

Discriminating Mild from Critical COVID-19 by Innate and Adaptive Immune Single-cell Profiling of Bronchoalveolar Lavages

Running title: Immune Atlas of COVID-19 Bronchoalveolar Lavages

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ABSTRACT

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

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 SPPI⁺ macrophages⁸. It is
100 unclear however whether this represents direct viral infection of myeloid cells, or phagocytosis of

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

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

RESULTS

scRNA-seq and cell typing of BAL samples

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

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

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

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

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

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

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

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

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

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

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

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

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

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

We then modelled gene expression along the T_{RM}- and T_{EX}-lineage, and identified 5 gene sets with 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*, *LEF1*, *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 *ITGAI*, *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 (*LEF1*, *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_{H1}) cells, expressing high levels of immune-checkpoints (*HAVCR2*,
228 *LAG3*, *PDCD1* and *CTLA4*), as well as CD4⁺ T-helper-17 (T_{H17}) and CD4⁺ regulatory T-cells (T_{REG})

(**Fig. 2b** for marker gene sets). Compared to non-COVID-19, we observed slightly less CD4⁺ T_{H17}-cells, but more T_{H1}-cells in COVID-19. Comparing mild *versus* critical COVID-19, we found T_{H1}-cells to be significantly increased in the latter.

Slingshot revealed additional phenotypic heterogeneity, identifying central-memory CD4⁺ T_{CM}-cells and stem cell-like memory CD4⁺ T_{SCM}-cells (**Fig. 4a, b**), and constructed 3 trajectories, which were independently confirmed based on shared TCR clonotypes (**Fig. 4c**). Briefly, T_N-cells connected closely with T_{CM}-cells followed by T_{EM}-cells, which branched-off into 3 different lineages to form T_{H1}-cells, T_{H17}-cells and T_{SCM}-cells. Profiling of marker genes along these trajectories confirmed their functional annotation (**Fig. 4d**). Density plots stratified for T-cells from each subgroup revealed that COVID-19 was enriched for T-cells early and late in the T_{H1}- and T_{SCM}-trajectory, while *vice versa* T-cells from non-COVID-19 and normal lung were enriched halfway these trajectories (**Fig. 4e, f**). In the T_{H17}-trajectory, COVID-19 BAL was strongly enriched for T-cells in the first half of the trajectory. Overall, CD4⁺ T-cells from mild COVID-19 behaved similarly as normal lung or non-COVID-19. Both TCR richness and evenness were reduced along the T_{H1}-lineage from COVID-19, but not from non-COVID-19 (**Fig. 4g**). Notably, this reduction was most prominent in mild, but not in critical COVID-19 (**Fig. 4h**). In contrast, T_{H17}-cells and T_{SCM}-cells were characterized by a modest decrease in TCR richness only at the very end of their lineage, suggesting that mainly T_{H1}-cells are selected for specific SARS-CoV-2 PAMPs/antigens.

Overall, this suggests that mild COVID-19 is characterized by more stable or differentiated T_{H17}-cells' activity, which is crucial for productive immunity against pathogens at mucosal surfaces¹⁷, whereas T_{H1}-cells become entangled halfway in their trajectory. In critical COVID-19, T_{H17} cells completely fail to differentiate, while T_{H1}-cells behave the opposite.

Gene expression modelling along the CD4⁺ T_{H1}- and T_{H17}-lineage

Differential gene expression analysis along both lineages identified gene sets with specific expression profiles. In the T_{H1}-lineage, the first gene set consisted of naïve (*LEF1*, *TCF7*) and undifferentiated (*CCR7*, *SIPRI*) T-cell markers (**Fig. 4i**; Supplementary information, **Table S5**). A second set was enriched for both pro- and anti-inflammatory markers (*CXCR4*, *CXCL2*, *ANXA1*, *SOCS2*, *LTB*), while a third set was characterized (halfway the trajectory) by an effector-like T_{H1}-program based on expression of *IFNG*, granzymes (*GZMA*, *GZMK*, *GZMB*), *CXCR3*, *PRFI*, *NGK7*, *CCL5*, as well as *CTLA4* and *HAVCR2*. Finally, a fourth gene set was characterized by high HLA expression, auto-regulatory markers (*LGALS1*, *CCL3*), partial activation markers (*CXCL13*) and stress-response markers (*PDIA6*, *HSBP1*, *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 (*MTIE*, *MTIX*), proteotoxic stress
 264 (*PSMB3/B6/D4/A7/C3*, *HSPB11*, *PARK7*, *EIF4EBP1*) and glycolysis (*PGAM1*) suggesting 'terminal
 265 exhaustion' at the end of the T_{H1} -trajectory^{18,19}. Overall, in mild COVID-19, the T_{H1} -lineage was
 266 enriched for cells halfway the trajectory where expression of set 2-3 genes was most dominant,
 267 indicating increased T_{H1} -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_{H17} -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_{H17} 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_{H17} -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_{H17} lineage.

