

1 Discriminating Mild from Critical COVID-19 by Innate and

2 Adaptive Immune Single-cell Profiling of Bronchoalveolar

3 Lavages

4 Running title: Immune Atlas of COVID-19 Bronchoalveolar Lavages

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7 Els Wauters^{1,2,14}, Pierre Van Mol^{2,3,4,14}, Abhishek D. Garg^{5,14}, Sander Jansen^{6,14}, Yannick
8 Van Herck⁷, Lore Vanderbeke⁸, Ayse Bassez^{3,4}, Bram Boeckx^{3,4}, Bert Malengier-
9 Devlies⁹, Anna Timmerman^{3,4}, Thomas Van Brussel^{3,4}, Tina Van Buyten⁶, Rogier
10 Schepers^{3,4}, Elisabeth Heylen⁶, Dieter Dauwe¹⁰, Christophe Dooms^{1,2}, Jan Gunst¹⁰,
11 Greet Hermans¹⁰, Philippe Meersseman¹¹, Dries Testelmans^{1,2}, Jonas Yserbyt^{1,2},
12 Patrick Matthys⁹, Sabine Teijpar¹², CONTAGIOUS collaborators, Johan Neyts^{6,14},
13 Joost Wauters^{11,14}, Junbin Qian^{3,4,13,14}, Diether Lambrechts^{3,4,14}

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15
16
17 1 Laboratory of Respiratory Diseases and Thoracic Surgery (BREATHE), Department of Chronic Diseases and
18 Metabolism, KU Leuven, Belgium; 2 Department of Pneumology, University Hospitals Leuven, Belgium; 3
19 Laboratory of Translational Genetics, Department of Human Genetics, KU Leuven, Belgium; 4 VIB Center for
20 Cancer Biology, VIB, Leuven, Belgium; 5 Laboratory for Cell Stress & Immunity (CSI), Department of Cellular
21 and Molecular Medicine (CMM), KU Leuven, Belgium; 6 Laboratory of Virology and Chemotherapy,
22 Department of Microbiology, Immunology and Transplantation, Rega Institute, KU Leuven, Belgium; 7
23 Laboratory of Experimental Oncology, Department of Oncology, KU Leuven, Belgium; 8 Laboratory of Clinical
24 Bacteriology and Mycology, Department of Microbiology, Immunology and Transplantation, KU Leuven,
25 Belgium; 9 Laboratory of Immunobiology, Department of Microbiology, Immunology and Transplantation, Rega
26 Institute, KU Leuven, Belgium; 10 Laboratory of Intensive Care Medicine, Department of Cellular and
27 Molecular Medicine, KU Leuven, Belgium; 11 Laboratory for Clinical Infectious and Inflammatory Disorders,
28 Department of Microbiology, Immunology and Transplantation, KU Leuven, Belgium; 12 Molecular Digestive
29 Oncology, Department of Oncology, KU Leuven, Belgium; 13 Women's Hospital, School of Medicine, Zhejiang
30 University, Hangzhou 31006, Zhejiang, China; 14 These authors contributed equally;

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34 Correspondence: Junbin Qian (Junbin.Qian@kuleuven.vib.be) and Diether Lambrechts
35 (Diether.Lambrechts@kuleuven.vib.be)

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43 ABSTRACT

44 How innate and adaptive lung immune responses to SARS-CoV-2 synchronize during COVID-19
45 pneumonitis and regulate disease severity is poorly established. To address this, we applied single-
46 cell profiling to bronchoalveolar lavages from 44 patients with mild or critical COVID-19 versus
47 non-COVID-19 pneumonia as control. Viral RNA-tracking delineated the infection phenotype to
48 epithelial cells, but positioned mainly neutrophils at the forefront of viral clearance activity during
49 COVID-19. In mild disease, neutrophils could execute their antiviral function in an immunologically
50 ‘controlled’ fashion, regulated by fully-differentiated T-helper-17 (T_{H17})-cells, as well as T-helper-1
51 (T_H)-cells, CD8⁺ resident-memory (T_{RM}) and partially-exhausted (T_{EX}) T-cells with good effector
52 functions. This was paralleled by ‘orderly’ phagocytic disposal of dead/stressed cells by fully-
53 differentiated macrophages, otherwise characterized by anti-inflammatory and antigen-presenting
54 characteristics, hence facilitating lung tissue repair. In critical disease, CD4⁺ T_{H1} - and CD8⁺ T_{EX} -
55 cells were characterized by inflammation-associated stress and metabolic exhaustion, while CD4⁺
56 T_{H17} - and CD8⁺ T_{RM} -cells failed to differentiate. Consequently, T-cell effector function was largely
57 impaired thereby possibly facilitating excessive neutrophil-based inflammation. This was
58 accompanied by impaired monocyte-to-macrophage differentiation, with monocytes exhibiting an
59 ATP-purinergic signalling-inflammasome footprint, thereby enabling COVID-19 associated fibrosis
60 and worsening disease severity. Our work represents a major resource for understanding the lung-
61 localised immunity and inflammation landscape during COVID-19.

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69 INTRODUCTION

70 SARS-CoV-2 has rapidly swept across the globe affecting >7 million people, with >400.000 fatal
71 cases¹. It is now well appreciated that while most COVID-19 patients (80%) remain asymptomatic
72 or experience only mild symptoms, 20% present with overt pneumonia; about a quarter of these
73 progress to a life-threatening state of Acute Respiratory Distress Syndrome (ARDS) and severe
74 or atypical systemic inflammation². Fever, increased acute phase reactants and coagulopathy with
75 decreased lymphocyte counts, pronounced myeloid inflammation and increased neutrophil-to-
76 lymphocyte ratio are predominant immunological hallmarks of severe COVID-19^{3,4}.

77 Wen *et al.* were the first to provide an immune atlas of circulating mononuclear cells from 10
78 COVID-19 patients based on single-cell RNA-sequencing (scRNA-seq). Lymphocyte counts were
79 globally decreased, while inflammatory myeloid cells, predominantly IL1 β -secreting classical
80 monocytes, were more abundant, suggesting COVID-19 immunopathology to be a myeloid-driven
81 process⁵. Conversely, the contribution of circulating classical monocytes to systemic inflammation
82 was put into question by Wilk *et al.* Based on scRNA-seq, they observed sparse expression of
83 inflammatory cytokines by peripheral monocytes in 7 COVID-19 patients versus 6 six healthy
84 controls. On the other hand, antigen presentation and the number of cytotoxic NK- and T-cells
85 were reduced, while plasmablasts and neutrophils were increased, especially in in COVID-19
86 patients experiencing ARDS⁶.

87 However, profiling the peripheral immune landscape in COVID-19 may not be as comprehensive
88 since immune characteristics in the periphery are different from those within the lungs, both in
89 terms of amplitude and qualitative characteristics, as well as duration of the immune response.
90 Thus, a better understanding of the immune interactions in COVID-19 lungs is needed. In their
91 seminal paper, Liao *et al.* applied single-cell T-cell receptor-sequencing (scTCR-seq) and scRNA-
92 seq on bronchoalveolar lavage (BAL) fluid from 3 mild and 6 critical COVID-19 patients, as well as
93 3 healthy controls. They observed an abundance of highly inflammatory monocytes and neutrophils
94 and T-cell depletion in critical versus mild COVID-19. The latter showed a more potent adaptive
95 immune response to SARS-CoV-2, evidenced by presence of CD8 $^{+}$ T-cells with tissue-resident
96 features displaying clonal expansion and increased effector function⁷. Subsequently, Bost *et al.* were
97 able to sort infected cells from bystander cells and investigate virus-induced transcriptional changes.
98 This Viral-Track pipeline showed ciliated and progenitor epithelial cells to be the main targets of
99 SARS-CoV-2, yet a strong enrichment of viral RNA was observed in SPP1 $^{+}$ macrophages⁸. It is
100 unclear however whether this represents direct viral infection of myeloid cells, or phagocytosis of

101 viral particles (or virus-infected cells). Moreover, due to the small sample size, in-depth
102 characterization of all the cellular phenotypes detected by scRNA-seq in mild versus critical
103 COVID-19 remained largely unexplored.

104 Here, we provide a comprehensive deep-immune atlas of COVID-19 pneumonitis, analyzing BAL
105 from 31 COVID-19 patients with mild or critical disease, while inclusion of 13 patients with non-
106 COVID-19 pneumonitis allowed us to reliably distinguish non-specific lung-localised inflammatory
107 signalling from COVID-19 specific lung-associated immune changes.

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109 **RESULTS**

110 **scRNA-seq and cell typing of BAL samples**

111 We performed scRNA-seq on BAL from 22 hospitalized patients with a positive qRT-PCR for
112 SARS-CoV-2 on a nasopharyngeal swab or a lower respiratory tract sample. We also collected
113 BAL from 13 patients with clinical suspicion of COVID-19 pneumonia, yet negative PCR on lower
114 respiratory tract sampling for SARS-CoV-2. These samples are referred to as non-COVID-19. We
115 further stratified COVID-19 patients by disease severity at the time of sampling, by discerning two
116 groups; a ‘mild’ (n=2) and a ‘critical’ (n=20) disease group, the latter requiring mechanical
117 ventilation or extracorporeal membrane oxygenation. Demographic and clinical data of the
118 prospectively recruited patient cohort are summarised in Supplementary information, **Table S1**.

119 BAL samples were immediately processed for scRNA-seq. After quality filtering (Methods), we
120 obtained ~186 millions of unique transcripts from 65,166 cells with >150 genes detected. Of these,
121 ~51% of cells were from COVID-19. Subsequent analysis involving dimensionality reduction and
122 clustering identified several clusters (**Fig. 1a**), which through marker genes (Supplementary
123 information, **Fig. S1a, b**) could be assigned to lung epithelial cells (including ciliated, inflammatory,
124 hillock, secretory and AT2 lung epithelial cells), myeloid cells (monocytes/macrophages,
125 neutrophils, mast cells, plasmacytoid dendritic cells/pDCs and conventional dendritic cells/cDCs),
126 lymphoid cells (CD4⁺ and CD8⁺ T-cells, natural killer cells (NK), B-cells and plasma cells). We
127 describe each cell type in more detail, highlighting the number of cells, read counts and transcripts
128 detected in Supplementary information, **Table S2**. There was no cluster bias between disease
129 status (COVID-19 versus non-COVID-19), disease severity (mild versus critical) or individual
130 patients (**Fig. 1b**).

131 To increase our resolution, we processed scRNA-seq data on COVID-19 BAL by Liao et al.,
132 consisting of 3 patients with ‘mild’ and 6 patients with ‘critical’ COVID-19 (n=51,631 cells)

133 (Supplementary information, **Fig. S1c**)⁷. We also retrieved 7 normal lung samples (n=64,876 cells)
134 profiled by Lambrechts et al. and 8 normal lung samples (n= 27,266 cells) by Reyfman et al. to
135 further enhance our resolution, specifically for T-cells and DCs^{9,10}. Datasets were integrated by
136 clustering cells from each dataset separately and assigning cell type identities to each cell. We then
137 pooled cells from each dataset based on cell type identities and performed canonical correlation
138 analysis (CCA), as described previously¹¹, followed by graph-based clustering to generate a UMAP
139 per cell type, displaying its phenotypic heterogeneity. Per cell type, we ruled out batch effects due
140 to different datasets.

141 After integration, COVID-19 BAL scRNA-seq data were derived from 5 mild and 26 critical
142 COVID-19 patients. Quantitatively, monocyte/macrophages and neutrophils were the most
143 abundant cell types, amounting up to 65.7% (n=55,825) of COVID-19 cells (**Fig. 1c**). When
144 evaluating the relative enrichment or depletion of these cell types, we found that monocytes and
145 neutrophils were indeed more frequent, while macrophages and epithelial cells were less abundant
146 in COVID-19 versus non-COVID-19. B-cells and NK-cells were slightly enriched in COVID-19.
147 Comparing mild versus critical COVID-19 revealed an increase in CD8⁺ T-cells, macrophages and
148 cDCs in the former (**Fig. 1d**).

149 Below, we describe the heterogeneity underlying each cell type in more detail.

150 **Phenotypic heterogeneity of CD8⁺ T-cells in COVID-19 BAL**

151 Altogether, we retrieved 23,468 T- and NK-cells, which were subclustered into 14 phenotypes
152 (**Fig. 2a, b**; Supplementary information, **Fig. S2**). Briefly, we identified 7 CD8⁺ T-cell clusters, 5
153 CD4⁺ T-cell clusters and 2 NK-cell clusters. While naïve CD8⁺ T-cells (T_N) expressed naïve T-cell
154 markers (CCR7, LEF1 and TCF7), effector-memory (T_{EM}) and partially-exhausted (T_{EX}) T-cells were
155 characterized by increased expression of effector markers (IFNG, PRFI, NKG7 and GZMB). Herein,
156 expression of (inflammation-driven) exhaustion-defining immune-checkpoints (LAG3, HAVCR2 and
157 PDCD1) distinguished T_{EX}-cells. Additionally, we identified CD8⁺ resident-memory T-cells (T_{RM})
158 based on ZNF683 and ITGAE, as well as CD8⁺ recently-activated effector-memory T-cells (T_{EMRA}).
159 Finally, we also identified mucosal-associated invariant T-cells (T_{MAIT}) and gamma-delta (T_{γδ}) T-cells.

