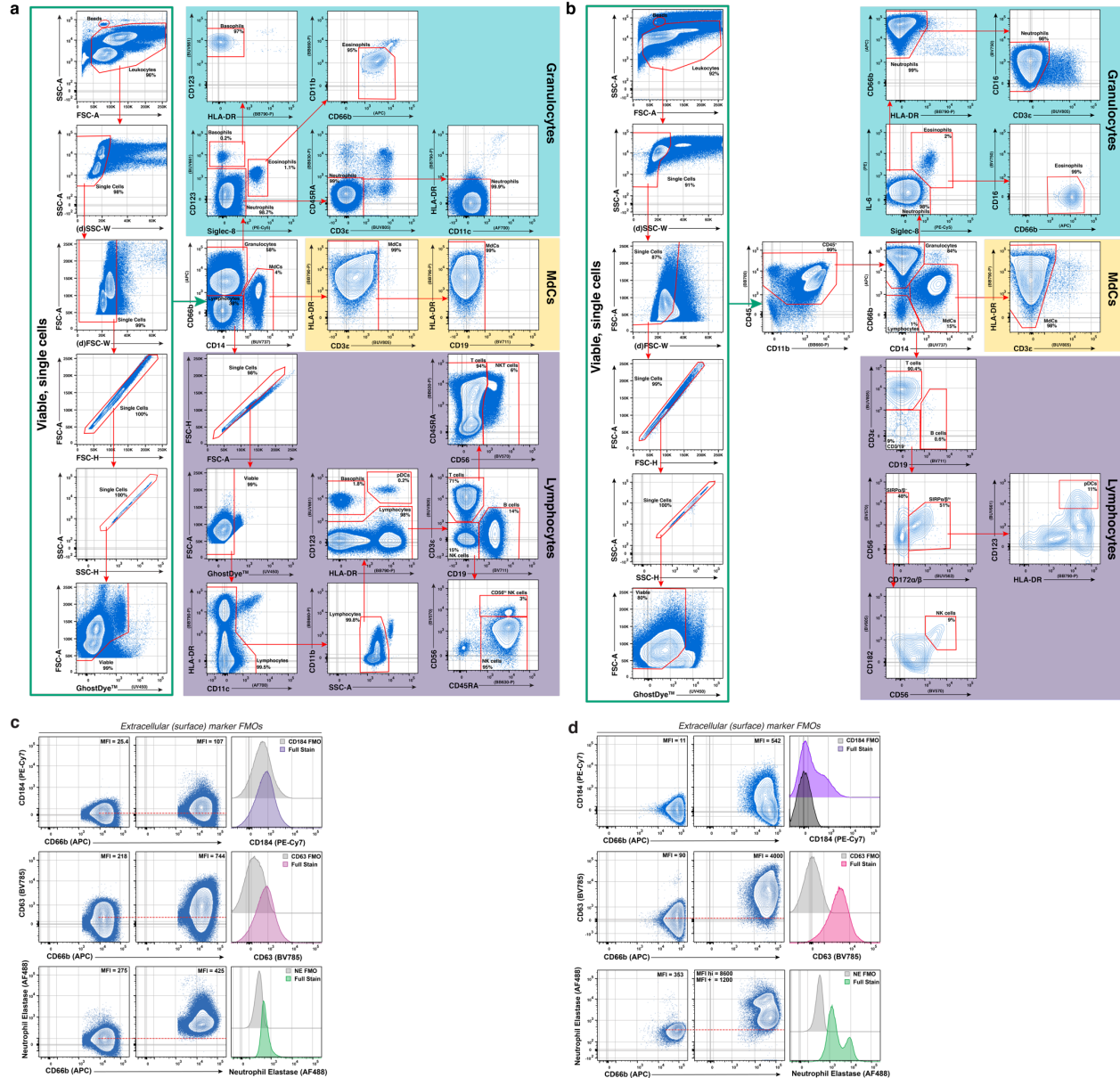
1201
1202 **Extended Data Figure 1.** *Representative gating strategy for Hi-D FACS data.*
1203 Representative gating of blood (a) and ETA (b) samples. Extracellular stain FMOs for markers
1204 used to interrogate neutrophil phenotype in blood (c) and ETA (d).