277 Overall, this clearly indicates that mild COVID-19 is characterized by improved T_{H1} - and T_{H17} -
 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 *MSR1*, *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-

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

We observed a significant increase in *FCN1*^{high} and *IL1B*^{high} monocytes in COVID-19 versus non-COVID-19, while *FABP4*^{medium} and *FABP4*^{high} alveolar macrophages were reduced (**Fig. 5c**). *FCN1*^{high} monocytes were significantly reduced in mild COVID-19, while alveolar macrophages were increased (**Fig. 5d**). Using Slingshot, we reconstructed two monocyte-to-macrophage lineages, consisting of a common branch of *FCN1*^{high} monocytes differentiating into *IL1B*^{high} monocytes, followed by *CCL2*^{high} and *CCL18*^{high} monocyte-derived macrophages. These subsequently branched into *RGS1*^{high} monocyte-derived macrophages (*RGS1*-lineage), or via *FABP4*^{medium} into *FABP4*^{high} tissue-resident macrophages (alveolar lineage; **Fig. 5e**). Monocyte marker expression decreased along both lineages, while macrophage marker expression increased (**Fig. 5f**). Density plots revealed that in COVID-19 cells were enriched in the first half of both lineages (**Fig. 5g**), confirming our above observations of monocyte enrichment in COVID-19. Comparing mild to critical COVID-19, we noticed that the former differentiated along both lineages, whereas in the latter monocytes completely failed to differentiate (**Fig. 5h**).

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

driven purinergic-inflammasome signalling; especially given the high likelihood of extracellular ATP release from damaged epithelium in the context of acute viral infection. Importantly, this ATP-driven purinergic-inflammasome signalling pathway is a danger signalling cascade, which has been shown to facilitate ARDS-associated lung fibrosis and thus acts disease-worsening in this context²²⁻²⁴. Finally, set 4 and 5 genes were expressed at the end of the trajectory. Set 4 was characterized by expression of chaperone-coding genes (*CALU*, *CALR*, *CANX*, *PDIA4*, *HSP90B1*), which are crucial for robust functioning of the antigen-loading machinery for MHC molecules, whereas in set 5 there were clear signs of antigen presentation (expression of numerous MHC class II genes). Furthermore, set 5 comprised genes involved in receptor-mediated phagocytosis and post-phagocytic lipid degradation/metabolism: *APOE* for lipid metabolism, scavenger receptors *MARCO* and *MSR1*, complement activation (*CIQA*, *CIQB*, *CIQC* and *CD46*; that can also facilitate phagocytosis), viral infection-relevant inflammatory orientation (*CD81*, *CD9*), as well as anti-inflammatory markers (*PPARG*, *FABP4*)^{25,26}. Similar gene sets were observed for the RGS1-lineage (Supplementary information, **Fig. S3c**), except for gene set 5, which exhibited expression of genes involved in chemokine signalling desensitization (*RGS1*), phagocytosis (*AXL*) and ATP clearance (*ENTPDI*)²⁷.

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

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

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

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

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

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

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

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

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

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

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

Finally, we retrieved 22,215 epithelial cells, which we subclustered into 7 distinct clusters (**Fig. 7a, b**; Supplementary information, **Fig. S5e, f**), the largest 3 clusters consisting of secretory, ciliated and hillock lung epithelial cells. The basal population (*KRT5*, *AQP3* and *SPARCL1*), representing stem cell epithelial cells responsible for epithelial remodelling upon lung injury, was significantly enriched in COVID-19 versus non-COVID-19, as well as ionocytes, which is another rare epithelial cell type that regulates salt balance (**Fig. 7c**). There were no significant differences between mild versus critical COVID-19 (**Fig. 7d**). Interestingly, *ACE2* and *TMPRSS2* expression was increased in COVID-19 versus non-COVID-19, with 21% and 2.3% of epithelial cells being positive, respectively (**Fig. 7e, f**). We then assessed in which cells we retrieved sequencing reads mapping to the SARS-CoV-2 genome, identifying 3,773 positive cells from 17 out of 31 COVID-19 patients. Surprisingly, this revealed a higher overall number of reads mapping to lymphoid and myeloid than epithelial cells (**Fig. 7g**). Stratification for each of the 11 SARS-CoV-2 open-reading frames (ORF) using Viral-Track revealed that the RNA encoding for spike protein (S), which interacts with ACE2 during viral entry of the cell, was almost exclusively detected in epithelial cells, which were also the only cells expressing *ACE2* and *TMPRSS2* (**Fig. 7g**). In contrast, the nucleocapsid protein (N), and to a lesser extent the ORF10 and ORF1a-encoding mRNAs were detected in myeloid and lymphoid cells at much higher levels than in epithelial cells (**Fig. 7h**). Further stratification into cell types revealed that neutrophils and to a limited extent also monocytes, contained most reads 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-**
429 **i**). Pathway analysis on differentially-expressed genes revealed IFN-signalling using REACTOME and
430 Response_to_virus using GO for genes upregulated in *N*-containing neutrophils (**Fig. 7k, l**).
431 Notably, amongst the different neutrophil phenotypes, *N* was most strongly enriched in
432 ‘inflammatory mature’ neutrophils expressing calgranulins (**Fig. 7m**). As expected, significantly
433 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**).