160 Next, we assessed prevalence of each T-cell phenotype in COVID-19 versus non-COVID-19
161 disease, but failed to observe differences in the CD8⁺ phenotypes (**Fig. 2c**). When comparing mild
162 to critical COVID-19 (**Fig. 2d**), we found T_{MAIT}-cells to be increased in the former. Interestingly,
163 T_{MAIT}-cells can actively co-opt for specific innate immune characteristics (e.g. proficient pattern-
164 recognition receptor-based signalling, and/or broad non-MHC antigenic surveillance), thereby

165 allowing them to rapidly respond to pathogenic agents possessing pathogen-associated molecular
166 patterns (PAMPs)¹².

167 The largest increase in mild versus critical COVID-19, however, was seen for CD8⁺ T_{RM}-cells. To
168 understand this difference, we used Slingshot to infer pseudotime trajectories (excluding CD8⁺
169 T_{MAIT}- and T_{γδ}-cells). We observed 3 distinct CD8⁺ T-cell trajectories (**Fig. 3a**): CD8⁺ T_N-cells
170 connected with T_{EM}-cells, which subsequently branched into 3 different (well-connected) lineages
171 i.e., T_{RM}-cells, T_{EX}-cells and T_{EMRA}-cells. Profiling of marker genes along these trajectories confirmed
172 their functional annotations (**Fig. 3b**). Notably, effector function peaked halfway in each lineage
173 and then stabilized or decreased, depending on the lineage (**Fig. 3c**). Next, density plots reflecting
174 the relative number of T-cells in each phenotypic state were created along these trajectories (**Fig.**
175 **3d**), and stratified for normal tissue, non-COVID-19, and mild or critical COVID-19. Non-COVID-
176 19 T-cells were enriched towards the end of the T_{RM}-lineage, while in COVID-19 they were more
177 frequent in the T_{EX}-lineage (**Fig. 3e**). In contrast, T_{EMRA}-cells were more differentiated in normal
178 lung. When comparing mild to critical disease, the T_{RM}-lineage was more differentiated in mild
179 COVID-19, while along the T_{EX}-lineage differentiation was most prominent in critical COVID-19
180 (**Fig. 3f**). There were no differences in the T_{EMRA}-lineage.

181 We also processed T-cells by scTCR-seq, obtaining 3,966 T-cells with a TCR sequence that were
182 also annotated by scRNA-seq (excluding NK-, T_{MAIT} and T_{γδ}-cells). Based on TCR sharing, we
183 could reinforce the 3 trajectories identified by Slingshot (**Fig. 3g**). Overall, CD8⁺ T_{RM}-cells
184 contained the highest number of T-cell clonotypes. Plotting TCR richness and evenness along the
185 trajectories, revealed that both parameters were reduced along the T_{RM}-lineage, specifically in
186 COVID-19 (**Fig. 3h**), likely indicating antigen-driven clonal expansion. Notably, this expansion was
187 more prominent in mild COVID-19 (**Fig. 3i**). In contrast, T_{EX}-cells were characterized by only a
188 modest decrease in TCR richness/evenness along their lineage. In the T_{EMRA} lineage, richness did
189 not decrease along the pseudotime.

190 Overall, this suggests that mild COVID-19 is characterized by fully-differentiated T_{RM}-cells
191 undergoing active (presumably antigen-driven) TCR expansion and selection, while T_{EX}-cells are
192 entangled halfway their trajectory. In critical COVID-19, T_{RM}-cells fail to differentiate or expand,
193 while T_{EX}-cells become more exhausted albeit without undergoing clonal expansion.

194 **Gene expression modelling along the CD8⁺ T_{RM}- and T_{EX}-lineage**

195 We then modelled gene expression along the T_{RM}- and T_{EX}-lineage, and identified 5 gene sets with
196 a specific expression pattern in each trajectory. In the T_{RM}-lineage, set 1 and 2 consisted of naive

197 T-cell markers (set 1: *CCR7*, *LEFI*, *TCF7*; set 2: *SELL*), whose expression decreased along the
198 trajectory (**Fig. 3j**; Supplementary information, **Table S3**). A third set was enriched for interferon
199 (IFN)-induced (anti-viral) genes (*IFI6*, *IFI44L*, *ISG15*, *ISG20*, *MX2*), activation-associated genes (*CD38*)
200 and genes mediating effector-memory functions (*GZMK*, *CD44*, *KLRG1*). Set 3 exhibited high
201 expression halfway of the trajectory. Genes from the last 2 sets were expressed at the end of the
202 trajectory and were mostly related to cytotoxicity and increased effector function (set 4: *GZMA*,
203 *GZMB*, *FASLG*, *CXCR3*, *CCL5*), a balance of pro-inflammatory and auto-regulatory genes (set 5:
204 *ITGA1*, *TNF*, *XCL2*, *CD7* versus *LGALS3*, *SLAMF1*, *S100A4*) and genes marking resident memory
205 formation (*ZNF683*, *ITGAE*)^{13,14}. In mild COVID-19, T_{RM} -cells mainly expressed set 3-5 genes,
206 indicating increased (but balanced) effector function.

207 In the T_{EX} -lineage, the first set contained naïve markers (*LEFI*, *CCR7*, *TCF7*), a second set IFN-
208 induced (anti-viral) genes (*MX1*, *MX2*, *ISG15*, *IFI44*, *IFIT5*, *IFI6*), while a third set besides *IFNG* and
209 IFN-induced genes (*IFI27*, *IFI27L2*) was also comprised of T-cell activation-related genes (*CD38*,
210 *GZMH*, *GZMA*), chemokines (*CCL3*, *CCL4* and *CCL5*), cytotoxicity- (*NKG7*, *GNLY*, *GZMB*) and
211 (inflammatory) exhaustion-related genes (*HAVCR2*) (**Fig. 3k**; Supplementary information, **Table**
212 **S4**). Set 4 was characterized by expression of pro-inflammatory (*CD70*, *COTL*, *HMGB1*) and anti-
213 inflammatory genes (*ENTPD1*, *ANXA5*, *SERPINB1*), suggesting these cells exhibit a chronic
214 dysregulated hyperinflammatory phenotype. We also noticed expression of the TIM auto-
215 regulatory protein family (*TIMD4*) and viral infection-induced auto-regulatory genes (*LGALS1*,
216 *LGALS3*)^{15,16}. In set 5, cell-cycle genes (*CDK1*, *KIFs*, *PCNA*, *CCNA/B2*), stress-associated genes (*HSPD1*,
217 *HSP90AA1*, *BIRC5*) and chromatin re-modelling related genes (e.g., *HMGB2*, *HMGB3*, *EZH2*) were
218 increased, suggesting that T-cells were largely adjusting to inflammation-driven stress (rather than
219 mounting any discernible effector or auto-regulatory responses). Notably, mild COVID-19 showed
220 a very prominent enrichment in cells characterized by set 3-associated genes, whereas critical
221 COVID-19 was enriched for set 4-5 T_{EX} -cells.

222 Overall, gene expression profiling along the trajectories confirmed that mild COVID-19 exhibits
223 $CD8^+$ T_{RM} - and T_{EX} -cells with good effector function, while in critical COVID-19 this effector
224 function is drastically reduced possibly due to (persistent) inflammation-associated stress.

225 **Phenotypic heterogeneity of $CD4^+$ T-cells in COVID-19 BAL**

226 Amongst the 5 $CD4^+$ T-cell clusters, we identified naïve $CD4^+$ T-cells (T_N), effector-memory T-
227 cells (T_{EM}), $CD4^+$ T-helper-1 (T_{HI}) cells, expressing high levels of immune-checkpoints (*HAVCR2*,
228 *LAG3*, *PDCD1* and *CTLA4*), as well as $CD4^+$ T-helper-17 (T_{HI7}) and $CD4^+$ regulatory T-cells (T_{REG})

229 (Fig. 2b for marker gene sets). Compared to non-COVID-19, we observed slightly less CD4⁺
230 T_{HI7}-cells, but more T_{HI}-cells in COVID-19. Comparing mild versus critical COVID-19, we found
231 T_{HI}-cells to be significantly increased in the latter.

232 Slingshot revealed additional phenotypic heterogeneity, identifying central-memory CD4⁺ T_{CM}-cells
233 and stem cell-like memory CD4⁺ T_{SCM}-cells (Fig. 4a, b), and constructed 3 trajectories, which
234 were independently confirmed based on shared TCR clonotypes (Fig. 4c). Briefly, T_N-cells
235 connected closely with T_{CM}-cells followed by T_{EM}-cells, which branched-off into 3 different lineages
236 to form T_{HI}-cells, T_{HI7}-cells and T_{SCM}-cells. Profiling of marker genes along these trajectories
237 confirmed their functional annotation (Fig. 4d). Density plots stratified for T-cells from each
238 subgroup revealed that COVID-19 was enriched for T-cells early and late in the T_{HI}- and T_{SCM}-
239 trajectory, while vice versa T-cells from non-COVID-19 and normal lung were enriched halfway
240 these trajectories (Fig. 4e, f). In the T_{HI7}-trajectory, COVID-19 BAL was strongly enriched for T-
241 cells in the first half of the trajectory. Overall, CD4⁺ T-cells from mild COVID-19 behaved similarly
242 as normal lung or non-COVID-19. Both TCR richness and evenness were reduced along the T_{HI}-
243 lineage from COVID-19, but not from non-COVID-19 (Fig. 4g). Notably, this reduction was most
244 prominent in mild, but not in critical COVID-19 (Fig. 4h). In contrast, T_{HI7}-cells and T_{SCM}-cells
245 were characterized by a modest decrease in TCR richness only at the very end of their lineage,
246 suggesting that mainly T_{HI}-cells are selected for specific SARS-CoV-2 PAMPs/antigens.

247 Overall, this suggests that mild COVID-19 is characterized by more stable or differentiated T_{HI7}-
248 cells' activity, which is crucial for productive immunity against pathogens at mucosal surfaces¹⁷,
249 whereas T_{HI}-cells become entangled halfway in their trajectory. In critical COVID-19, T_{HI7} cells
250 completely fail to differentiate, while T_{HI}-cells behave the opposite.

251 **Gene expression modelling along the CD4⁺ T_{HI}- and T_{HI7}-lineage**

252 Differential gene expression analysis along both lineages identified gene sets with specific
253 expression profiles. In the T_{HI}-lineage, the first gene set consisted of naïve (*LEF1*, *TCF7*) and
254 undifferentiated (*CCR7*, *S1PR1*) T-cell markers (Fig. 4i; Supplementary information, Table S5). A
255 second set was enriched for both pro- and anti-inflammatory markers (*CXCR4*, *CXCL2*, *ANXA1*,
256 *SOCS2*, *LTB*), while a third set was characterized (halfway the trajectory) by an effector-like T_{HI}-
257 program based on expression of *IFNG*, granzymes (*GZMA*, *GZMK*, *GZMB*), *CXCR3*, *PRF1*, *NGK7*,
258 *CCL5*, as well as *CTLA4* and *HAVCR2*. Finally, a fourth gene set was characterized by high HLA
259 expression, auto-regulatory markers (*LGALS1*, *CCL3*), partial activation markers (*CXCL13*) and
260 stress-response markers (*PDIA6*, *HSBPI*, *VDAC3*, *PARP1*) at the end of the trajectory, suggesting a

261 complex mixture of a pro- and anti-inflammatory phenotype coupled with early-stress modulation.
262 In a final fifth set, we noticed mitochondrial stress (*LDHA*, *PKM*, *COX17*, *VDAC1*, *COX8A*), an *IL2*
263 withdrawal-associated stressed phenotype (*MT1E*, *MT1X*), proteotoxic stress
264 (*PSMB3/B6/D4/A7/C3*, *HSPB11*, *PARK7*, *EIF4EBP1*) and glycolysis (*PGAM1*) suggesting ‘terminal
265 exhaustion’ at the end of the T_{HI} -trajectory^{18,19}. Overall, in mild COVID-19, the T_{HI} -lineage was
266 enriched for cells halfway the trajectory where expression of set 2-3 genes was most dominant,
267 indicating increased T_{HI} -effector function. In critical COVID-19, expression of sets 4-5 pre-
268 dominated, suggesting inflammation-driven terminal exhaustion and severe dysregulation.

269 In the T_{HI7} -lineage, we also identified 5 gene sets (Fig. 4j; Supplementary information, Table S6):
270 the first 2 sets with high expression early in the trajectory did not express markers indicative of
271 T_{HI7} function. Three other gene sets with high expression at the end of the trajectory were
272 characterized by T-cell effector function (set 3: *PDCD1*, *CCL5*, *CXCR2*, *CCR2* and *GZMA/B*),
273 expression of cytotoxic-activity genes (set 4: *NKG7* and *PRF1*) and T_{HI7} -cell associated interleukins
274 (set 5: *IL17A*, *IL17F*, *IL23R*, as well as *IFNG* and *CCL4*). Notably, in mild COVID-19, cells were
275 characterized by expression of genes belonging to set 3-5, while critical patients only expressed
276 set 1-2 genes, completely failing to differentiate along the T_{HI7} lineage.

277 Overall, this clearly indicates that mild COVID-19 is characterized by improved T_{HI} - and T_{HI7} -
278 effector functions that together mediate a highly controlled antiviral immune response, whose
279 absence underlies critical COVID-19.