47
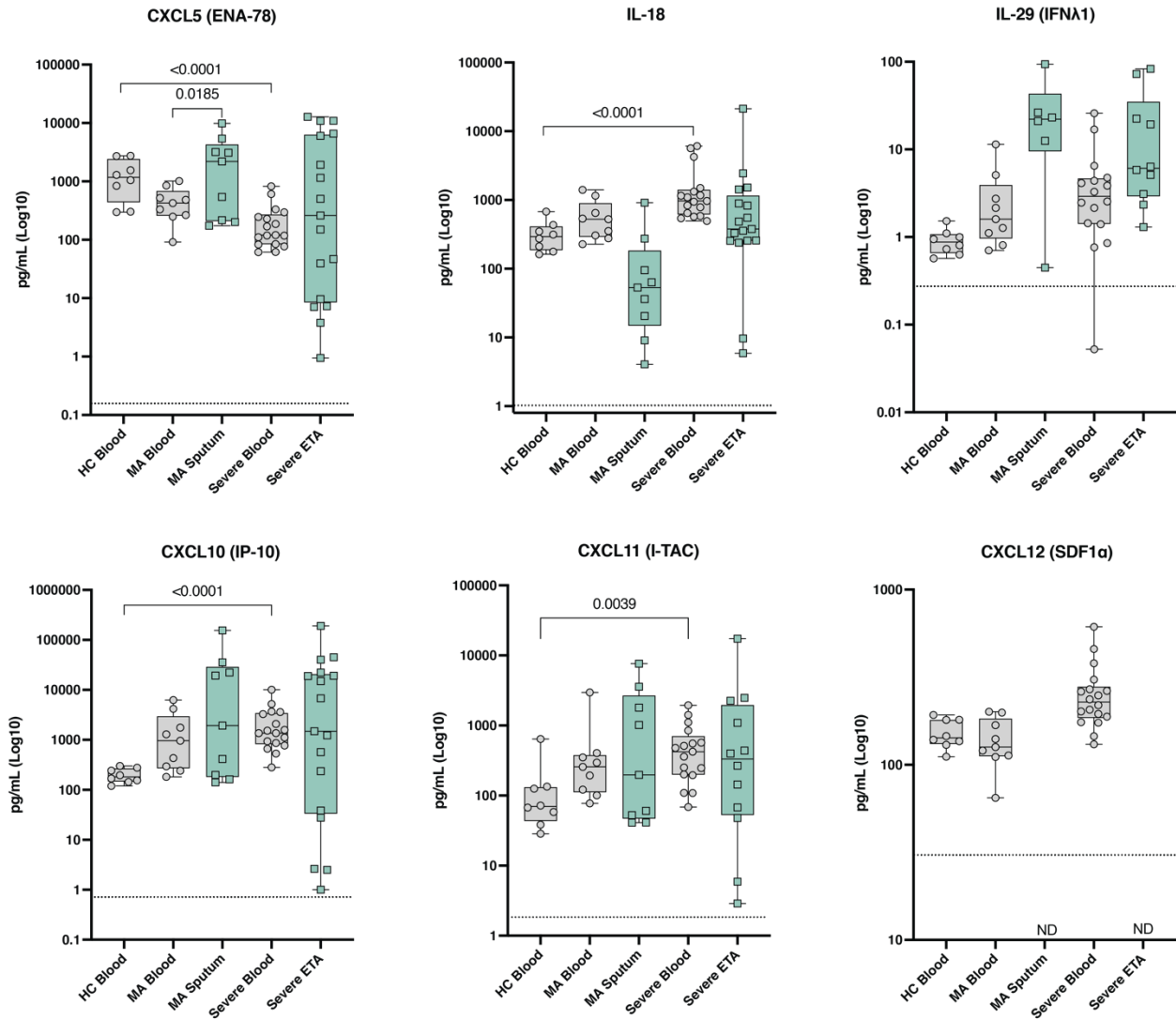
Extended Data Figure 2. *Additional cytokine assessment in blood and lungs.*
Concentration (pg/mL) of remaining 6 analytes interrogated by Mesoscale analyses in plasma
(gray circles) and respiratory supernatant (Resp. SNT; green squares) from healthy control
(HC), mild-acute (MA), and severe COVID-19 patients. Dotted line = assay limit of detection
(LOD).
