Tocilizumab treatment in severe COVID-19 patients attenuates the inflammatory storm incited by monocyte centric immune interactions revealed by single-cell analysis

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ABSTRACT

Coronavirus disease 2019 (COVID-19) has caused more than 40,000 deaths worldwide[1]. Approximately 14% of patients with COVID-19 experienced severe disease and 5% were critically ill[2]. Studies have shown that dysregulation of the COVID-19 patients' immune system may lead to inflammatory storm and cause severe illness and even death[3,4]. Tocilizumab treatment targeting interleukin 6 receptor has shown inspiring clinical results of severe COVID-19 patients[5]. However, the immune network with Tocilizumab treatment at single cell resolution has not been uncovered. Here, we profiled the single-cell transcriptomes of 13,289 peripheral blood mononuclear cells isolated at three longitudinal stages from two severe COVID-19 patients treated with Tocilizumab. We identified a severe stage-specific monocyte subpopulation and these cells centric immune cell interaction network connected by the inflammatory cytokines and their receptors. The over-activated inflammatory immune response was attenuated after Tocilizumab treatment, yet immune cells including plasma B cells and $CD8^+$ T cells still exhibited an intense humoral and cell-mediated anti-virus immune response in recovered COVID-19 patients. These results provided critical insights into the immunopathogenesis of severe COVID-19 and revealed fundamentals of effectiveness in Tocilizumab treatment.

Keywords : Coronavirus disease 2019 (COVID-19); Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); Tocilizumab; Single-cell RNA sequencing (scRNA-seq); Inflammatory storm; Monocyte

Main

As of Apr 1, 2020, WHO reported 40,598 deaths out of 823,626 confirmed cases infected by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and these numbers are still growing rapidly[1]. Approximately 14% of patients with COVID-19 experienced severe disease, and 5% were critically ill suffered from 49% fatality rate[2], which may be caused by patients' abnormal immune system activation[3,4,6]. Hence, there is an urgent need for researchers to understand how the immune system respond to the viral infections at severe stage and thereby provide effective treatment strategies.

Studies have shown that the inflammatory storm caused by excessive immune responses was associated with the crucial cause for mortality in COVID-19[7,8]. Plasma concentrations of a series of inflammatory cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6[4], tumor necrosis factor α (TNF-α), IL-2, 7, 10 and granulocyte colony-stimulating factor (G-CSF)[9] were increased after SARS-CoV-2 infections. Further investigation demonstrated peripheral inflammatory monocytes and pathogenic T cells may incite cytokine storm in severe COVID-19 patients[4,7]. To calming inflammatory storm, Tocilizumab, which targeting IL-6 receptors and has proved its effectiveness in the treatment of cytokine release syndrome that is severe or life-threatening[10,11], was used in the treatment of severe COVID-19. After receiving Tocilizumab, the body temperature of the patients returned to normal after 24 hours. The concentration of oxygen inhalation was significantly decreased on the 5th day[5]. However, the immune network arousing the inflammatory storm in severe or recovery stage during Tocilizumab therapy at single cell level has not been uncovered.

Here, we profiled the peripheral immune cells of COVID-19 patients using single-cell transcriptome sequencing. We obtained 5 peripheral blood samples from 2 severe COVID-19 patients at 3 consecutive time-points from the severe to recovery stages during Tocilizumab treatment (Fig. 1a). Specifically, the blood samples at severe stage were collected within 12 hours of Tocilizumab was given. The blood samples at recovery stage were collected at the 5th and 7th day after Tocilizumab treatment. The

patients at severe stage had decreased number of lymphocytes, increased percentage of neutrophil and concentrations of C-reaction protein, and increased expression of IL-6 (Supplementary Table 1). Peripheral blood mononuclear cells (PBMCs) were isolated and subjected to single-cell mRNA sequencing (scRNA-seq) using the 10X platform (Fig. 1a, Supplementary Table 2). After filtering low quality cells, we retained a total of 13,289 single transcriptomes of PBMCs. Of these, 4,364 cells were from severe stage and 8,925 were from patients at recovery stage.