280 **Trajectory of monocyte-to-macrophage differentiation in COVID-19 BAL**

281 In the 63,114 myeloid cells derived from BAL, we identified 9 phenotypes (Fig. 5a). Monocytes
282 clustered separately from macrophages based on the absence of macrophage markers (*CD68*,
283 *MSRI*, *MRC1*) and presence of monocyte markers (*IL1RN*, *FCN1*, *LILRA5*). Monocytes could be
284 further divided into *FCN1*^{high}, *IL1B*^{high} and *HSPA6*^{high} monocytes (Fig. 5b; Supplementary
285 information, Fig. S3a), respectively, characterized by expression of classical monocyte markers
286 (*IL1RN*, *S100A8/9*), pro-inflammatory cytokines (*IL1B*, *IL6*, *CCL3*, *CCL4*) and heat-shock proteins
287 (*HSPA6*, *HSPA1A/B*). Based on *CSF1R*, *CSF3R* and *SPPI*, 3 monocyte-derived macrophage
288 phenotypes could further be delineated, including *CCL2*^{high}, *CCL18*^{high} and *RGS1*^{high} (Supplementary
289 information, Fig. S3b). *CCL2*^{high} clusters were characterized by the pro-migratory cytokine *CCL2*,
290 but also by several pro- (*CCL7*, *CXCL10*) and anti-inflammatory (*CCL13*, *CCL22*) genes, suggesting
291 existence of an intermediate population of cells. In contrast, *CCL18*^{high} and *RGS1*^{high} cells expressed
292 mainly anti-inflammatory genes (*CCL13*, *CCL18*, *PLD4*, *FOLR2*), as well as genes involved in receptor-

293 mediated phagocytosis (*MERTK*, *AXL*). Finally, we identified *MTIG*^{high} macrophages (expressing
294 numerous metallothioneins suggestive of oxidative stress or immune cell's growth factor-
295 withdrawal), a monocyte-derived (*FABP4*^{medium}) and tissue-resident (*FABP4*^{high}) alveolar macrophage
296 cluster. The latter two populations were characterized by high expression of resident markers
297 (*FABP4*, *PPARG*), anti-inflammatory (*CCL18*, *CCL22*) and antigen-presentation relevant MHC-I/II
298 genes.

299 We observed a significant increase in *FCNI*^{high} and *IL1B*^{high} monocytes in COVID-19 versus non-
300 COVID-19, while *FABP4*^{medium} and *FABP4*^{high} alveolar macrophages were reduced (**Fig. 5c**). *FCNI*^{high}
301 monocytes were significantly reduced in mild COVID-19, while alveolar macrophages were
302 increased (**Fig. 5d**). Using Slingshot, we reconstructed two monocyte-to-macrophage lineages,
303 consisting of a common branch of *FCNI*^{high} monocytes differentiating into *IL1B*^{high} monocytes,
304 followed by *CCL2*^{high} and *CCL18*^{high} monocyte-derived macrophages. These subsequently branched
305 into *RGS1*^{high} monocyte-derived macrophages (*RGS1*-lineage), or via *FABP4*^{medium} into *FABP4*^{high}
306 tissue-resident macrophages (alveolar lineage; **Fig. 5e**). Monocyte marker expression decreased
307 along both lineages, while macrophage marker expression increased (**Fig. 5f**). Density plots
308 revealed that in COVID-19 cells were enriched in the first half of both lineages (**Fig. 5g**),
309 confirming our above observations of monocyte enrichment in COVID-19. Comparing mild to
310 critical COVID-19, we noticed that the former differentiated along both lineages, whereas in the
311 latter monocytes completely failed to differentiate (**Fig. 5h**).

312 Modelling gene expression along the alveolar lineage revealed 5 gene sets (**Fig. 5i**; Supplementary
313 information, **Table S7**). Sets 1 and 2 were characterized by inflammatory markers (*CXCL1-3*,
314 *CCL20*, *CXCL8*, *CXCL10*, *CCR1*, *IL1B*), survival factors (*RAC1*, *JAK1*, *ZEB2*, *CDKN1A*), IFN-induced
315 (anti-viral) genes (*IFITM1-3*, *IFIT1-3*, *IRF1*, *MX1/2*), hypoxia (*HIF1A*) and NF- κ B (*NFKB1/2*, *NFKBIZ*)
316 signalling early in the lineage, suggesting these monocytes to be characterized by a
317 hyperinflammatory state, in which they prioritized inflammation rather than committing toward
318 differentiation into macrophages. The third gene set was characterized by a possible CD47-based
319 macrophage-suppressive phenotype, potentially aimed at dysregulating macrophage-activation
320 (since CD47 is a well-established 'don't eat me' signal striving to avoid auto-immunity)^{20,21}.
321 Moreover, based on expression of purinergic signalling (*P2RX7*), inflammasome or IL1-modulating
322 factors (*NLRP3*, *IL1B*, *IL10RA*, *CTSL*, *CALM1*, *NFKB1*), endoplasmic reticulum (ER) stress capable of
323 enabling ATP secretion (*UBC*, *PSMB9*, *SEC61G*, *ATF5*, *ATF3*), unconventional trafficking (*VAMP5*),
324 fibrosis-related factors (*FGL2*, *TGFB1*, *COTL1*) and vascular inflammation (*TNF*, *AIF1*, *RNF213*, *CCL2*,
325 *CCL8*) across sets 2 and 3 of these monocytes, we strongly suspect presence of extracellular ATP-

326 driven purinergic-inflammasome signalling; especially given the high likelihood of extracellular ATP
327 release from damaged epithelium in the context of acute viral infection. Importantly, this ATP-
328 driven purinergic-inflammasome signalling pathway is a danger signalling cascade, which has been
329 shown to facilitate ARDS-associated lung fibrosis and thus acts disease-worsening in this context²²-
330 ²⁴. Finally, set 4 and 5 genes were expressed at the end of the trajectory. Set 4 was characterized
331 by expression of chaperone-coding genes (*CALU*, *CALR*, *CANX*, *PDIA4*, *HSP90BI*), which are crucial
332 for robust functioning of the antigen-loading machinery for MHC molecules, whereas in set 5 there
333 were clear signs of antigen presentation (expression of numerous MHC class II genes).
334 Furthermore, set 5 comprised genes involved in receptor-mediated phagocytosis and post-
335 phagocytic lipid degradation/metabolism: *APOE* for lipid metabolism, scavenger receptors *MARCO*
336 and *MSR1*, complement activation (*CIQA*, *CIQB*, *CIQC* and *CD46*; that can also facilitate
337 phagocytosis), viral infection-relevant inflammatory orientation (*CD81*, *CD9*), as well as anti-
338 inflammatory markers (*PPARG*, *FABP4*)^{25,26}. Similar gene sets were observed for the RGS1-lineage
339 (Supplementary information, **Fig. S3c**), except for gene set 5, which exhibited expression of genes
340 involved in chemokine signalling desensitization (*RGS1*), phagocytosis (*AXL*) and ATP clearance
341 (*ENTPD1*)²⁷.

342 Overall, this indicates that mild COVID-19 is characterized by functional pro-phagocytic and
343 antigen-presentation facilitating functions in myeloid cells, whereas critical COVID-19 is
344 characterized by disease-worsening characteristics related to monocyte-based macrophage
345 suppression and ATP-purinergic signalling-inflammasome that may enable COVID-19 associated
346 fibrosis and can worsen patient prognosis.

347 **Qualitative assessment of T-cell and monocyte/macrophage function in COVID-19**

348 Next, although pseudotime inference usually allocates cells with a similar expression to the same
349 pseudotime on the trajectory, we explored specific differences in gene expression along the
350 pseudotime. We scored each cell using REACTOME pathway signatures and when comparing
351 COVID-19 versus non-COVID-19 BAL, we observed consistently decreased IFN-signalling in non-
352 COVID-19 T-cell and myeloid lineages (Supplementary information, **Fig. S4a**). In mild versus
353 critical COVID-19, we observed that amongst several other pathways, IFN- (type I and II),
354 interleukin (e.g., *IL12* and *IL6*) and oligoadenylate synthetase (OAS) antiviral response signalling
355 was increased in $CD8^+$ T_{RM} - and T_{EX} -lineages (**Fig. 5j**; Supplementary information, **Fig. S4b-e**).
356 The $CD4^+$ T_{HI} -lineage was similarly characterized by increased IFN- (type I and II) and interleukin
357 (*IL6*, *IL12*, *IL21*) signalling in mild COVID-19 (**Fig. 5k**; Supplementary information, **Fig. S4f, g**).

358 Additionally, TRAF6-induced NF- κ B and IRF7 activation, as well as TGFBR complex activation were
359 increased. Similar effects were observed in the T_{H17} -lineage (Supplementary information, **Fig. S4h**,
360 **i**). The alveolar macrophage lineage was characterized by increased phagocytosis-related pathways
361 (scavenging receptors, synthesis of lipoxins or leukotrienes) and IFN-signalling in mild COVID-19
362 (**Fig. 5l**; Supplementary information, **Fig. S4j-m**). Vice versa, IL10-signalling (which inhibits the IFN-
363 response), chemokine receptor binding and ATF4-mediated ER stress response were increased in
364 critical COVID-19.

365 Overall, while our trajectory and cell density analyses already indicated quantitative shifts in various
366 cellular phenotypes comparing mild versus critical COVID-19, we noticed that also qualitatively
367 immune cells from critical COVID-19 were severely dysfunctional.

368 **scRNA-seq of neutrophils, DCs and B-cells in COVID-19**

369 We retrieved 14,154 neutrophils, which were subclustered into 5 phenotypes (**Fig. 6a, b**;
370 Supplementary information, **Fig. S5a**). A first cluster consisted of 'progenitor' neutrophils based
371 on CXCR4 and CD63, and was also characterized by expression of the angiogenic factor VEGFA and
372 cathepsins (CTSA, CTSD) (**Fig. 6c**). A second cluster consisted of few 'immature' neutrophils
373 expressing LTF, LCN2, MMP8/9, PADI4 and ARG1. Cluster 3 and 4 consisted of 'inflammatory mature'
374 neutrophils, both expressing a signature footprint that highlights anti-pathogenic orientation of
375 neutrophils²⁸: cluster 3 expressed IFN-induced genes and calgranulins (S100A8/9, S100A9 and
376 S100A12), which can modulate inflammation, while cluster 4 expressed high levels of cytokines
377 (IL1B) and chemokines (CXCL8, CCL3, CCL4). A final subset was characterized as 'hybrid'
378 neutrophils due to their macrophage-like characteristics, i.e., expression of MHC class II and
379 complement activation genes (C1QB, C1QC, CD74), cathepsins (CTSB, CTSL) and APOE. All
380 neutrophil subclusters were more frequent in COVID-19 than non-COVID-19, but most significant
381 changes were noticed for the 'progenitor' and 'inflammatory mature' neutrophils (**Fig. 6d**). Similar
382 trends were observed in mild versus critical COVID-19, albeit non-significantly (**Fig. 6e**).

383 We also identified 1,410 dendritic cells (DCs), which we could subcluster into 6 established
384 populations (**Fig. 6f, g**; Supplementary information, **Fig. S5b, c**). None of these differed
385 significantly between COVID-19 and non-COVID-19, while migratory DCs and Langerhans-cell-
386 like DC were more frequent in mild versus critical COVID-19 (**Fig. 6h, i**).

387 Within the 1,397 B-cells, we obtained 4 separate clusters (**Fig. 6j**; Supplementary information, **Fig.**
388 **S5d**). Follicular B-cells were composed of mature-naïve (CD27⁻) and memory (CD27⁺) B-cells.
389 The former were characterized by a unique CD27⁻/IGHD⁺(IgD)/IGHM⁺(IgM) signature and give

390 rise to the latter by migrating through the germinal center to form CD27⁺/IGHD⁻(IgD)/IGHM⁻(IgM)
391 memory B-cells (**Fig. 6k, l**). Memory B-cells then further differentiate into antibody-secreting
392 plasma cells (*IGHA1*, *IGHG1*, *JCHAIN*). A first cluster of ‘active’ plasma cells expressed high levels
393 of *PRDM1*(Blimp-1) and *XBP1*, indicating high antibody-secretion capacity, while the latter was
394 enriched for *CLL2* and *CCL5*, but also characterized by a reduced G2M and S cell cycle score and
395 increased expression of mitochondrial genes, indicating ongoing stress (**Fig. 6m**). Notably, this
396 population of ‘terminal’ plasma cells was also characterized by increased BCR clonality and reduced
397 BCR evenness (**Fig. 6n**). Compared to non-COVID-19, mature-naïve B-cells and active plasma
398 cells were increased in COVID-19, while terminal B-cells were reduced in COVID-19, albeit non-
399 significantly (**Fig. 6o**). There were no significant differences between mild versus critical COVID-
400 19 (**Fig. 6p**). Overall, this suggests terminal B-cells in COVID-19 to be characterized by sub-
401 optimal differentiation or activation, which may cause defective or counter-productive (possibly
402 low-quality) antibody responses in COVID-19.