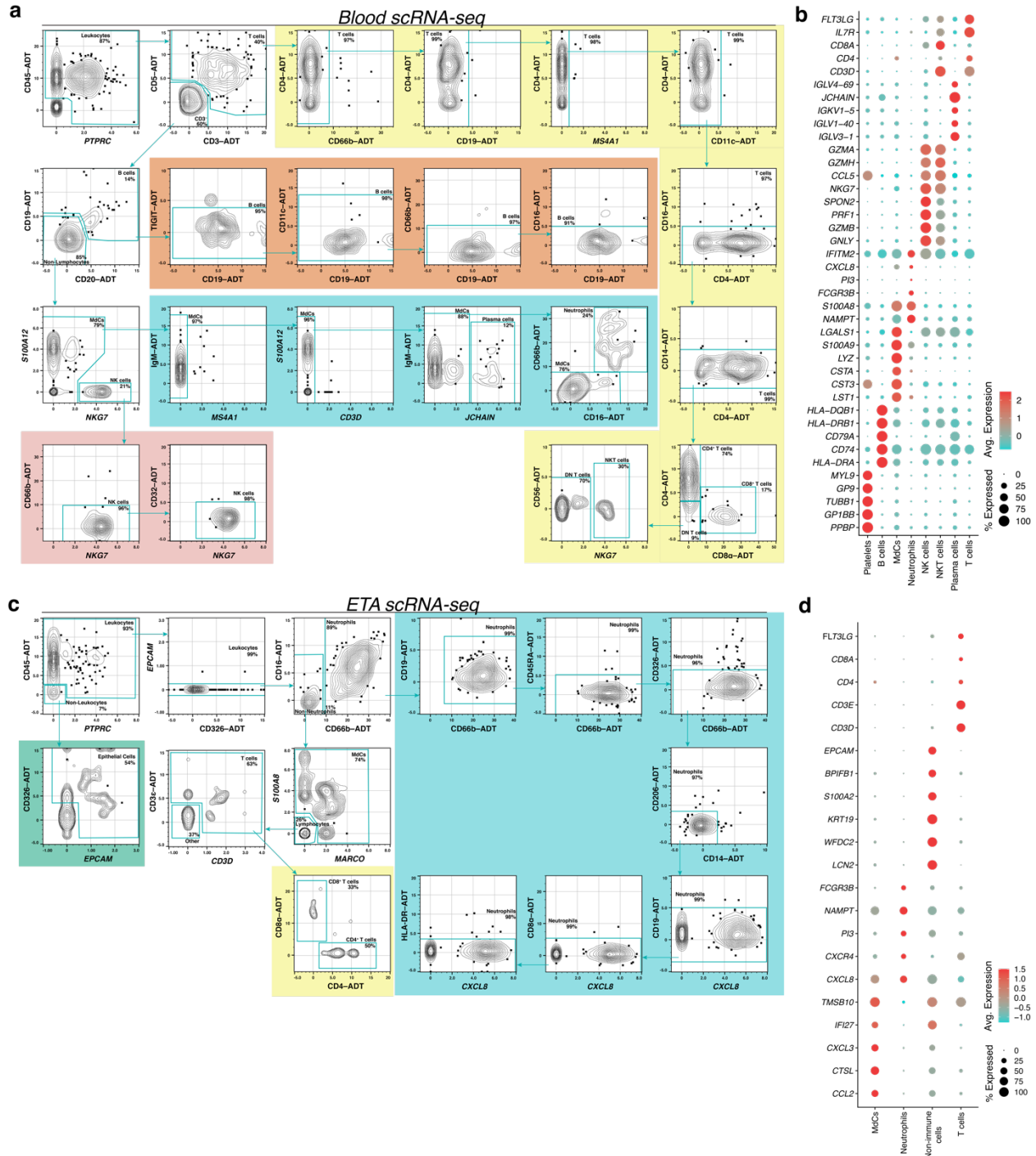
Extended Data Figure 3. *Multi-omic scRNA-seq analysis of leukocytes in the blood and lung.* Gating strategy (a and c) employed to classify major lineages of immune cells by surface antibody-derived tag (ADT) and dot plots of the intersection of the top differentially expressed genes sorted by average log-fold change across cell populations (b and d) from the blood (a-b) and ETA (c-d) of severe COVID-19 patients using the SuPPER-seq pipeline previously described[30].