A very different scenario was observed in mild COVID-19 (**Fig. 8c-g**). Amongst the numerous interactions between monocytes/macrophages and neutrophils, we noticed interleukin signalling (bi-directional *IL1B*, *IL1A*, *IL1RN/A* signalling, *IL7/IL7R*, *CXCR2/CXCL1* or *CXCL8*), but also *MRC1/PTPR* (phagocytosis) and *LTBR/LTB* (pro-inflammation). Between T-cells and neutrophils specific interactions involved *CCR1/CCL3* or *CCL3L1* (pro-inflammation), *CD2/CD58* (co-stimulatory/immunogenic pathway) and *CD94:NKG2E/HLA-F* (anti-viral immune-surveillance), whereas between epithelial cells and neutrophils, *IL1R/IL1A* or *IL1B* or *IL1R* interactions were most pronounced (which can facilitate productive neutrophil immunity in an immune-controlled/immunogenic context)^{28,33,34}. Numerous interactions were also observed between epithelial cells and monocyte/macrophages: *GAS6* or *PROS1/AXL* (receptor-mediated phagocytosis), *ADORA2B/ENTPD1* (extracellular ATP degradation/suppression), *CD83/PECAM1* (immune activation) and semaphorins interacting with their plexin and NRP receptors (tissue re-modelling and repair). Between epithelial cells and T-cells, we observed mainly co-stimulatory (*CD46/JAG1*, *CD40LG/CD40*, *IL7R/IL7*, *MICA* or *RAET1/1/NKG2D* receptor) and tissue repair interactions (*TGFB1/TGFR2* and *TGFB1/TGFR3*), while amongst T-cells and monocytes/macrophages, there were amongst others, co-stimulatory (*LTA/TNFRSF1A* or *TNFRSF1B* or *TNFRSF14*, *TNFSF10/TNFRSF10B*) or tissue-repair factors (*CSF1/CSFRI*, *TGFR3/TGFB1*, *IL15RA/IL15*), mediators of T-cell homeostasis and cytotoxicity (*FASLG/FAS*) and antiviral immune surveillance (*NKG2D II receptor/MICB* or *MICA*).

474

475 **DISCUSSION**

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

thereby generating data at a much greater resolution. Overall, this allowed us to draw the following key conclusions regarding what distinguishes a critical from a mild COVID-19 disease course:

Firstly, CD8⁺ T-cells exhibited good effector functions along their resident-memory and partially-exhausted lineages in mild COVID-19, while also CD4⁺ T-cells showed increased effector or disease-resolving functions in T_{HI}- and T_{HI17}-lineages. In critical COVID-19, T-cells were highly dysregulated, either failing to differentiate (T_{I7}- and T_{RM}-lineage) or exhausting excessively, thereby leading to metabolic disparities, dysregulation of their immunological interface with myeloid cells and/or a dysregulated chronic hyper-inflammatory phenotype (T_{HI} and T_{EX}-lineage). Notably, we observed that mild *versus* critical COVID-19, not only differed quantitatively in terms of the number of T-cells exhibiting a good T-cell effector function, but also qualitatively, in terms of consistently lower activation levels of the type I and II IFN (anti-viral) signalling pathways (amongst several other pathways). Overall, this showed that T-cells in mild COVID-19, unlike those in critical COVID-19, were cross-talking better with their lung-localised microenvironment thereby facilitating 'ordered' immune reactions capable of resolving, rather than exacerbating, inflammation and tissue repair³⁵.

Secondly, in mild COVID-19 monocytes exhibited a pronounced pro-inflammatory phenotype, but then differentiated into macrophages characterized by anti-inflammatory, pro-phagocytic and antigen-presentation facilitating functions. This suggests that in these patients, macrophages might be immunologically 'silently' cleaning the dying/dead epithelial cells (as well as other immune cells meeting their demise due to inflammation), hence contributing to degradation and dilution of the viral load in COVID-19 BAL. Such pro-homeostatic activity of macrophages is well-established to aid in disease amelioration and inflammation resolution³⁶. In critical COVID-19, monocytes were instead characterized by a chronically hyper-inflamed phenotype with characteristics of an ATP-inflammasome-purinergic signalling-based fibrosis, which can promote worse disease outcome by contributing to development of ARDS. This danger signalling pathway is hypothesized to be part of the chronology of events during SARS-CoV-2 infection, but its genetic footprints have not been documented as we report here³⁷. Considering that fully-differentiated macrophages are much more efficient in clearing large debris or cellular corpses (e.g. infected dead/dying lung epithelia or dead neutrophils) than monocytes or neutrophils, their dysfunction in critical COVID-19 may explain the excessive accumulation of lung epithelial (as well as dead immune cell) debris and alveolar 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 outcome^{48–50}. 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

MATERIALS AND METHODS

Patient cohort, sampling and data collection

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

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

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

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

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

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

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

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

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

Single-cell gene expression analysis

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

including hemoglobin and immunoglobulin genes, as well as T-cell receptor (TRAVs, TRBV, TRDV, TRGV) and B-cell receptor (IGLVs, IGKV, IGHV) genes, before downstream analyses.