403 **SARS-CoV-2 viral particles in epithelial and immune cells**

404 Finally, we retrieved 22,215 epithelial cells, which we subclustered into 7 distinct clusters (**Fig. 7a,**
405 **b**; Supplementary information, **Fig. S5e, f**), the largest 3 clusters consisting of secretory, ciliated
406 and hillock lung epithelial cells. The basal population (*KRT5*, *AQP3* and *SPARCL1*), representing stem
407 cell epithelial cells responsible for epithelial remodelling upon lung injury, was significantly enriched
408 in COVID-19 versus non-COVID-19, as well as ionocytes, which is another rare epithelial cell type
409 that regulates salt balance (**Fig. 7c**). There were no significant differences between mild versus
410 critical COVID-19 (**Fig. 7d**). Interestingly, *ACE2* and *TMPRSS2* expression was increased in
411 COVID-19 versus non-COVID-19, with 21% and 2.3% of epithelial cells being positive, respectively
412 (**Fig. 7e, f**). We then assessed in which cells we retrieved sequencing reads mapping to the SARS-
413 CoV-2 genome, identifying 3,773 positive cells from 17 out of 31 COVID-19 patients. Surprisingly,
414 this revealed a higher overall number of reads mapping to lymphoid and myeloid than epithelial
415 cells (**Fig. 7g**). Stratification for each of the 11 SARS-CoV-2 open-reading frames (ORF) using
416 Viral-Track revealed that the RNA encoding for spike protein (S), which interacts with ACE2
417 during viral entry of the cell, was almost exclusively detected in epithelial cells, which were also
418 the only cells expressing *ACE2* and *TMPRSS2* (**Fig. 7g**). In contrast, the nucleocapsid protein (N),
419 and to a lesser extent the *ORF10* and *ORF1a*-encoding mRNAs were detected in myeloid and
420 lymphoid cells at much higher levels than in epithelial cells (**Fig. 7h**). Further stratification into cell
421 types revealed that neutrophils and to a limited extent also monocytes, contained most reads
422 mapping to N (**Fig. 7i**). This might suggest that neutrophils are the main cell type interacting with

423 SARS-CoV-2 viral particles/infected cells and account for the highest procurement of viral material,
424 in line with their role as first innate immune responders to infection²⁹. Differential gene expression
425 of *N*-positive versus *N*-negative neutrophils identified upregulation of transcription factor *BCL6*,
426 which promotes neutrophils survival and inflammatory response following virus infection, and
427 numerous IFN-induced genes (*IFITM3*, *IFIT1-3*, *MX1/2*, *ISG15*, *RSAD2*; **Fig. 7j**)³⁰. No such
428 enrichment was observed in monocytes nor macrophages (Supplementary information, **Fig. S5g-i**). Pathway analysis on differentially-expressed genes revealed IFN-signalling using REACTOME and
429 Response_to_virus using GO for genes upregulated in *N*-containing neutrophils (**Fig. 7k, l**).
430 Notably, amongst the different neutrophil phenotypes, *N* was most strongly enriched in
431 ‘inflammatory mature’ neutrophils expressing calgranulins (**Fig. 7m**). As expected, significantly
432 more *N* was present in critical versus mild COVID-19³¹.

434 **Cell-to-cell communication to unravel the immune context of COVID-19 BAL**

435 Since, our data on the one hand reveal that neutrophils were involved in cleaning up viral
436 particles/virus-infected cells, yet T-cell and monocyte-to-macrophage lineages were significantly
437 disrupted in critical COVID-19, we explored the (predicted) interactome between these cell types
438 to gain more refined insights. First, we calculated interactions between cell types ($P \leq 0.05$)
439 separately for mild and critical COVID-19, then we assessed differences in the number of specific
440 interactions. Neutrophils were characterized by a low number of specific interactions that were
441 slightly more frequent in critical versus mild COVID-19. Vice versa, numerous specific interactions
442 were predicted between all other immune and epithelial cells, especially in mild COVID-19 (**Fig.**
443 **8a, b**; Supplementary information, **Fig. S6**).

444 In critical COVID-19, specific interactions between monocytes/macrophages and neutrophils
445 almost always involved pro-migratory interactions (*FLT1*, *NRPI* or *NRP2/VEGFA*, *CXCL1* or *CXCL2*
446 or *CXCL8/CXCR2*, *CCL3* or *CCL7/CCR1*), coupled with immune-inhibitory interactions, such as
447 *LILRB1* or *LILRB2/HLA-F* and *RPS19/C5AR1*, which also induce neutrophil dysfunction (**Fig. 8c**)³². A
448 few stimulatory T-cell to neutrophil interactions were observed, including *IFNG*/type II *IFNR*,
449 *PDCD1/CD274*, *LTA/TNFRSF1A* or *TNFRSF1B* (**Fig. 8d**), while specific epithelial cell-to-neutrophil
450 interactions were limited to a mixture of myeloid immunosuppression (*RPS19/C5AR1*) and viral
451 infection-relevant pro-inflammatory signals (*TNFRSF14/TNFSF14*) (**Fig. 8e**). Amongst T-cell and
452 monocytes/macrophages, some immune-stimulatory or auto-regulatory interactions were seen
453 (*CTLA4* or *CD28/CD80* or *CD86*, *CCL5/CCR5*) (**Fig. 8f**), but specific epithelial to T-cell interactions
454 in critical COVID-19 were limited to pro-inflammatory *ICAM1*-mediated interactions (**Fig. 8g**).

455 A very different scenario was observed in mild COVID-19 (**Fig. 8c-g**). Amongst the numerous
456 interactions between monocytes/macrophages and neutrophils, we noticed interleukin signalling
457 (bi-directional *IL1B*, *IL1A*, *IL1RN/A* signalling, *IL7/IL7R*, *CXCR2/CXCL1* or *CXCL8*), but also
458 *MRC1/PTPR* (phagocytosis) and *LTBR/LTB* (pro-inflammation). Between T-cells and neutrophils
459 specific interactions involved *CCR1/CCL3* or *CCL3L1* (pro-inflammation), *CD2/CD58* (co-
460 stimulatory/immunogenic pathway) and *CD94:NKG2E/HLA-F* (anti-viral immune-surveillance),
461 whereas between epithelial cells and neutrophils, *IL1R/IL1A* or *IL1B* or *IL1R* interactions were most
462 pronounced (which can facilitate productive neutrophil immunity in an immune-
463 controlled/immunogenic context)^{28,33,34}. Numerous interactions were also observed between
464 epithelial cells and monocyte/macrophages: *GAS6* or *PROS1/AXL* (receptor-mediated phagocytosis),
465 *ADORA2B/ENTPD1* (extracellular ATP degradation/suppression), *CD83/PECAM1* (immune
466 activation) and semaphorins interacting with their plexin and NRP receptors (tissue re-modelling
467 and repair). Between epithelial cells and T-cells, we observed mainly co-stimulatory (*CD46/JAG1*,
468 *CD40LG/CD40*, *IL7R/IL7*, *MICA* or *RAET1I/NKG2D* receptor) and tissue repair interactions
469 (*TGFB1/TGFR2* and *TGFB1/TGFB3*), while amongst T-cells and monocytes/macrophages, there
470 were amongst others, co-stimulatory (*LTA/TNFRSF1A* or *TNFRSF1B* or *TNFRSF14*,
471 *TNFSF10/TNFRSF10B*) or tissue-repair factors (*CSF1/CSFR1*, *TGFB3/TGFB1*, *IL15RA/IL15*),
472 mediators of T-cell homeostasis and cytotoxicity (*FASLG/FAS*) and antiviral immune surveillance
473 (*NKG2D II receptor/MICB* or *MICA*).
474

475 **DISCUSSION**

476 Based on scRNA-seq data obtained from BAL fluid, we were able to perform deep-immune
477 profiling of the adaptive and innate immune cell landscape within the main locale of COVID-19
478 pathology. A particular strength of our study is the profiling of BAL from a fairly large cohort of
479 COVID-19 patients (n=31), enabling statistically meaningful and robust comparisons between mild
480 and critical disease severity subgroups (in contrast to initial COVID-19 publications that profiled
481 <10 patients)⁷. Importantly, our control group also consisted of non-COVID-19 pneumonia cases
482 (n=13), instead of healthy controls. Since the latter are likely to differ on almost every
483 immunological parameter-level relative to COVID-19, our strategy enhances qualitative clarity of
484 immunological conclusions. Finally, due to the fact that we profiled >116,000 single-cells, we could
485 infer pseudotime trajectories for both T-cells and myeloid cells. Such method is particularly
486 attractive since it allows modelling of gene expression changes along the inferred trajectories,

487 thereby generating data at a much greater resolution. Overall, this allowed us to draw the following
488 key conclusions regarding what distinguishes a critical from a mild COVID-19 disease course:
489 Firstly, CD8⁺ T-cells exhibited good effector functions along their resident-memory and partially-
490 exhausted lineages in mild COVID-19, while also CD4⁺ T-cells showed increased effector or
491 disease-resolving functions in T_{HI}- and T_{H17}-lineages. In critical COVID-19, T-cells were highly
492 dysregulated, either failing to differentiate (T_{H17}- and T_{RM}-lineage) or exhausting excessively, thereby
493 leading to metabolic disparities, dysregulation of their immunological interface with myeloid cells
494 and/or a dysregulated chronic hyper-inflammatory phenotype (T_{HI} and T_{EX}-lineage). Notably, we
495 observed that mild versus critical COVID-19, not only differed quantitatively in terms of the number
496 of T-cells exhibiting a good T-cell effector function, but also qualitatively, in terms of consistently
497 lower activation levels of the type I and II IFN (anti-viral) signalling pathways (amongst several
498 other pathways). Overall, this showed that T-cells in mild COVID-19, unlike those in critical
499 COVID-19, were cross-talking better with their lung-localised microenvironment thereby
500 facilitating 'ordered' immune reactions capable of resolving, rather than exacerbating, inflammation
501 and tissue repair³⁵.
502 Secondly, in mild COVID-19 monocytes exhibited a pronounced pro-inflammatory phenotype, but
503 then differentiated into macrophages characterized by anti-inflammatory, pro-phagocytic and
504 antigen-presentation facilitating functions. This suggests that in these patients, macrophages might
505 be immunologically 'silently' cleaning the dying/dead epithelial cells (as well as other immune cells
506 meeting their demise due to inflammation), hence contributing to degradation and dilution of the
507 viral load in COVID-19 BAL. Such pro-homeostatic activity of macrophages is well-established to
508 aid in disease amelioration and inflammation resolution³⁶. In critical COVID-19, monocytes were
509 instead characterized by a chronically hyper-inflamed phenotype with characteristics of an ATP-
510 inflammasome-purinergic signalling-based fibrosis, which can promote worse disease outcome by
511 contributing to development of ARDS. This danger signalling pathway is hypothesized to be part
512 of the chronology of events during SARS-CoV-2 infection, but its genetic footprints have not been
513 documented as we report here³⁷. Considering that fully-differentiated macrophages are much more
514 efficient in clearing large debris or cellular corpses (e.g. infected dead/dying lung epithelia or dead
515 neutrophils) than monocytes or neutrophils, their dysfunction in critical COVID-19 may explain
516 the excessive accumulation of lung epithelial (as well as dead immune cell) debris and alveolar
517 dyshomeostasis coupled with dysregulated coagulopathy³⁸⁻⁴².

518 Lastly, based on the presence of sequencing reads mapping to the gene encoding for viral protein
519 S, which is needed to infect cells via ACE2 and TMPRSS2 receptors, we propose that SARS-CoV-
520 2 largely infects epithelial cells (as primary targets of excessive pathological replication and
521 propagation), but not necessarily lymphoid or myeloid cells (although we cannot exclude yet that
522 some virions might be capable of 'latently' entering these cells without showing pathological
523 replication or propagation). Interestingly, we also detected reads mapping to the nucleocapsid
524 protein (N) encoding gene mainly in neutrophils, but to some extent also in other lymphoid or
525 myeloid cells, especially monocytes. This suggests that neutrophils might be heavily involved in viral
526 clearance of SARS-CoV-2 – as is the case in most viral pathologies²⁹. Indeed, we observed that
527 'inflammatory mature' neutrophils, which exhibited an anti-pathogenic orientation with
528 pronounced degranulating activity, contained most of the viral N sequences (amongst all other
529 neutrophil phenotypes). Moreover, N-positive neutrophils exhibited increased expression of IFN-
530 induced (anti-viral) genes, compared to N-negative neutrophils. Some sequencing reads also
531 mapped to *ORF10* and *ORF1ab*, but not the other viral protein-encoding genes. We suspect this is
532 due to increased stability of N, *ORF10* and *ORF1ab* RNA compared to other viral ORFs. In
533 conclusion, these data suggest that the neutrophil's positioning in an immune-inhibitory (adverse)
534 environment, with disrupted T-cell effector/regulatory function as well as mostly inhibitory or
535 dysregulated interactions with other (myeloid) immune cells, might explain their failure in
536 controlling disease progression, thereby leading to critical COVID-19 pathology.

537 Our findings bear important therapeutic relevance. The RECOVERY trial recently claimed that
538 dexamethasone reduces death by one-third in hospitalised patients with critical COVID-19
539 (unpublished data). Dexamethasone has indeed been shown to dampen myeloid inflammatory
540 signalling (notably IL-1 and IL-6 release), reduce neutrophil inflammation⁴³, promote an 'M2-like'
541 macrophage phenotype, which has anti-inflammatory and phagocytic traits⁴⁴, as well as to maintain
542 clonal balance in T-cells⁴⁵. Given the findings we report here, the therapeutic effects of
543 dexamethasone are not entirely unexpected. Our data also suggest that neutrophils are key players
544 in the acute phase of the infection. However, prolonged neutrophil inflammation might also cause
545 excessive collateral lung damage and be detrimental to the host, as suggested by autopsy reports⁴⁶.
546 In this regard, the immunomodulatory antibiotic azithromycin might be a promising therapy for
547 COVID-19 when administered early in the disease course. Acutely administered azithromycin
548 enhances degranulation and the oxidative burst by neutrophils in response to a stimulus, yet this
549 is followed by a subsequent decrease of oxidative burst capacity and increase in neutrophil

550 apoptosis⁴⁷. We are therefore eagerly awaiting results from large-scale randomised trials with
551 azithromycin for COVID-19.