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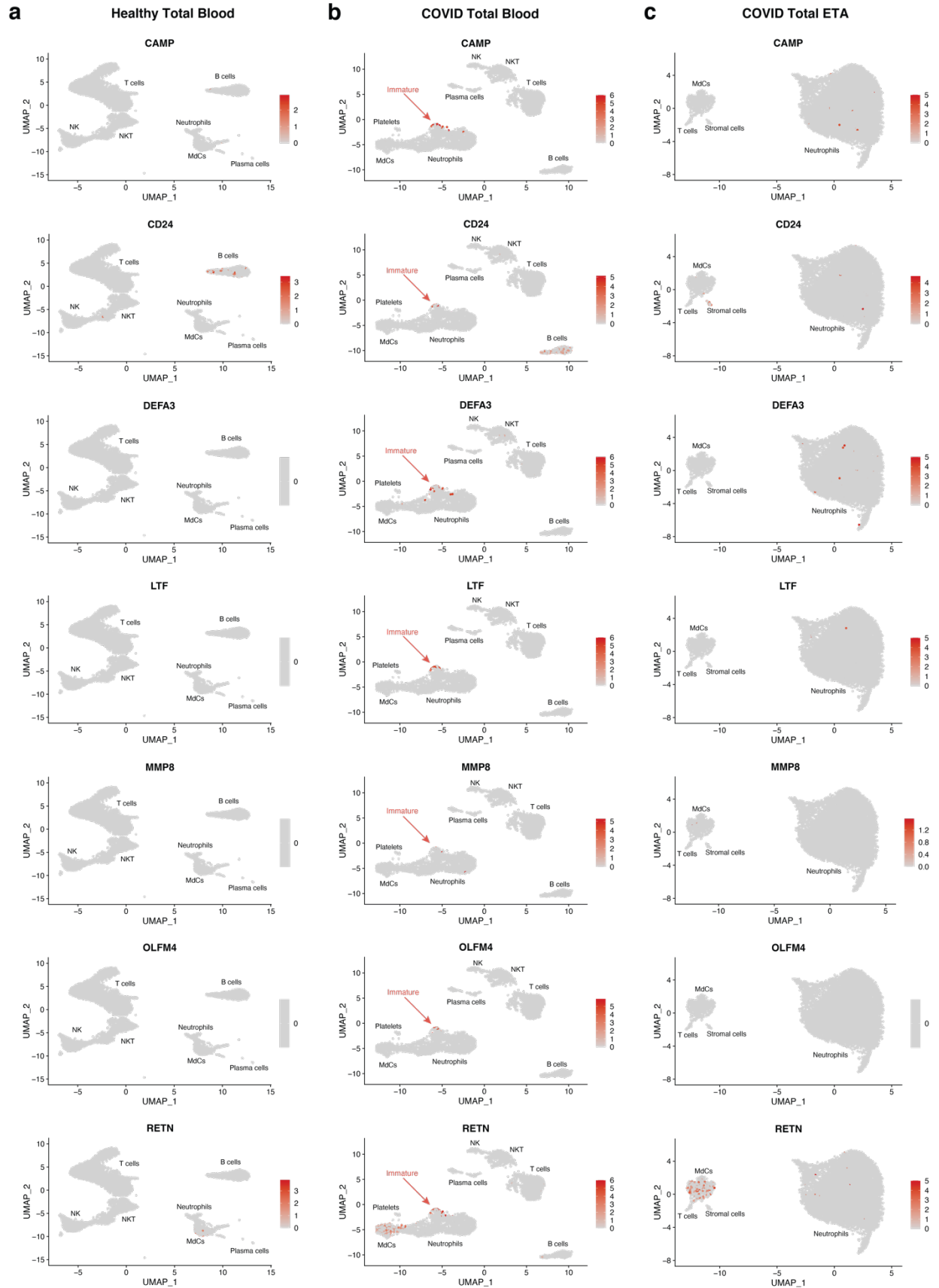
1222 **Extended Data Figure 4.** *Gene signature for immature neutrophils is lacking in healthy*
1223 *donors and lungs of COVID-19 patients.* UMAP visualizations of genes that identify immature
1224 neutrophils in the blood healthy individuals (a) and severe COVID-19 patients (b), along with
1225 cells from the lungs of severe COVID-19 patients (c).