To investigate the heterogeneity and differences of PBMCs between COVID-19 patients and healthy controls, we integrated our COVID-19 single cell transcriptomes with the published single-cell profiles of healthy PBMC from the 10X official website[12] and obtained a total of 69,237 cells (See **Methods**) (Fig. 1b-d). We then applied Seurat[13] to normalize and cluster the gene expression matrix, and identified 20 unique cell subsets, which were visualized via uniform manifold approximation and projection (UMAP) (Fig. 1b-d). Cell lineages, including monocytes, $CD4^+$ and $CD8^+$ T, γδT, NK, B, plasma B and myeloid dendritic cells (mDC), plasmacytoid dendritic cells (pDC), platelet and $CD34^+$ progenitor cells were identified based on the expression of known marker genes (Fig. 1e, Extended Data Fig.1a). With that, we delineated the landscape of circulating immune cells in severe COVID-19 patients.

We next explored the distribution of immune cells from the severe, recovery and healthy stage in each cell subpopulation (Fig. 1f, Extended Data Fig.1b). We observed that a monocyte subpopulation (cluster 9) existed only in patients at severe stage. Plasma B cells (cluster 11), effector $CD8^+$ T (cluster 6), proliferative $MKI67^+CD8^+$ T cells (cluster 12) and NK cells (cluster 7) were significantly enriched in patients versus control. However, a number of subpopulations, such as γδ T cells (cluster 8), pDCs (cluster 15) and mDCs (cluster 10 and 19), most monocytes (cluster 2, 13 and 14) existed only in patients at recovery stage and healthy controls, indicating that these cell types gradually become normal after the treatment. No significant differences were observed in $CD4^+$ T (cluster 1 and 4), naïve $CD8^+$ T (cluster 3) and B cells (cluster 5)

in patients versus control.

Monocytes were reported to play a vital role in CAR-T induced cytokine-release syndrome[14] and SARS-CoV-2 infection caused inflammatory storm[4], therefore we explored the features and functions of the monocyte from COVID-19 patients. We detected 1,737 monocytes in patients, 927 from severe stage and 810 from recovery stage, and integrated 9,787 monocytes from health control. The UMAP plot displayed two main clouds of monocytes that were clearly segregated (Fig 2a). One monocyte subpopulation (cluster 9) was almost exclusively consisted of cells from severe stage and others (cluster 2, 13, 14, 17) were dominated by cells from the recovery and healthy stages (Fig 2b), suggesting a severe-stage specific monocyte subpopulation.

We then investigated the expressions of several selected inflammatory cytokines and observed that these genes were all significantly enriched in severe stage-specific monocytes (Fig. 2c, $P < 0.001$, Wilcoxon rank-sum test). To further explore the transcriptional differences among the monocytes' subtypes, we performed a pairwise comparison of the gene expressions in severe, recovery and healthy stages. We obtained 2,335 differentially expressed genes (DEGs) enriched in each stage, within which reported cytokine storm related genes, such as *TNF*[9], *IL10*[9], *CCL3*[9] and *IL6*[4] were found significantly higher expressed in severe stage-specific monocytes (Fig 2d, Supplementary Table 3). In addition, we also discovered a large number of significant and inflammatory related genes that were less reported (Fig 2d, fold change > 2, $P < 10^{-3}$), including chemokine genes *CCL4*, *CCL20*, *CXCL2*, *CXCL8* and *CXCL9*, inflammasome activation associated genes *NLRP3* and *IL1B*, and complement pathway genes *C1QA*, *C1QB* and *C1QC* (Extended Data Fig.2a-c, Supplementary Table 4). These results indicated that this monocyte subpopulation may contribute to the inflammatory storm in severe COVID-19 patients.

We also observed that genes involved in "acute inflammatory response" and "leukocyte chemotaxis" were significantly decreased at recovery and healthy stage (Fig.2e, f, Supplementary Table 5), suggesting that the inflammatory storm caused by

this monocyte subpopulation was suppressed after the Tocilizumab treatment.