552 Nevertheless, there are also limitations to our study. For instance, we observed evidence of
553 counter-productive (possibly low-quality) antibody response-related signatures in COVID-19, but
554 failed to perform an in-depth study in this area. Additional studies performing scRNA- and scBCR-
555 seq on serially-collected samples during disease are needed to reinforce this observation. Also,
556 several COVID-19 patients were treated with the antiviral drugs remdesivir, which targets the viral
557 RNA-dependent RNA polymerase, or hydroxychloroquine, which has immunomodulatory traits
558 and is still controversial with respect to its therapeutic effects on disease outcome⁴⁸⁻⁵⁰. Of note,
559 we did not detect major patient-specific cell clusters nor other type of outliers during our analyses.

560 In conclusion, we used single-cell transcriptomics to characterize the innate and adaptive lung
561 immune response to SARS-CoV-2. We observed marked changes in the immune cell compositions,
562 phenotypes as well as immune cross-talks during SARS-CoV-2 infection and identified several
563 distinguishing immunological features of mild versus critical COVID-19. We also documented
564 genetic footprints of several crucial immunological pathways that have been extensively
565 hypothesized, but not always systematically confirmed, to be associated with COVID-19 pathology
566 and SARS-CoV-2 infection biology. We believe that this work represents a major resource for
567 understanding lung-localised immunity during COVID-19 and holds great promise for the study of
568 COVID-19 immunology, immune-monitoring of COVID-19 patients and relevant therapeutic
569 development.

570

571 **MATERIALS AND METHODS**

572

573 *Patient cohort, sampling and data collection*

574 22 COVID-19 patients and 13 non-COVID-19 pneumonitis patients in this study were enrolled
575 from the University Hospitals Leuven, between March 31th 2020 and May 4th 2020. Disease severity
576 was defined as 'mild' or 'critical', based on the level of respiratory support at the time of sampling.
577 Specifically, 'mild' patients required no respiratory support or supplemental oxygen through a nasal
578 cannula, whereas 'critically ill' patients were mechanically ventilated or received extracorporeal
579 membrane oxygenation.

580 The demographic and disease characteristics of the prospectively recruited patients studied by
581 scRNA-seq are listed in Supplementary Table I. Diagnosis of COVID-19 was based on clinical
582 symptoms, chest imaging and SARS-CoV-2 RNA-positive testing (qRT-PCR) on a nasopharyngeal
583 swab and/or BAL fluid sample. Non-COVID-19 pneumonitis cases all tested negative for SARS-
584 CoV-2 RNA using a qRT-PCR assay on BAL.

585 All 35 patients underwent bronchoscopy with BAL as part of the standard of medical care, because
586 of i) high clinical suspicion of COVID-19 yet negative SARS-CoV-2 qRT-PCR on nasopharyngeal
587 swab ii) established COVID-19 with clinical deterioration, to rule out opportunistic (co-)infection
588 and/or to remove mucus plugs. Lavage was performed instilling 20cc of sterile saline, with an
589 approximate retrieval of 10cc. 2-3cc of the retrieved volume was used for clinical purposes. The
590 remaining fraction was used for scRNA-seq.

591 The retrieved BAL volume was separated into two aliquots, as explained above, at the bedside by
592 the performing endoscopist. The aliquot used for scRNA-seq was immediately put on ice and
593 transported to a Biosafety Level 3 Laboratory (REGA Institute, KU Leuven) for scRNA-seq.

594 Demographic, clinical, treatment and outcome data from patient electronic medical records were
595 obtained through a standardized research form in Research Electronic Data Capture Software
596 (REDCAP, Vanderbilt University). This study was conducted according to the principles expressed
597 in the Declaration of Helsinki. Ethical approval was obtained from the Research Ethics Committee
598 of KU / UZ Leuven (S63881). All participants provided written informed consent for sample
599 collection and subsequent analyses.

600 *scRNA-seq, scTCR-seq and scBCR-seq profiling*

601 BAL fluid was centrifuged and the supernatant was frozen at -80°C for further experiments. The
602 cellular fraction was resuspended in ice-cold PBS and samples were filtered using a 40µm nylon
603 mesh (ThermoFisher Scientific). Following centrifugation, the supernatant was decanted and

604 discarded, and the cell pellet was resuspended in red blood cell lysis buffer. Following a 5-min
605 incubation at room temperature, samples were centrifuged and resuspended in PBS containing
606 UltraPure BSA (AM2616, ThermoFisher Scientific) and filtered over Flowmi 40µm cell strainers
607 (VWR) using wide-bore 1 ml low-retention filter tips (Mettler-Toledo). Next, 10 µl of this cell
608 suspension was counted using an automated cell counter to determine the concentration of live
609 cells. The entire procedure was completed in less than 1.5 h.

610 Single-cell TCR/BCR and 5' gene expression sequencing data for the same set of cells were
611 obtained from the single-cell suspension using the Chromium™ Single Cell 5' library and Gel Bead
612 & Multiplex Kit with the Single Cell V(D)J Solution from 10x Genomics according to the
613 manufacturer's instructions. Up to 5,000 cells were loaded on a 10x Genomics cartridge for each
614 sample. Cell-barcoded 5' gene expression libraries were sequenced on an Illumina NovaSeq6000,
615 and mapped to the GRCh38 human reference genome using CellRanger (10x Genomics, v3.1).
616 V(D)J enriched libraries were sequenced on an Illumina HiSeq4000 and TCR and BCR alignment
617 and annotation was achieved with CellRanger VDJ (10x Genomics, v3.1).

618 *Single-cell gene expression analysis*

619 Raw gene expression matrices generated per sample were merged and analysed with the Seurat
620 package (v3.1.4)⁵¹. Cell matrices were filtered by removing cell barcodes with <301 UMIs, <151
621 expressed genes or >20% of reads mapping to mitochondrial RNA. We opted for a lenient filtering
622 strategy to preserve the neutrophils, which are transcriptionally less active (lower transcripts and
623 genes detected). The remaining cells were normalized and the 3000 most variable genes were
624 selected to perform a PCA analysis after regression for confounding factors: number of UMIs,
625 percentage of mitochondrial RNA, patient ID and cell cycle (S and G2M phase scores calculated
626 by the CellCycleScoring function in Seurat), interferon response (BROWNE_INTERFERON_RE-
627 SPONSIVE_GENES in the Molecular Signatures Database or MSigDB v6.2), sample dissociation-
628 induced stress signatures⁵², hypoxia signature⁵³. This PCA and graph-based clustering approach
629 however resulted in some highly patient specific clusters, which prompted us to perform data
630 integration using anchor-based CCA in Seurat (v3) package between patients to reduce the patient-
631 specific bias. And this was performed after excluding cells from an erythrocyte cluster (primarily
632 from a single patient) and a low-quality cell cluster. After data integration, 3000 most variable genes
633 were calculated by FindVariableFeatures function, and all the mitochondrial, cell cycle, hypoxia,
634 stress and interferon response genes (Pearson correlation coefficient > 0.1 against scores of the
635 above-mentioned signatures calculated by AddModuleScore function in Seurat) were removed
636 from the variable genes. In addition, we also removed common ambient RNA contaminant genes,

637 including hemoglobin and immunoglobulin genes, as well as T-cell receptor (TRAVs, TRBVs,
638 TRDVs, TRGVs) and B-cell receptor (IGLVs, IGKVs, IGHVs) genes, before downstream analyses.

639 *scRNA-seq clustering for cell type identification*

640 For the clustering of all cell types, principal component analysis (PCA) was applied to the variable
641 genes of dataset to reduce dimensionality. The selection of principal components was based on
642 elbow and Jackstraw plots (usually 25-30). Clusters were calculated by the FindClusters function
643 with a resolution between 0.2 and 2, and visualised using the Uniform Manifold Approximation and
644 Projection for Dimension Reduction (UMAP) reduction. Differential gene-expression analysis was
645 performed for clusters generated at various resolutions by both the Wilcoxon rank sum test and
646 Model-based Analysis of Single-cell Transcriptomics (MAST) using the FindMarkers function⁵¹. A
647 specific resolution was selected when known cell types were identified as a cluster at a given
648 resolution, but not at a lower resolution with the minimal constraint that each cluster has at least
649 10 significantly differentially expressed genes (FDR <0.01, 2-fold difference in expression compared
650 to all other clusters). Annotation of the resulting clusters to cell types was based on the expression
651 of marker genes.

652 *Integration of publicly available datasets and identification of cell subtypes*

653 We additionally processed scRNA-seq data on COVID-19 BAL fluid by Liao et al. and on normal
654 lung samples by Reyfman et al. and Lambrechts et al. as described above^{7,9,10}. The former two
655 datasets were *de novo* clustered and annotated, and cell type annotation of the last dataset was
656 used as previously described¹¹. For cell subtype identification, the main cell types identified from
657 multiple datasets were pooled, integrated, and further subclustered using the similar strategy,
658 except that the constant immunoglobulin genes were not excluded for B-cell and plasma cell
659 subclustering. Finally, doublet clusters were identified based on: 1) expression of marker genes
660 from other cell (sub)clusters, 2) higher average UMIs as compared to other (subclusters), and 3) a
661 higher than expected doublets rate (> 20%), as predicted by both DoubletFinder (v2)⁵⁴ and
662 Scrublet⁵⁵ and the clustering was re-performed in the absence of the doublet clusters.

663 *Trajectory inference analysis*

664 The R package Slingshot was used to explore pseudotime trajectories/potential lineages in T- and
665 myeloid cells⁵⁶. The analyses were performed for CD8⁺ and CD4⁺ cell phenotypes separately, with
666 T_{MAIT}-, T_{γδ}- and T_{REG}-cells excluded due to their unique developmental origin. For each analysis,
667 PCA-based dimension reduction was performed with differentially expressed genes of each
668 phenotype, followed by two-dimensional visualization with UMAP. Graph-based clustering (Louvain)

669 identified additional heterogeneity for some phenotypes, as described in the manuscript for CD4⁺
670 T-cells. Next, this UMAP matrix was fed into SlingShot, with naïve T-cells as a root state for
671 calculation of lineages and pseudotime. Similar approach was applied to the monocyte-macrophage
672 differentiation trajectory inferences.

673 *Assessing the TCR and BCR repertoires*

674 We only considered productive TCR/BCRs, which were assigned by the CellRanger VDJ pipeline.
675 Relative clonotype richness⁵⁷, defined as the number of unique TCRs/BCRs divided by the total
676 number of cells with a unique TCR/BCR, was calculated to assess clonotype diversity. Relative
677 clonotype evenness⁵⁸, was defined as inverse Simpson index divided by species richness (number
678 of unique clonotypes).

679 *Inflammatory pathways and gene set enrichment analysis and tradeSeq*

680 The REACTOME pathway activity of individual cells was calculated by AUCell package (v1.2.4)⁵⁹.
681 And the differential activity between lineages along the trajectories were calculated using
682 TradeSeq⁶⁰. Pathways with median fold change >3 and an adjusted p-value < 0.01 were considered
683 as significantly changed. GO and REACTOME geneset enrichment analysis were performed using
684 hperR package⁶¹; geneset over-representation was determined by hypergeometric test.

685 *SARS-CoV-2 viral sequence detection*

686 Viral-Track was used to detect SARS-CoV-2 reads from BAL scRNA-seq data (reference
687 genome NC_045512.2), as previously described⁸. The initial application was aimed to identify
688 SARS-CoV-2 reads against thousands of other viruses, and thus the STAR indexes for read
689 alignment were built by combining the human (GRCh38) genome reference with thousands of virus
690 reference genomes from viruSITE. Since the likelihood of co-infection with multiple viruses (>2) is
691 low in COVID-19 patients⁸, we adapted the Viral-Track pipeline to reduce computation time and
692 increase sensitivity. Briefly, instead of directly processing raw fastq reads, we took advantage of
693 BAM reads generated for scRNA-seq data, which mapped to human genome by the CellRanger
694 pipeline as described above. The BAM files were filtered to only keep reads with cell barcodes
695 annotated in the scRNA-seq analysis using subset-bam tools (10x Genomics). Then the
696 corresponding unmapped BAM reads were extracted using samtools and converted to fastq files
697 using bamtofastq tool to be further processed by UMI-tools for cell barcode assignment before
698 feeding into Viral-Track pipeline. These unmapped reads, which contain potential viral sequences,
699 were aligned using STAR to SARS-CoV-2 reference genome, with less stringent mapping parameter
700 (outFilterMatchNmin 25-30), as compared to the original Viral-Track pipeline. Our approach

701 identified 17 SARS-CoV-2 positive patients from a total of 31 COVID-19 patients, including 3
702 patients that were previously not detected using original Viral-Track pipeline by Bost et al. None
703 of the patients among the 13 non-COVID-19 patients were detected as SARS-CoV-2 positive,
704 suggesting our adapted pipeline does not result in major false-positive detection. For the detection
705 of 11 SARS-CoV-2 ORFs or genes, a GTF annotation file was generated according to
706 NC_045512.2⁶² for counts matrix using Viral-Track. The viral gene counts of each barcoded cells
707 were integrated into the scRNA-seq gene count matrix and normalized together using
708 NormalizeData function in Seurat.