scRNA-seq clustering for cell type identification

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

Integration of publicly available datasets and identification of cell subtypes

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

Trajectory inference analysis

The R package Slingshot was used to explore pseudotime trajectories/potential lineages in T- and myeloid cells⁵⁶. The analyses were performed for CD8⁺ and CD4⁺ cell phenotypes separately, with T_{MAIT}⁺, T_{γδ}⁺ and T_{REG}⁺-cells excluded due to their unique developmental origin. For each analysis, PCA-based dimension reduction was performed with differentially expressed genes of each 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 hypeR 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

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

Cell-to-cell communication of scRNA-seq data

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

Quantification and statistical analysis

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

DATA AVAILABILITY

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

ADDITIONAL RESOURCES

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

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AUTHORS CONTRIBUTIONS

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

CONFLICT OF INTEREST

The authors declare no competing interests

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Figure Legends

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

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

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

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

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

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

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

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

highlighted T-cell effector function. **e** Density plots reflecting the number of T-cells along the 3 CD4⁺ T-cell lineages stratified for non-COVID-19, COVID-19 and normal lung. **f** Density plots reflecting the number of T-cells along the 3 CD4⁺ T-cell lineages stratified for mild versus critical COVID-19. **g-h** TCR richness and TCR evenness along the 3 CD4⁺ T-cell lineages comparing non-COVID-19 versus COVID-19 (**g**) and mild versus critical COVID-19 (**h**). **i-j** Gene expression dynamics along the CD4⁺ T_{H1}- (**i**) and T_{H17}-lineage (**j**). Genes cluster into 5 gene sets, each of them characterized by specific expression profiles, as depicted by a selection of marker genes characteristic for each set. Differences in trajectories were assessed by Mann-Whitney test. For CD4⁺ T_{H1} and CD4⁺ T_{SCM}: COVID-19 versus non-COVID-19 and lung normal ($P = 1.4E-6$ and $5.9E-37$), For CD4⁺ T_{H17}: COVID-19 versus non-COVID-19 ($P = 9.7E-12$), mild versus critical COVID-19 ($P = 1.3E-121$).

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

a Subclustering of myeloid cells into 9 phenotypes, as indicated by the color-coded legend. **b** Heatmap showing myeloid cell phenotypes with corresponding functional gene sets. **c** Relative contribution of each cell type (in %) to COVID-19 versus non-COVID-19 BAL. **d** Relative contribution of each cell type (in %) to mild versus critical COVID-19 BAL. **e** Pseudotime trajectories for myeloid cells based on Slingshot, showing the common branch of FCN1^{hi} monocytes differentiating into either RGS1^{hi} monocyte-derived macrophages (RGS1^{hi}-lineage) or FABP4^{hi} tissue-resident alveolar macrophages (alveolar lineage). **f** Profiling of marker genes along these trajectories to confirm their functional annotation: *FCN1*, *SI00A12*, *CCL2*, *CCL18* for the common branch, *FABP4* and *PPARG* for the alveolar lineage, *RGS1* and *GPR183* for the RGS1-lineage. **g** Density plots reflecting the number of myeloid cells along the 2 lineages stratified for non-COVID-19 versus COVID-19. **h** Density plots reflecting the number of myeloid cells along the 2 lineages stratified for mild versus critical COVID-19. **i** Gene expression dynamics along the alveolar lineage. Genes cluster into 5 gene sets, each of them characterized by specific expression profiles, as depicted by a selection of genes characteristic for each cluster. **j-l** Profiling of IFN type I and II signalling along the 3 CD8⁺ (**j**) and CD4⁺ (**k**) T-cell lineages, and along the monocyte-macrophage lineage (**l**), comparing mild versus critical COVID-19. All P values were assessed by a Mann-Whitney test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. P values comparing COVID-19 versus non-COVID-19, and mild versus critical COVID-19 for density plots were all $< 10E-50$.