Next, we explored the transcription factors (TFs) that may be involved in the regulation of inflammatory storm in monocytes. We used SCENIC[15] and predicted 15 TFs that may regulate genes which were enriched in severe stage-specific monocyte (Fig 2g). By constructing a gene regulatory network, we found 3 of them, namely ETS2, NFIL3 and PHLDA2 were regulating the cytokine storm relevant genes (Extended Data Fig.2d). In addition, we found the expressions of *ETS2*, *NFIL3* and *PHLDA2* and their target genes were enhanced in severe-specific monocyte subpopulation (Fig 2h), suggesting these 3 TFs may regulate the inflammatory storm in monocytes.

Given that monocytes in the severe stage may be involved in the regulation of a variety of immune cell types, we used the accumulated ligand/receptor interaction database[16] CellPhoneDB (www.cellphonedb.org) to identify alterations of molecular interactions between monocytes and all the immune cell subsets (Supplementary Table 6). We found 15 pairs of cytokines and their receptors whose interaction were significantly altered in severe versus recovery and healthy stages (Fig 3a). Consistent with previous study[4], we found monocytes interacted with $CD4^+$ T cells and plasma B cells in patients at severe stage through the ligand/receptor pairs of IL-6/IL-6R. This interaction, together with many other cytokine storm relevant cell-cell communications were then extensively attenuated after the treatment of Tocilizumab (Fig 3b), suggesting that this drug may functioning by effectively blocking the inflammatory storm in severe COVID-19 patients.

In addition, we also discovered many other ligand/receptor pairs involved in a broader spectrum of immune cell communications enriched at the severe stage. For example, TNF-α and its receptors, which connected monocytes with many other immune cells. Others like IL-1β and its receptor, which connected monocytes with $CD8^+$ T cells. Chemokines, such as CCL4L2, CCL3 and CCL4 and their receptors were also found enriched at severe stage. These cytokines and their receptors may serve as potential drug targets to treat COVID-19 patients at severe stage, and some of their

179 inhibitors are undergoing clinical trials in multiple places around the world
180 (Supplementary Table 7). Collectively, these findings illustrated the molecular basis of
181 cell-cell interactions at the peripheral blood of COVID-19 patients, leading to a better
182 understanding of the mechanisms of inflammatory storm of the disease.

183 Robust multi-factorial immune responses can be elicited against viral infection,
184 such as avian H7N9 disease[17,18]. A recent report has found effective immune responses
185 from a non-severe COVID-19 patient[19]. However, it is not clear whether the anti-virus
186 immune response would be affected after Tocilizumab treatment. Therefore, the anti-
187 virus immune responses from the humoral and cell-mediated immunity in COVID-19
188 patients at severe stage were compared with recovery stage and healthy controls. As
189 expected, we found plasma B cells were barely existing in healthy controls (Fig. 4a).
190 By contrast, significantly higher proportion of plasma B cells was exclusively increased
191 in both severe and recovery stages (Fig. 4a, b), suggesting powerful anti-virus humoral
192 immune responses during Tocilizumab treatment.

193 $CD8^+$ T cells are a critical component of cell-mediated immunity against viral
194 infections by killing infected cells and secreting proinflammatory cytokines. To identify
195 the anti-virus immune responses from the cell-mediated immunity during Tocilizumab
196 treatment, we detected 12,121 $CD8^+$ T cells from our analysis. Clustering these cells
197 revealed 3 subtypes: naïve $CD8^+$ T cells (cluster 3), effector $CD8^+$ T cells (cluster 6)
198 and a subset of $CD8^+$ T cells with proliferation characteristics (cluster 12) (Fig. 4c, d).
199 Among them, the cells from the severe patients were mainly distributed in the effector
200 $CD8^+$ T cell cluster (Fig. 4c, d). We then conducted pairwise comparisons to identify
201 DEGs of $CD8^+$ T cells between the severe, recovery and healthy stages (Fig. 4e,
202 Supplementary Table 8). We found that genes enriched in severe stage showed
203 "regulation of cell activation" signatures (Fig. 4f, Supplementary Table 9; $P < 10^{-10}$).
204 Meanwhile, genes involved in "leukocyte mediated cytotoxicity" and "positive
205 regulation of cytokine production" were highly enriched in $CD8^+$ T cells from COVID-
206 19 patients at both severe and recovery stage (Fig. 4g, Supplementary Table 9; $P < 10^-$

[5]). We further detected elevated expression of the 108 and 449 genes involved in these GO terms (Fig. 4h, i, Supplementary Table 10). Together, these results demonstrated the critical evidence that robust adaptive immune responses against SARS-CoV-2 infection exist in severe stage and remain after Tocilizumab treatment.