709 *Cell-to-cell communication of scRNA-seq data*

710 The CellPhoneDB algorithm was used to infer cell-to-cell interactions⁶³. Briefly, the algorithm
711 allows to detect ligand-receptor interactions between cell types in scRNA-seq data. We assessed
712 the amount of interactions that are shared and specific for *i*) COVID-19 versus non-COVID-19 and
713 *ii*) mild versus critical COVID-19.

714 *Quantification and statistical analysis*

715 Descriptive statistics are presented as median [interquartile range; IQR] (or median [range] if
716 dataset contained only 2 variables) and n (%) for continuous and categorical variables, respectively.
717 Statistical analyses were performed using R (version 3.6.3, R Foundation for Statistical Computing,
718 R Core Team, Vienna, Austria). Statistical analyses were performed with a two-sided alternative
719 hypothesis at the 5% significance level.

720 **DATA AVAILABILITY**

721 Raw sequencing reads of the scRNA-seq and scTCR-seq experiments generated for this study will
722 be deposited in the EGA European Genome-Phenome Archive database. Based on SScope, which
723 is an interactive web server for scRNA-seq data visualisation, a download of the read count matrix
724 will be made available at <http://blueprint.lambrechtslab.org>. The publicly available datasets that
725 supported this study are available from GEO GSE145926⁷, GEO GSE122960¹⁰ and from
726 ArrayExpress E-MTAB-6149/E-MTAB-6653⁹.

727

728 **ADDITIONAL RESOURCES**

729 The findings outlined above are part of the COntAGlouS observational clinical trial:
730 <https://clinicaltrials.gov/ct2/show/NCT04327570>.

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743 **AUTHORS CONTRIBUTIONS**

744 E.W., P.V.M., A.D.G., J.W., D.L. and J.Q. designed the experiments, developed the methodology,
745 analysed and interpreted data and wrote the manuscript. S.J. and J.N. designed the experiments,
746 developed the methodology and performed experiments. Y.V.H. and L.V. designed the experiments,
747 collected and interpreted data. D.T., G.H., D.D., J.Y., J.G. and C.D. performed sample collection.
748 A.B., B.B., B.M.D., P.M. and S.J. performed experiments and data analysis and interpretation. T.V.B.,
749 R.S., T.V.B. and E.H. provided technical assistance and performed experiments. E.W. and J.W.
750 supervised the clinical study design and were responsible for coordination and strategy. All authors
751 have approved the final manuscript for publication.

752 **CONFLICT OF INTEREST**

753 The authors declare no competing interests

754 **CONTAGIOUS co-authors:**

755 Francesca Maria Bosisio, Michael Casaer, Frederik De Smet, Paul De Munter, Stephanie Humblet-
756 Baron, Adrian Liston, Natalie Lorent, Kim Martinod, Paul Proost, Jeroen Raes, Karin Thevissen,
757 Robin Vos, Birgit Weynand, Carine Wouters
758

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911 **Figure Legends**

912

913 **Fig. 1. Annotation of cell types by scRNA-seq in COVID-19 and non-COVID-19 BAL**

914 **a** UMAP representation of 65,166 cells (obtained from BAL from n=13 non-COVID-19, n=2 mild and n=22
915 critical COVID-19 patients) by scRNA-seq color-coded for the indicated cell type. pDC: plasmacytoid
916 dendritic cell, cDC: conventional dendritic cell, NK: natural-killer cell, Md_Mac: monocyte-derived
917 macrophage. Alveolar_Mac: alveolar macrophage. AT2: alveolar type II epithelial cell. **b** UMAP panels
918 stratified per individual patient, COVID-19 versus non-COVID-19 and mild versus critical COVID-19. **c**
919 Relative contribution of each cell type (in %) in COVID-19 versus non-COVID-19. **d** Relative contribution
920 of each cell type (in %) in mild versus critical COVID-19. P values were assessed by Mann-Whitney test. *
921 P<0.05, ** P<0.01, ***P<0.001

922

923 **Fig. 2. 14 T-cell phenotypes in mild and critical COVID-19 BAL**

924 **a** Subclustering of 23,468 T-/NK-cells into 14 T-/NK-cell phenotypes, as indicated by the color-coded
925 legend. NK cyto: cytotoxic NK cell; NK inflam: inflammatory NK cell. **b** Heatmap showing T-/NK-cell
926 phenotypes with corresponding marker genes and functional gene sets. **c** Relative contribution of each T-
927 /NK-cell phenotype (in %) in COVID-19 versus non-COVID-19. **d** Relative contribution of each T-/NK-cell
928 phenotype (in %) in mild versus critical COVID-19. P values were assessed by Mann-Whitney test. * P<0.05,
929 ** P<0.01, ***P<0.001

930

931 **Fig. 3. CD8⁺ T-cell phenotypes in mild and critical COVID-19 BAL**

932 **a** Pseudotime trajectories for CD8⁺ T-cells based on Slingshot, showing 3 lineages (T_{RM}-lineage, T_{EX}-lineage
933 and T_{EMRA}-lineage), color-coded for the CD8⁺ T-cell phenotypes (left panel), the pseudotime (middle panel)
934 and the number of clonotypes (right panel). **b** Profiling of marker genes along these trajectories to confirm
935 their functional annotation: ZNF683 and ITGAE for the T_{RM}-lineage, HAVCR2 and CTLA4 for the T_{EX}-lineage,
936 FGFBP2 and CX3CR1 for the T_{EMRA}-lineage. **c** Genes involved in T-cell effector function and cytotoxicity
937 (GZMB, IFNG, GNLY) and related transcription factor (TOX2) modelled along the CD8⁺ T-cell lineages. **d**
938 Density plots reflecting the number of T-cells along the 3 CD8⁺ T-cell lineages. **e** Density plots reflecting
939 the number of T-cells along the 3 CD8⁺ T-cell lineages stratified for non-COVID-19, COVID-19 and normal
940 lung. **f** Density plots reflecting the number of T-cells along the 3 CD8⁺ T-cell lineages stratified for mild
941 versus critical COVID-19. **g** Analysis of clonotype sharing (thickness indicates proportion of sharing)
942 between the CD8⁺ T-cells. **h-i** TCR richness and TCR evenness along the 3 T-cell lineages for non-COVID-
943 19 versus COVID-19 (**h**), and mild versus critical COVID-19 (**i**). **j-k** Gene expression dynamics along the
944 CD8⁺ T_{RM}- (**j**) and T_{EX}-lineage (**k**). Genes cluster into 5 gene sets, each of them characterized by specific
945 expression profiles, as depicted by a selection of marker gene characteristic for each set. Differences in
946 trajectories were assessed by Mann-Whitney test. For CD8⁺ T_{RM}: COVID-19 versus non-COVID-19 (P
947 =1.0E-6), mild versus critical COVID-19 (P=5.9E-102). For CD8⁺ T_{EX}: COVID-19 versus non-COVID-19 and
948 normal lung (P=2.3E-67), mild versus critical (P=1.1E-39). For CD8⁺ T_{EMRA}: normal lung versus COVID-19
949 and non-COVID-19 (P=3.8E-39).

950

951 **Fig. 4. CD4⁺ T-cell developmental trajectories in mild and critical COVID-19 BAL**

952 **a** UMAP with pseudotime trajectories based on Slingshot, showing 3 lineages (T_{HI}-lineage, T_{HI7}-lineage and
953 T_{SCM}-lineage), color-coded for the CD4⁺ T-cell phenotypes (left), the pseudotime (middle) and the number
954 of clonotypes (right). **b** Naïve and memory-related marker gene expression (left), and cell cycle scoring
955 (right) reveal additional CD4⁺ T-cell subclusters. T_{SCM}-cells are characterized by naïve marker genes (CCR7,
956 TCF7), memory markers (CD27), cell proliferation but no GZMA expression. **c** Analysis of clonotype sharing
957 (thickness indicates proportion of sharing) between the CD4⁺ T-cell subclusters. **d** Profiling of marker genes
958 along these trajectories to confirm their functional annotation: GZMB and IFNG for the T_{HI}-lineage, IL17A
959 and RORC for the T_{HI7}-lineage, TCF7 and CCR7 for the T_{SCM}-lineage, while GNLY and PRF1 were plotted to

960 highlighted T-cell effector function. **e** Density plots reflecting the number of T-cells along the 3 CD4⁺ T-cell
961 lineages stratified for non-COVID-19, COVID-19 and normal lung. **f** Density plots reflecting the number of
962 T-cells along the 3 CD4⁺ T-cell lineages stratified for mild versus critical COVID-19. **g-h** TCR richness and
963 TCR evenness along the 3 CD4⁺ T-cell lineages comparing non-COVID-19 versus COVID-19 (**g**) and mild
964 versus critical COVID-19 (**h**). **i-j** Gene expression dynamics along the CD4⁺ T_{HI}- (**i**) and T_{HI7}-lineage (**j**).
965 Genes cluster into 5 gene sets, each of them characterized by specific expression profiles, as depicted by a
966 selection of marker genes characteristic for each set. Differences in trajectories were assessed by Mann-
967 Whitney test. For CD4⁺ T_{HI} and CD4⁺ T_{SCM}: COVID-19 versus non-COVID-19 and lung normal (P = 1.4E-6
968 and 5.9E-37), For CD4⁺ T_{HI7}: COVID-19 versus non-COVID-19 (P=9.7E-12), mild versus critical COVID-
969 19 (P=1.3E-12).
970

971 **Fig. 5. Monocyte-to-macrophage differentiation in COVID-19 BAL**

972 **a** Subclustering of myeloid cells into 9 phenotypes, as indicated by the color-coded legend. **b** Heatmap
973 showing myeloid cell phenotypes with corresponding functional gene sets. **c** Relative contribution of each
974 cell type (in %) to COVID-19 versus non-COVID-19 BAL. **d** Relative contribution of each cell type (in %) to
975 mild versus critical COVID-19 BAL. **e** Pseudotime trajectories for myeloid cells based on Slingshot, showing
976 the common branch of FCN1^{hi} monocytes differentiating into either RGS1^{hi} monocyte-derived macrophages
977 (RGS1^{hi}-lineage) or FABP4^{hi} tissue-resident alveolar macrophages (alveolar lineage). **f** Profiling of marker
978 genes along these trajectories to confirm their functional annotation: FCN1, S100A12, CCL2, CCL18 for the
979 common branch, FABP4 and PPARG for the alveolar lineage, RGS1 and GPR183 for the RGS1-lineage. **g**
980 Density plots reflecting the number of myeloid cells along the 2 lineages stratified for non-COVID-19 versus
981 COVID-19. **h** Density plots reflecting the number of myeloid cells along the 2 lineages stratified for mild
982 versus critical COVID-19. **i** Gene expression dynamics along the alveolar lineage. Genes cluster into 5 gene
983 sets, each of them characterized by specific expression profiles, as depicted by a selection of genes
984 characteristic for each cluster. **j-l** Profiling of IFN type I and II signalling along the 3 CD8⁺ (**j**) and CD4⁺ (**k**)
985 T-cell lineages, and along the monocyte-macrophage lineage (**l**), comparing mild versus critical COVID-19.
986 All P values were assessed by a Mann-Whitney test. * P<0.05, ** P<0.01, ***P<0.001. P values comparing
987 COVID-19 versus non-COVID-19, and mild versus critical COVID-19 for density plots were all <10E-50.
988

989 **Fig. 6. Neutrophil, dendritic cell and B-cell phenotypes in COVID-19 BAL**

990 **a** Subclustering of neutrophils into 5 phenotypes, as indicated by the color-coded legend. **b** UMAP showing
991 expression of a marker gene for each neutrophil phenotype. **c** Heatmap showing neutrophil phenotypes
992 with corresponding marker genes and functional gene sets. **d** Relative contribution of each neutrophil
993 phenotype (in %) to COVID-19 versus non-COVID-19. **e** Relative contribution of each neutrophil phenotype
994 (in %) to mild versus critical COVID-19. **f** Subclustering of DC into 6 phenotypes, as indicated by the color-
995 coded legend. **g** Heatmap showing DC phenotypes with corresponding marker genes and functional gene
996 sets. **h** Relative contribution of each DC phenotype (in %) to COVID-19 versus non-COVID-19. **i** Relative
997 contribution of each DC phenotype (in %) to mild versus critical COVID-19. **j** Subclustering of B-cells and
998 plasma cells into 4 phenotypes, as indicated by the color-coded legend. **k** Heatmap showing B-cell and
999 plasma cell phenotypes with corresponding marker genes and functional gene sets. **l** Feature plots of marker
1000 gene expression for each B-cell and plasma cell subcluster. **m** Violin plots showing cell cycle scores and
1001 mitochondrial gene expression by plasma cell subcluster. **n** B-cell receptor evenness in B-cell and plasma
1002 cell subclusters. **o** Relative contribution of each B-cell and plasma cell phenotype (in %) to COVID-19 versus
1003 non-COVID-19. **p** Relative contribution of each B-cell and plasma cell phenotype (in %) to mild versus
1004 critical COVID-19. P values were assessed by a Mann-Whitney test. * P<0.05, ** P<0.01, ***P<0.001.
1005

1006 **Fig. 7. SARS-CoV-2 RNA detection in epithelial and immune cells**

1007 **a** Subclustering of epithelial cells into 7 phenotypes, as indicated by the color-coded legend. **b** Heatmap
1008 showing epithelial cell phenotypes with corresponding marker genes. **c** Relative contribution of each
1009 epithelial cell phenotype (in %) to COVID-19 versus non-COVID-19. **d** Relative contribution of each
1010 epithelial cell phenotype (in %) to mild versus critical COVID-19. **e-f** Expression level of ACE2 (**e**) and

1011 **f** by epithelial cell subclusters, comparing COVID-19 versus non-COVID-19. **g** Expression levels
1012 of ACE2, TMPRSS2 and SARS-CoV-2 (cells with viral reads) RNA in epithelial, myeloid and lymphoid cells from
1013 COVID-19. **h** Detection of 11 SARS-CoV-2 open-reading frames in epithelial, myeloid and lymphoid cells
1014 from COVID-19. **i** Detection of spike protein (S) and nucleocapsid protein (N) encoding viral RNA in
1015 epithelial cells and immune cell subclusters from COVID-19. Cell types with <50 positive cells are not
1016 shown. **j** Differential gene expression of N-positive versus N-negative neutrophils from 17 COVID-19
1017 patients in which viral reads were detected. **k-l** REACTOME (**k**) and GO (**l**) pathway analysis on IFN-
1018 signalling and response-to-virus signalling, comparing N-positive versus N-negative neutrophils from 17
1019 COVID-19 patients in which viral reads were detected. **m** Detection of reads mapping to SARS-CoV-2 and
1020 to N in neutrophil subclusters from COVID-19 BAL. P values were assessed by a Mann-Whitney test. *
1021 P<0.05, ** P<0.01, ***P<0.001.