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

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

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

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

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

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

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

Figure 1

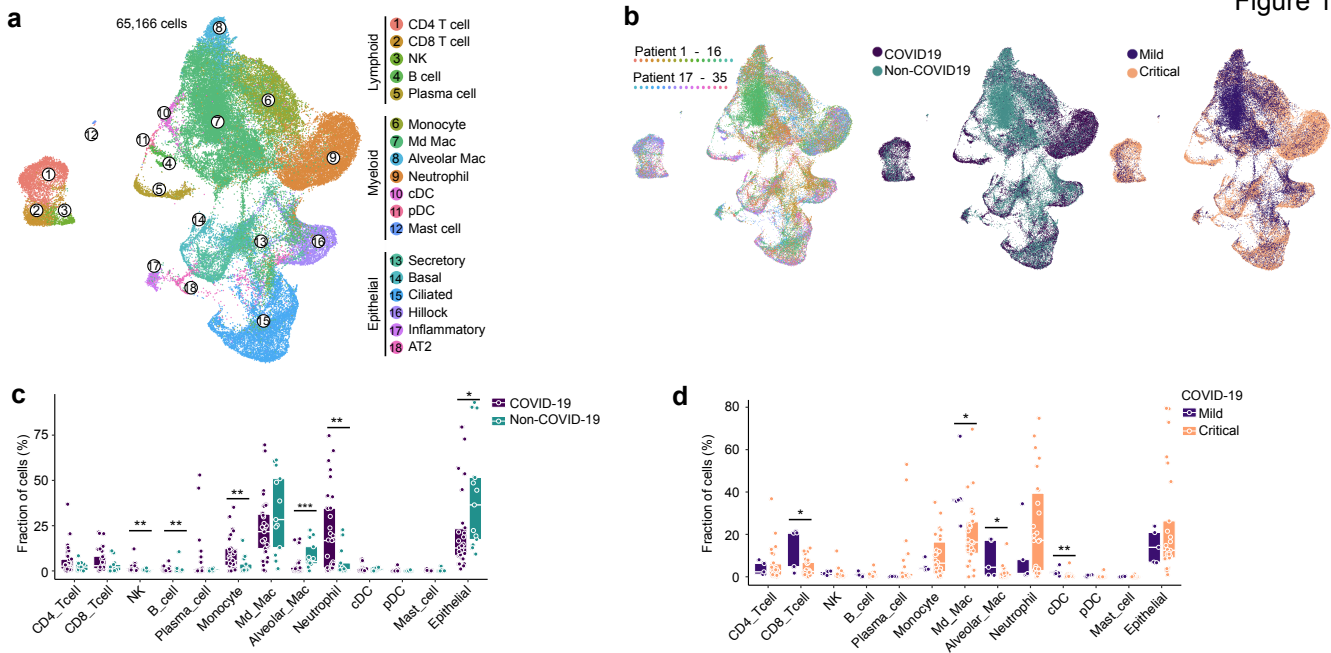


Figure 2

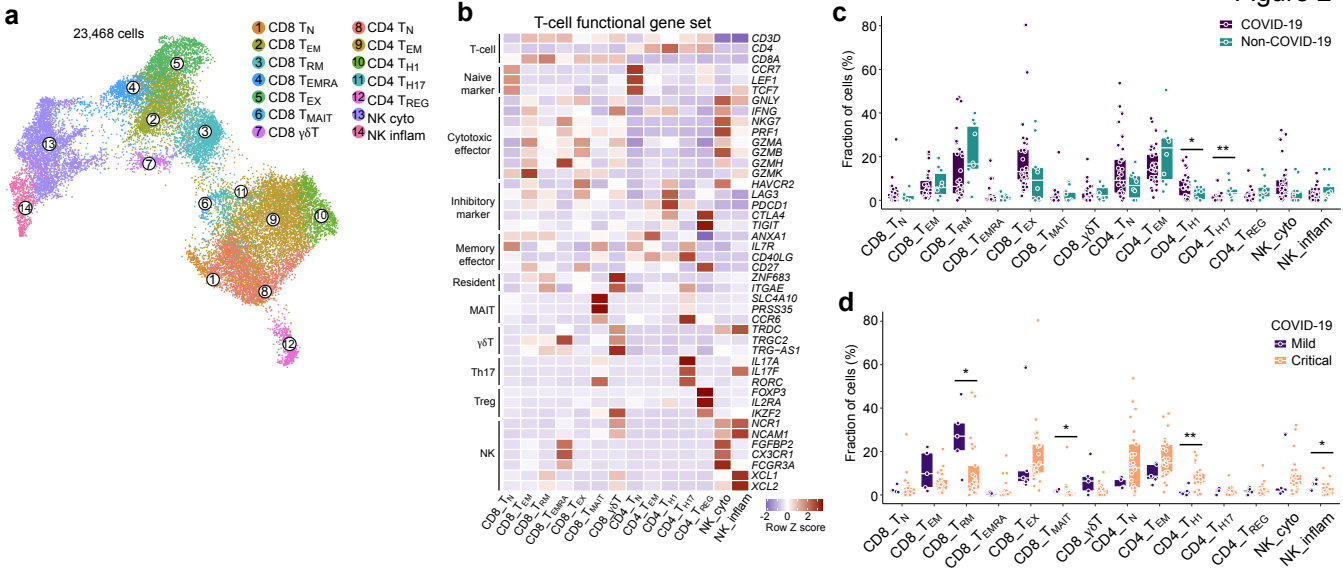
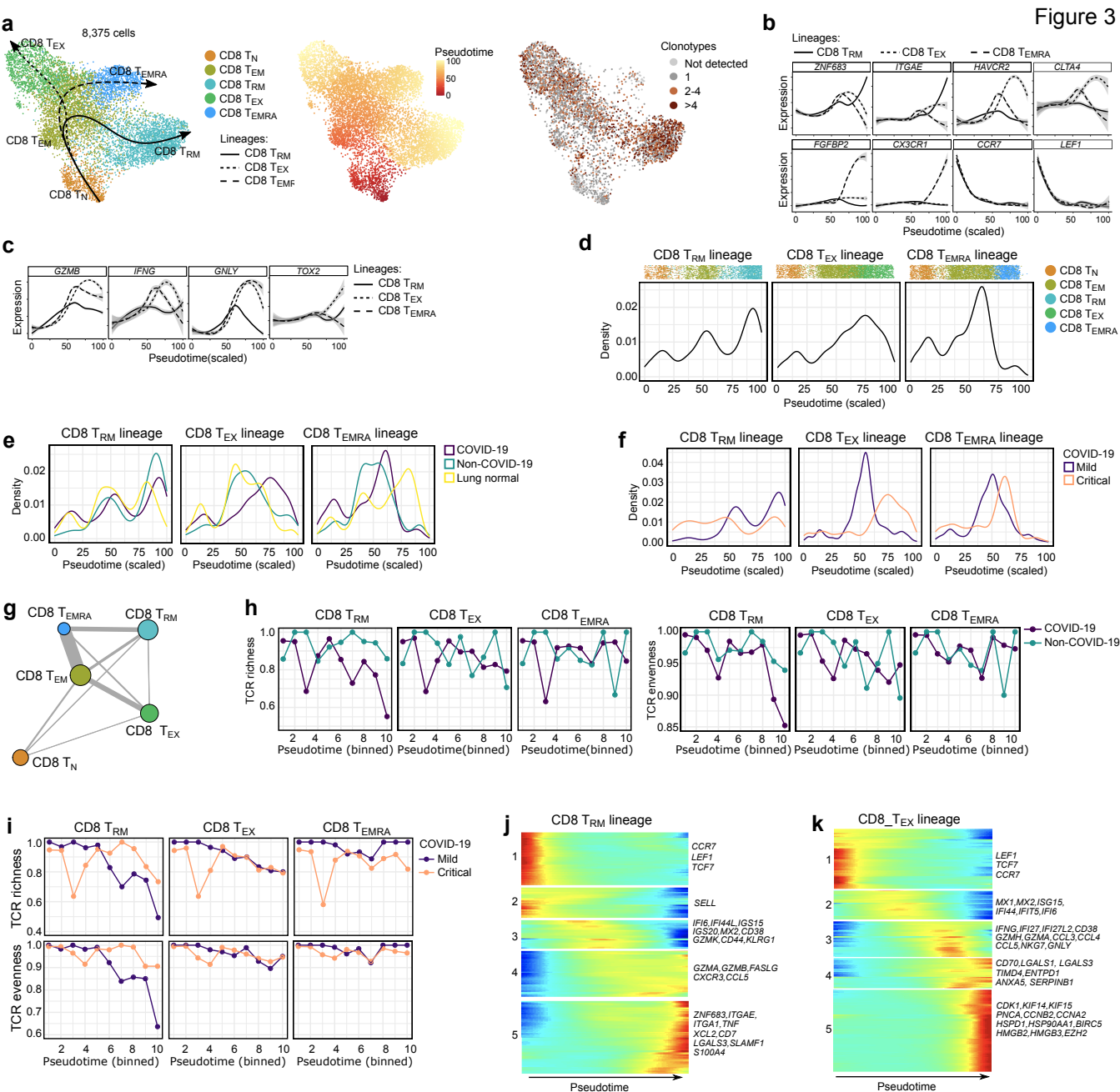