The immune system is crucial to fight off viral infection[20,21]. Recent studies have illustrated that monocytes may be the main cause of exacerbation and even death of COVID-19 patients through inflammatory storms[4]. In this study, we discovered a specific monocyte subpopulation that may lead to the inflammatory storm in patients at severe stage through single-cell mRNA sequencing. By analyzing the monocyte-centric ligand/receptor interactions, we revealed a severe stage-specific landscape of peripheral immune cell communication that may drive the inflammatory storm in COVID-19 patients. With that we obtained a list of cytokine storm relevant ligand/receptors that can serve as candidate drug targets to treat the disease, and provided mechanistic insights of the immunopathogenesis of COVID-19.

There are always questions about whether Tocilizumab treatment may affect the antiviral effect of the body[22,23]. Our single cell profiles illustrated a sustained humoral and cell-mediated anti-virus immune response of COVID-19 patients at both severe and recovery stage. For example, the proportion of plasma B cells with antibody-secreting function were keeping at high levels and the cytotoxicity and cytokine production of effector $CD8^+$ T cells were also remained stable in severe COVID-19 patients after Tocilizumab treatment.

The distributions of NK cells were significantly different in the two patients (Extended Data Fig.1b), and the analysis of gene expression differences did not enrich significant biological functions, therefore we did not discuss them in depth. The normal functions of other cell types, such as γδT cells and DCs, were almost lost under severe conditions, and the contribution of these cells to the progression of the disease requires further investigation.

Methods

Human samples

Peripheral blood samples were obtained from two severe COVID-19 patients. The patient severity was defined by the "Diagnosis and Treatment of COVID-19 (Trial Version 6)" which was released by The General Office of the National Health Commission and the Office of the National Administration of Traditional Chinese Medicine. Patient PZ was defined as a severe patient for his peripheral capillary oxygen saturation (SPO2) <93%. Patient PZ provided 2 blood samples at severe stage (Day 1) and recovery stage (Day 5). Patient PW was defined as critical ill for respiratory failure, multiple organ dysfunction (MOD) and SPO2 <93% under high flow oxygen (50 L/min, FIO2 50%). Patient PW provided 3 blood samples at severe stage (Day 1) and recovery stage (Day 5 and Day 7). All samples were collected from the First Affiliated Hospital of University of Science and Technology of China. Before blood draw, informed consent was obtained from each patient. Ethical approvals were obtained from the ethics committee of the First Affiliated Hospital of the University of Science and Technology of China (No. 2020-XG(H)-020).

Cell Isolation

We collected 2ml peripheral blood each time from the COVID-19 patients. Peripheral blood mononuclear cells (PBMC) were freshly isolated from the whole blood by using a density gradient centrifugation using Ficoll-Paque and cryopreserved for subsequent generation of single-cell RNA library.

Single-cell RNA-seq

We generated single-cell transcriptome library following the instructions of single-cell 3' solution v2 reagent kit (10x Genomics). Briefly, after thawing, washing and counting cells, we loaded the cell suspensions onto a chromium single-cell chip along with partitioning oil, reverse transcription (RT) reagents, and a collection of gel beads that

contain 3,500,000 unique 10X Barcodes. After generation of single-cell gel bead-in-emulsions (GEMs), RT was performed using a C1000 Touch^{TM} Thermal Cycler (Bio-Rad). The amplified cDNA was purified with SPRIselect beads (Beckman Coulter). Single-cell libraries were then constructed following fragmentation, end repair, polyA-tailing, adaptor ligation, and size selection based on the manufacturer's standard parameters. Each sequencing library was generated with unique sample index. Libraries were sequenced on the Illumina NovaSeq 6000 system.