1022

1023 **Fig. 8. Cell-to-cell communication between epithelial and immune cells**

1024 **a** Number of predicted interactions ($P \leq 0.05$) between monocytes, macrophages, T-cells, neutrophils and
1025 epithelial cells based on CellPhoneDB in critical (left panel) and mild (right panel) COVID-19. **b** Differences
1026 in the number of predicted interactions, comparing mild versus critical COVID-19, showing generally more
1027 interactions in mild COVID-19. **c** Predicted interactions between monocytes/macrophages and neutrophils,
1028 comparing critical versus mild COVID-19. **d** Predicted interactions between T-cells and neutrophils,
1029 comparing critical versus mild COVID-19. **e** Predicted interactions between epithelial and myeloid cells,
1030 comparing critical versus mild COVID-19. **f** Predicted interactions between T-cells and
1031 monocytes/macrophages, comparing critical versus mild COVID-19. **g** Predicted interactions between T-
1032 cells and epithelial cells, comparing critical versus mild COVID-19.

1033

1034

Figure 1

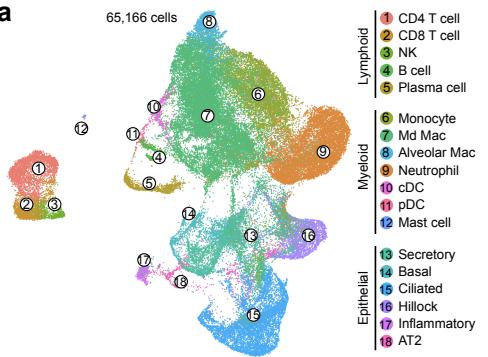
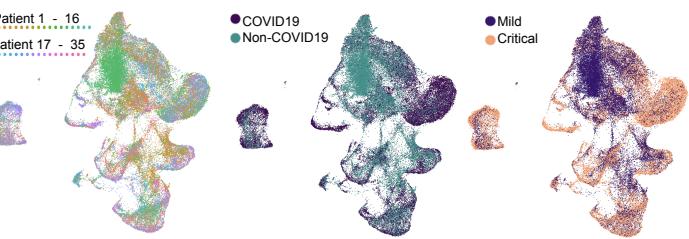
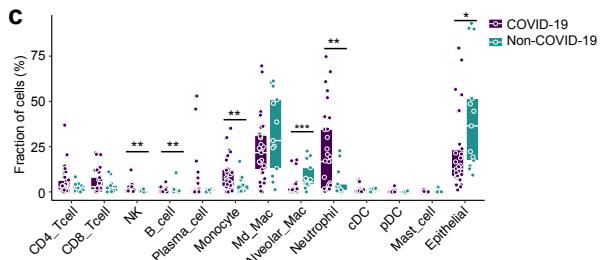
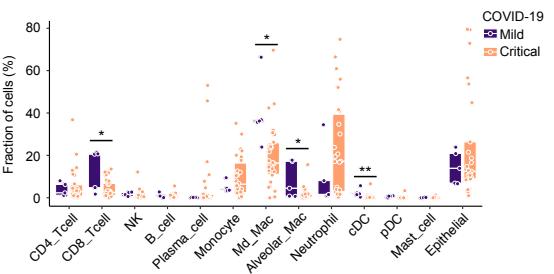
a**b****c****d**

Figure 2

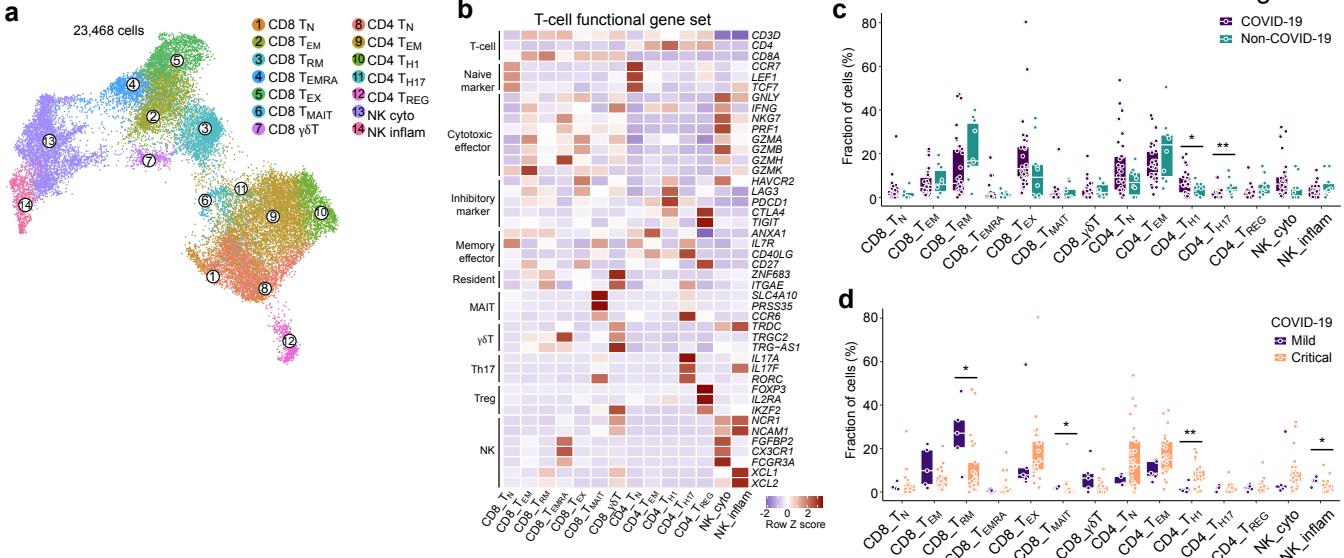


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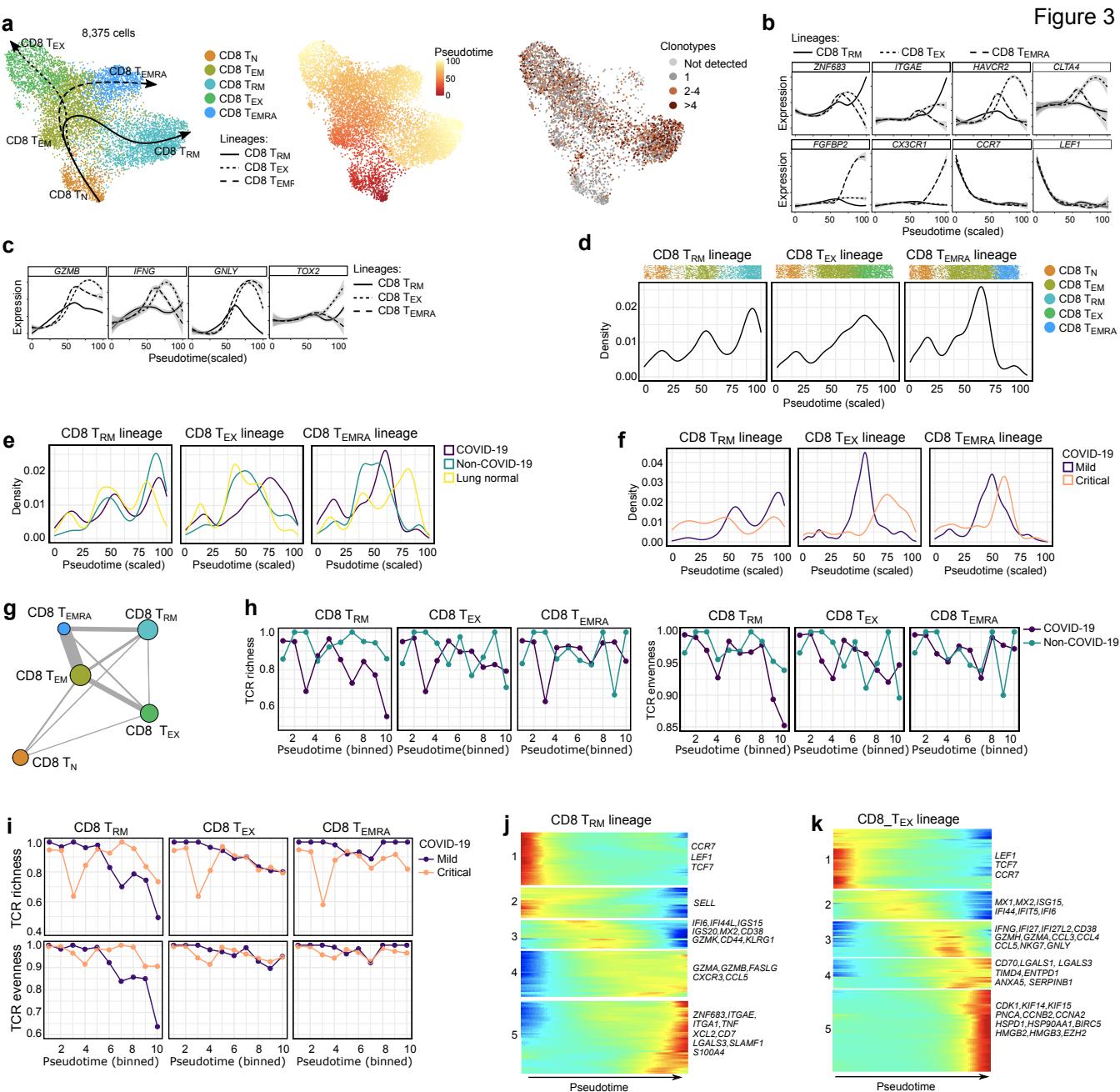


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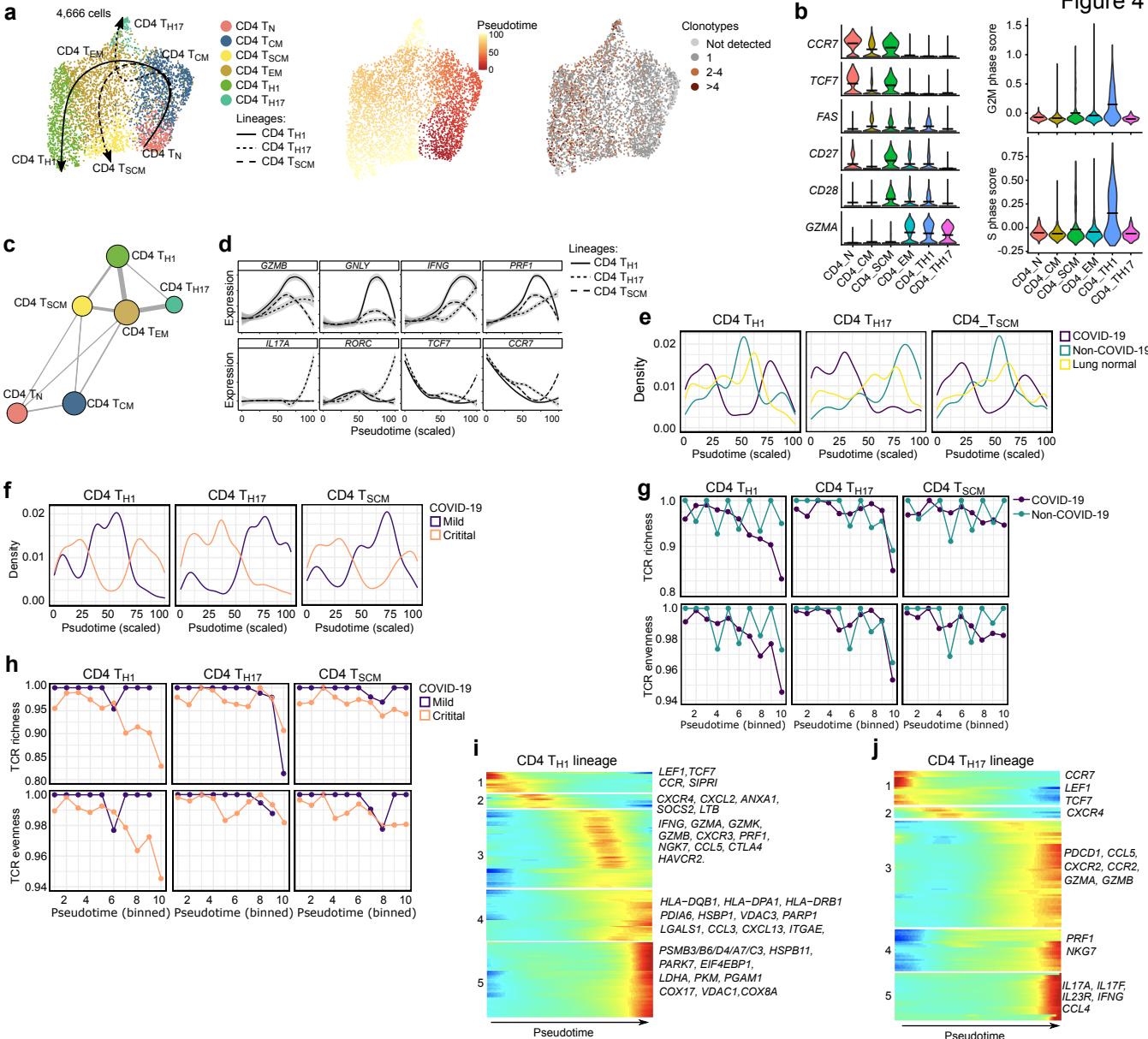