Figure 3



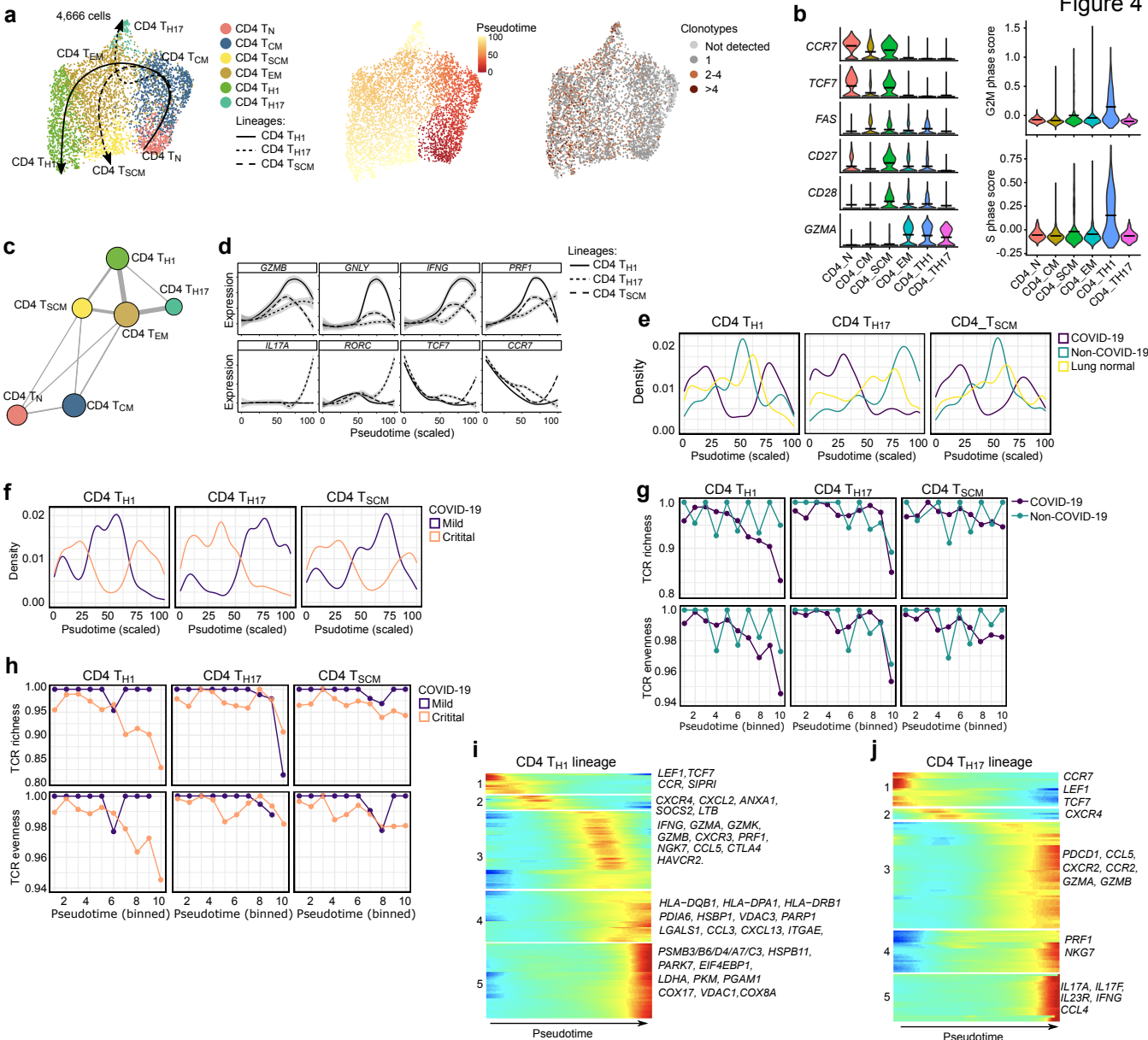


Figure 5

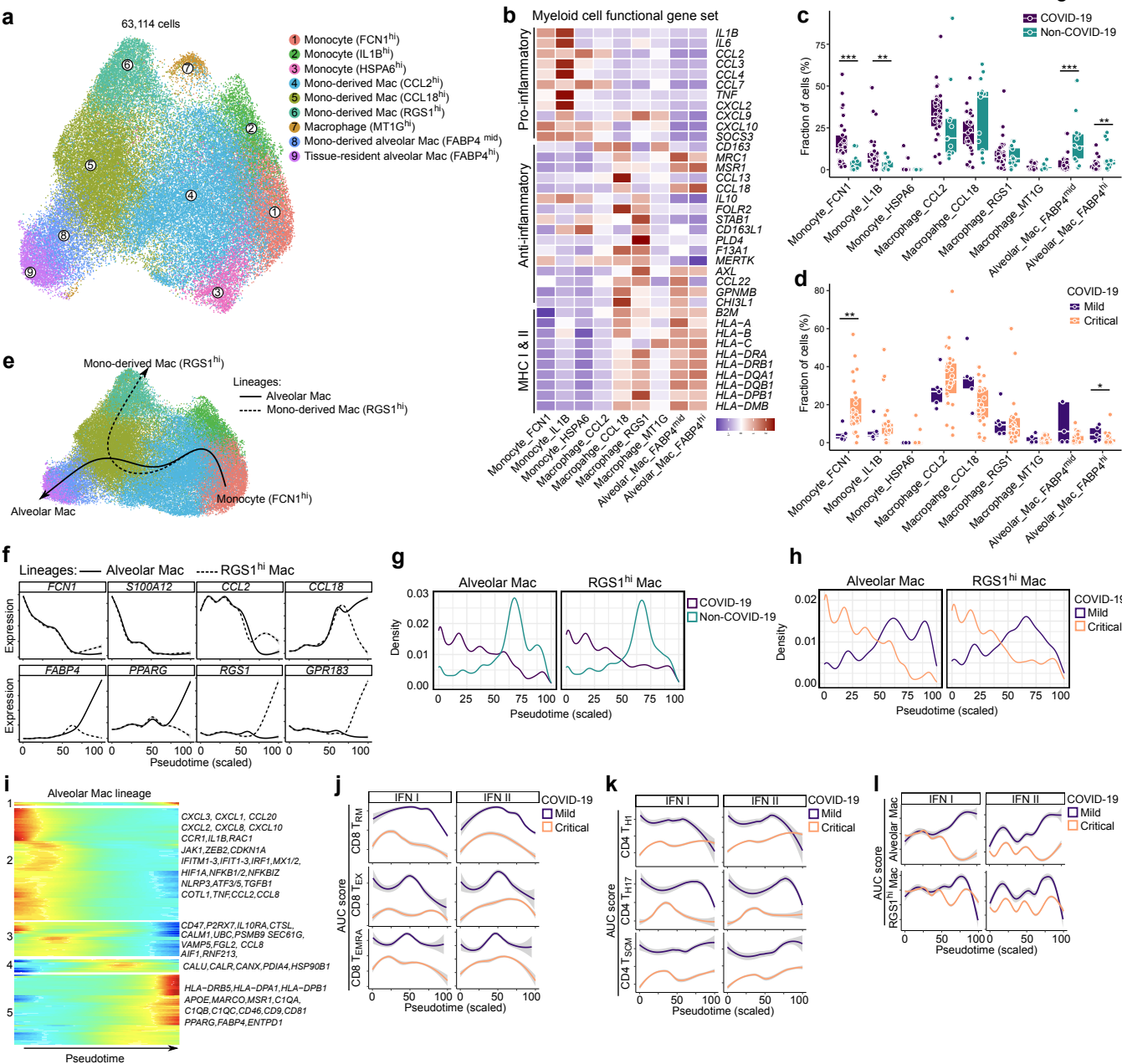