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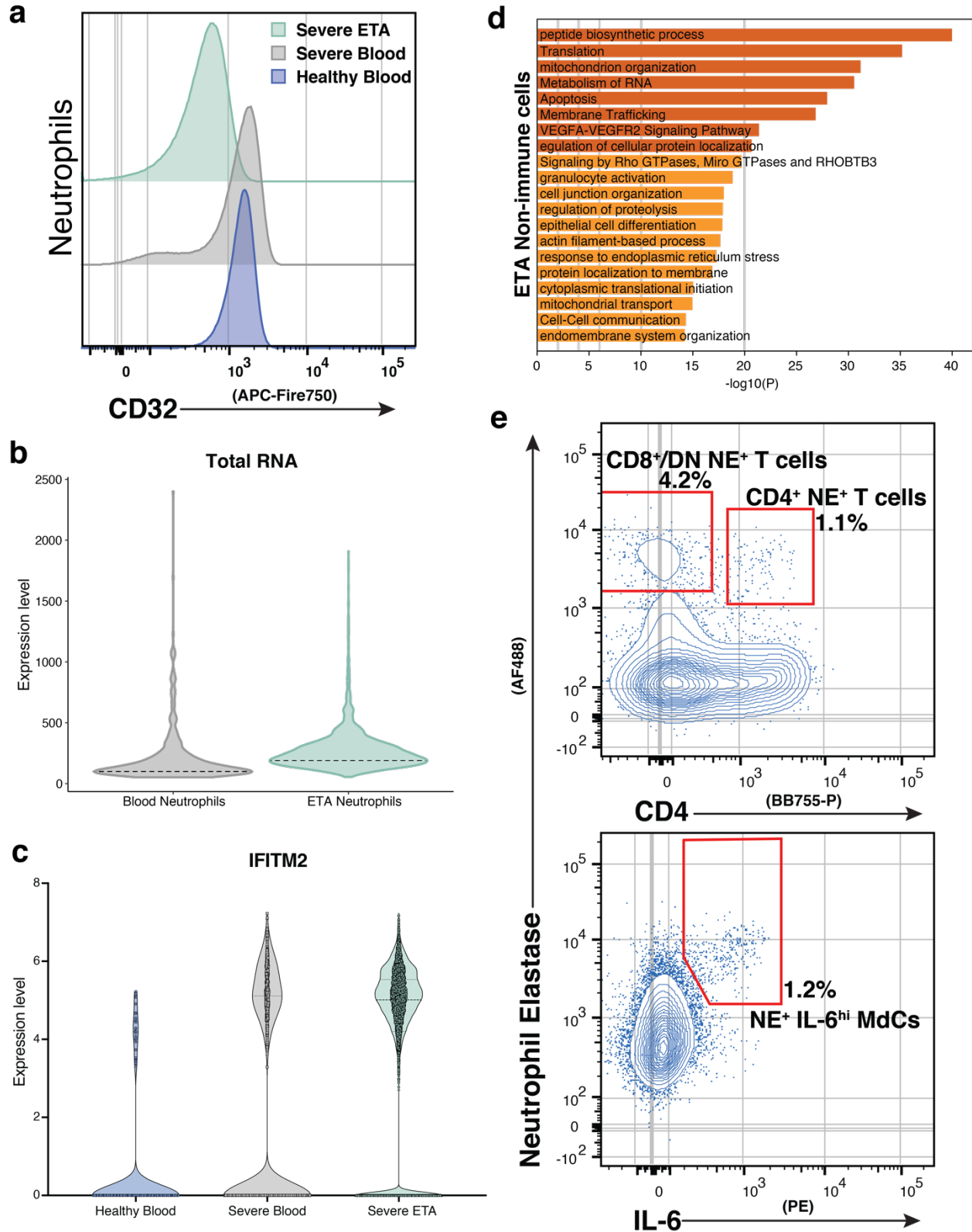
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1229 **Extended Data Figure 5.** *Cellular signatures during SARS-CoV-2 pathogenesis.* (a)
1230 Pulmonary neutrophils have a reduction in CD32 (FcγRII) expression as compared to circulating

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) Metascape[85] 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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