Single-cell RNA-seq data processing

The raw sequencing data of patients and health donors were processed using Cell Ranger (version 3.1.0) against the GRCh38 human reference genome with default parameters, and data from different patients and disease stages were combined by the Cell Ranger 'aggr' function. We are uploading the scRNA-seq data of PBMCs from the 2 severe COVID-19 patients to the Genome Sequence Archive at BIG Data Center and the accession number will be available upon request. We also used the scRNA-seq data of PBMCs from 2 healthy donors, which can be downloaded from the 10X genomics official website. Firstly, we filtered low quality cells using Seurat[13] (version 3.1.4). For cells from COVID-19 patients (PZ and PW), we retained cells with detected gene numbers between 500 and 6,000 and mitochondrial UMIs less than 10%. For cells from healthy donors, we retained cells with detected gene numbers between 300 and 5,000 and mitochondrial UMIs less than 10%. Subsequently we normalized gene counts for each cell using the "NormalizeData" function of Seurat with default parameters.

In downstream data processing, we used canonical correlation analysis (CCA) and the top 40 canonical components to find the "anchor" cells between patients and healthy controls. We then used the "IntegrateData" function in Seurat to integrate all the cells. We clustered cells based on the integrated dataset using Seurat with parameter "resolution=0.3" and generated 20 clusters. To display cells in a 2-dimensional space, we ran the principal component analysis (PCA) on the integrated dataset and adopted

the first 50 principal components for the uniform manifold approximation and projection (UMAP) analysis.

Differential expression analysis

To search for the differentially expressed genes (DEGs), we first assign the negative elements in the integrated expression matrix to zero. We used Wilcoxon rank-sum test to search for the DEGs between each pair of the 3 stages of cells (i.e. severe stage, recovery stage and healthy control). We applied multiple thresholds to screen for DEGs, including mean fold-change >2, *P* value <0.001, and were detected in >10% of cells in at least one stage.

We defined stage A specific-DEGs as the intersections between the DEGs in stage A versus stage B and the DEGs in stage A versus stage C. We defined stage A and B common-DEGs as the intersections of the DEGs in stage A versus stage C and the DEGs in the stage B versus stage C, minus the DEGs between stage A and B. In this way, we obtained the specific-DEGs for each stage, and the common-DEGs for each pair of the 3 stages. We then uploaded these DEG groups to the Metascape[24] website (https://metascape.org/gp/index.html#/main/step1), and used the default parameters to perform Gene Ontology (GO) analysis for each stage.

Motif enrichment and regulatory network

We adopted SCENIC[15] (version 1.1.2) and RcisTarget database to build the gene regulatory network of $CD14^+$ monocytes. Since the number of $CD14^+$ monocytes from healthy control (N = 9,618) was more than those from the severe and recovery stages (N = 1,607), to balance their contributions in the motif analysis, we randomly sampled 2,000 $CD14^+$ monocytes from the healthy control for calculation. We selected 13,344 genes that were detected in at least 100 monocytes or included in the DEGs of the 3 stages as the input features for SCENIC. With default parameters, SCENIC generated the enrichment scores of 427 motifs. We used the student's t-test to calculate the *P*

values of these motifs between severe stage and healthy control, and selected severe-specific enriched motifs with fold change >1.5 and *P* value < 10^{-100}.

We then applied the enrichment scores of the severe-specific enriched motifs and the expressions of their targeted genes to Cytoscape[25] to construct a connection map for the gene regulatory network, as shown in Extended Data Fig. 2b. The thickness of line connecting TFs and target genes represented the weight of regulatory link predicted by SCENIC.

Ligand/receptor interaction analysis

To identify potential cellular communications between monocytes and other cell types ($CD4^+$ T, $CD8^+$ T, B, plasma B and NK cells), we applied the CellphoneDB[16] algorithm to the scRNA-seq profiles from the severe, recovery and healthy stages. CellphoneDB evaluated the impact of a ligand/receptor interactions based on the ligand expression in one cell type and its corresponding receptor expression in another cell type. To identify the enriched ligand/receptor interactions in patients at severe stage, we selected the ligand/receptor interactions with more significant (*P* value < 0.05) cell-cell interaction pairs in the severe stage than that in the recovery and healthy stages. We also included ligand/receptor pairs which were highly expressed in severe stage.