Figure 6

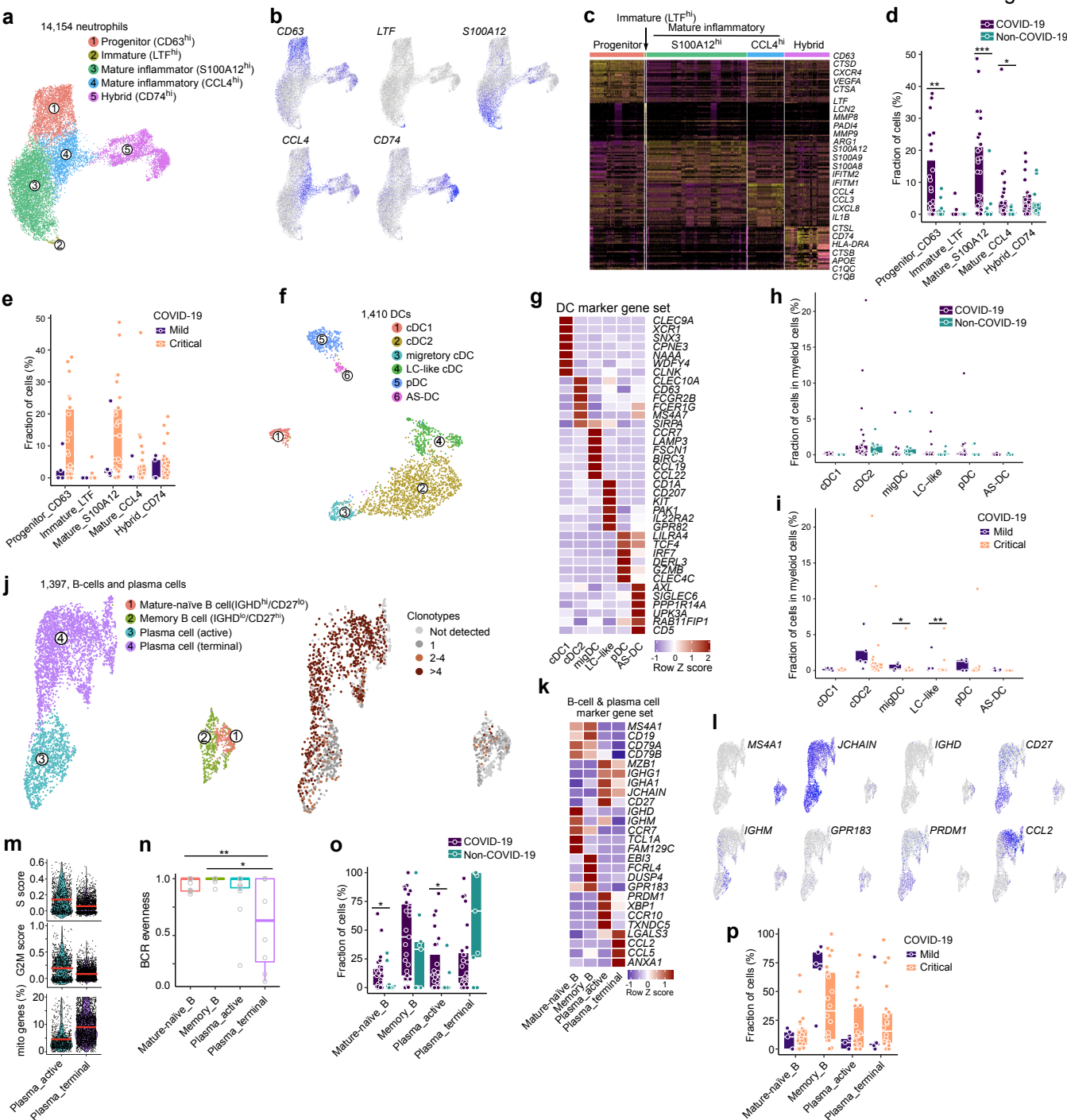


Figure 7