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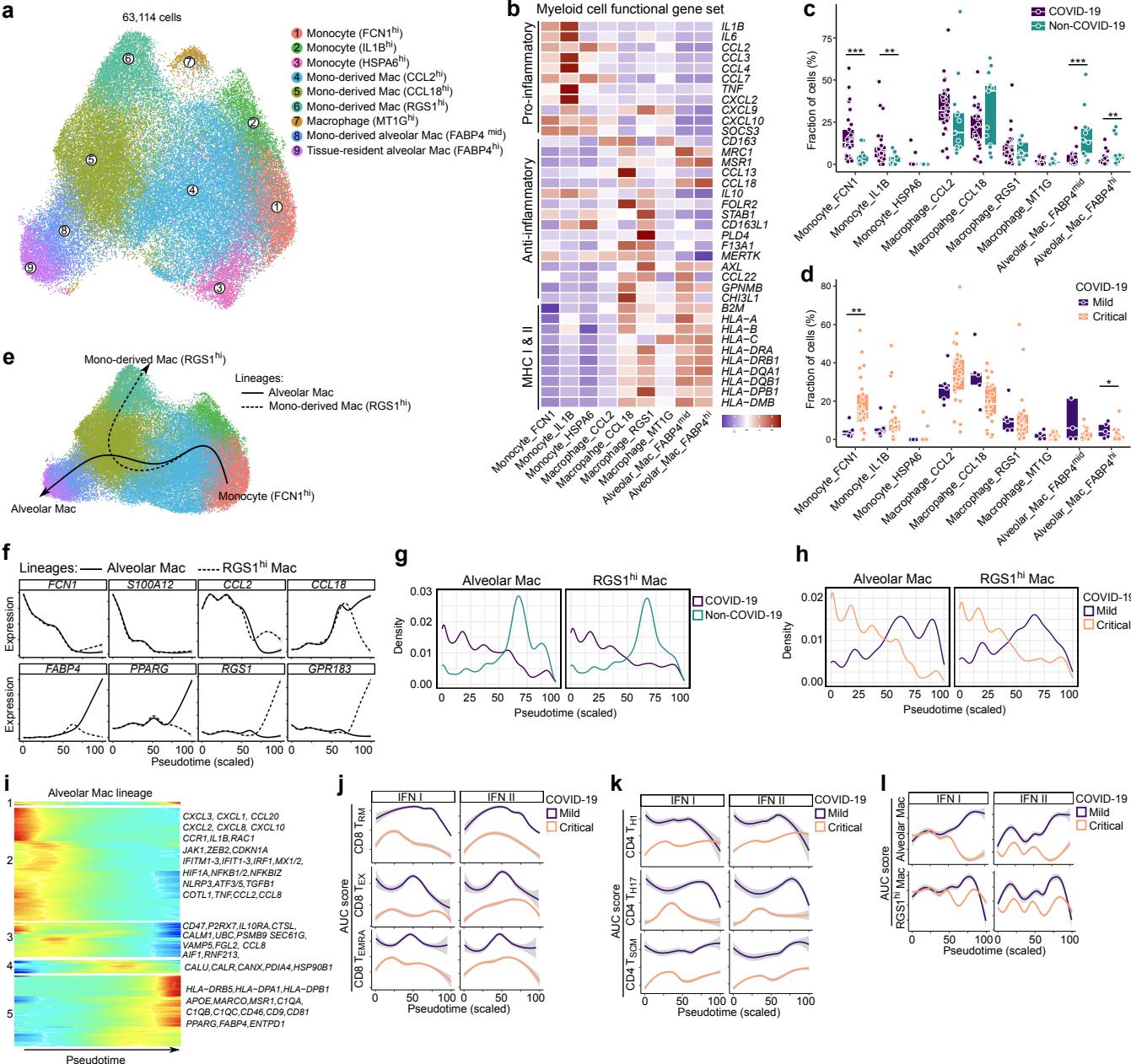


Figure 6

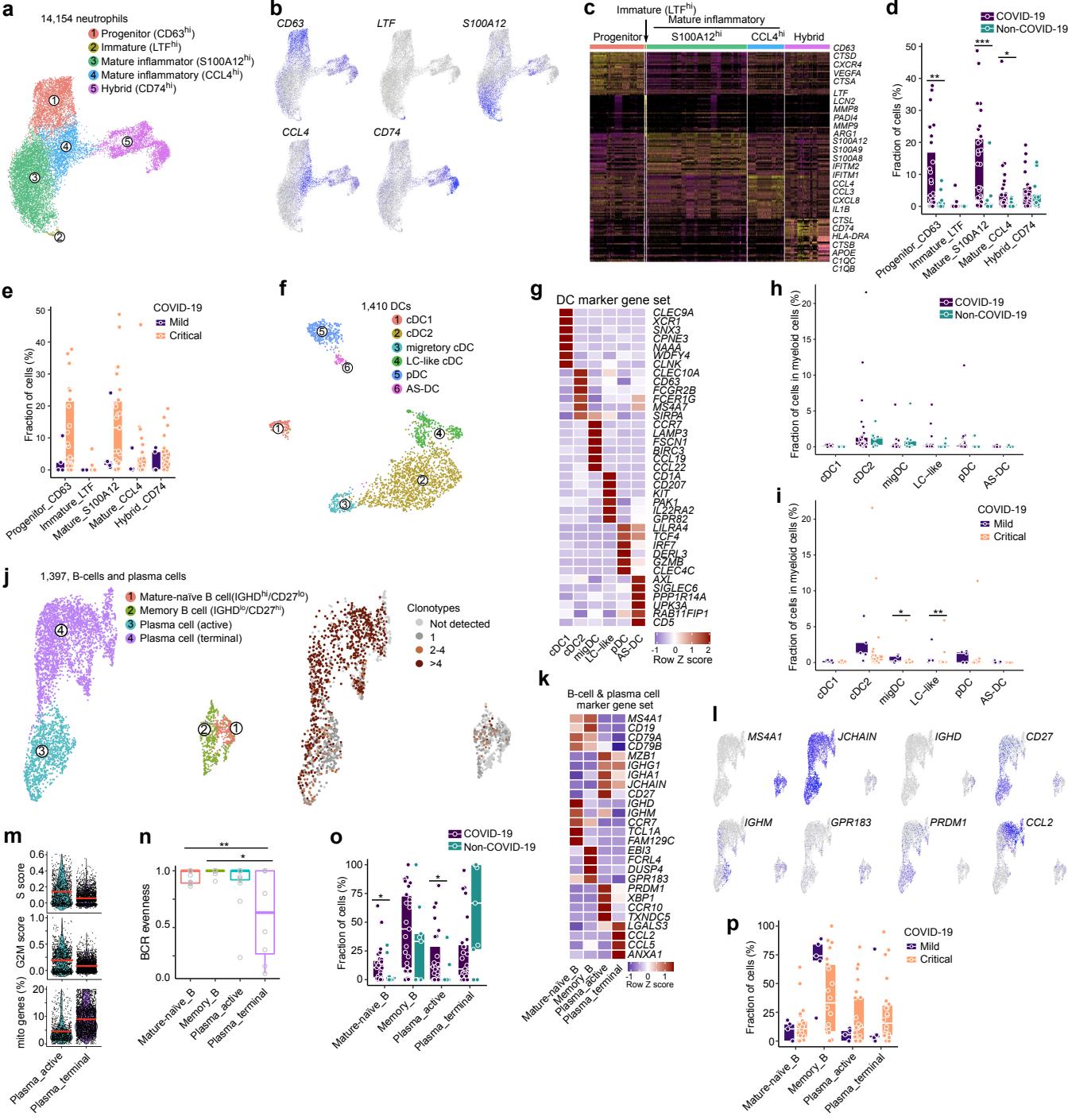


Figure 7

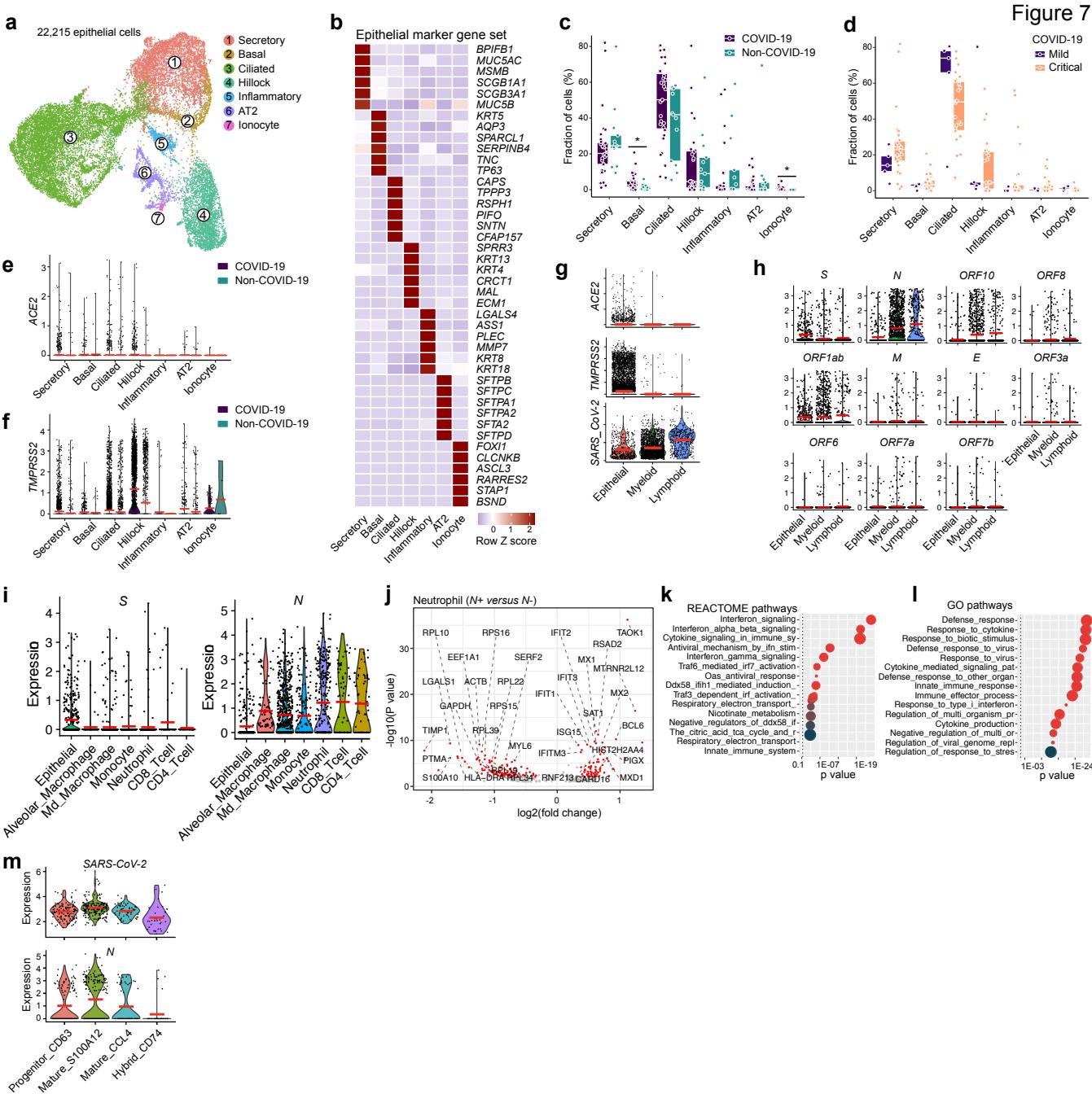


Figure 8