Data Availability

We are uploading the scRNA-seq data of PBMCs from the 2 severe COVID-19 patients to the Genome Sequence Archive at BIG Data Center and the accession number will be available upon request. We also used the scRNA-seq data of PBMCs from 2 healthy donors, which can be downloaded from the 10X genomics official website.

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Author Contributions

K.Q. conceived and supervised the project; K.Q., C.G. and J.L. designed the experiments; C.G. and J.L. performed the experiments and conducted all the sample preparation for NGS with the help from H.M. and T.C.; B.L. performed the data analysis with the help from P.C., Q.Y., L.Z., L.J., C.J., Q.L., D.Z., W.Z., Y.L., K.L., X.G. and J.F; T.C., X.W., L.L. and X.M. provided COVID-19 blood samples and clinical information. K.Q., C.G., J.L. and B.L. wrote the manuscript with the help of B.F., H.W. and all the other authors.

Competing interests

Jingwen Fang is the chief executive officer of HanGen Biotech.

Figure Legends

Figure 1 | An atlas of peripheral immune cells in severe COVID-19 patients. a, Flowchart depicting the overall design of the study. Blood draws from patient PZ were performed at 2 time points (Day1 and Day5), and PW at 3 time points (Day1, Day5, and Day7). Patients at Day 1 were at severe stage and Day 5 and Day 7 were at recovery stages. Samples were collected within 12 hours of Tocilizumab was given at Day 1. **b-d**, UMAP representations of single-cell transcriptomes of 13,289 PBMCs. Cells are color-coded by clusters **(b)**, disease stages **(c)**, and the corresponding patient or healthy control **(d)**. Dotted circles represented cell types with > 5% proportion of PBMCs in **(b)** and clusters significantly enriched in patients versus control in **(c, d)**. Mono, monocyte; NK, natural killer cells; mDC, myeloid dendritic cells; pDC, plasmacytoid dendritic

cells. **e**, Violin plots of selected marker genes (upper row) for multiple cell subpopulations. The left column are cell subtypes identified by combination of marker genes. **f**, Bar chart showing the proportion of immune cells from the severe, recovery and healthy stage in each cell subpopulation.

Figure 2 | A unique monocyte subpopulation contributes to the inflammatory storm in COVID-19 patients at severe stage. a, UMAP plot showing 4 clusters of $CD14^+$ monocytes and 1 cluster of $CD16^+$ monocyte. Cells are color-coded by clusters. **b**, Bar plot of the proportion of monocytes in cluster 9 at the severe, recovery and healthy stages. **c**, UMAP plots showing the expressions of selected cytokines in all monocytes clusters. **d**, Heatmap of differentially expressed genes (DEGs) in monocytes from pairwise comparison between severe, recovery and healthy stages. **e,f,** Box plot of the average expressions of genes involved in the signaling pathway "Acute inflammatory response" and "Leukocyte chemotaxis" in monocytes from severe, recovery and healthy stages. Center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range; points, outliers; **** represents *P* value < 10^{-100}, student's t-test. **g**, Heatmap of the area under the curve (AUC) scores of expression regulation by transcription factors (TFs) estimated using SCENIC. Shown are the top differential TFs. **h**, UMAP plots showing the expressions of genes *ETS2*, *NFIL3* and *PHLDA2* in monocytes (top) and the AUC of the estimated regulon activity of the corresponding TFs, indicating the degree of expression regulation of their target genes (bottom).

Figure 3 | The monocyte-centric molecular interactions of peripheral immune cells in COVID-19 patients at severe stage. a, Dot plot of predicted interactions between monocytes and indicated immune cell types in the severe, recovery and healthy stages. *P* values were measured by circle sizes. The expression levels of the interacted genes were indicated by colors, scales on the right. **b**, Summary illustration depicting the

cytokine/receptor interactions between monocytes and other types of peripheral immune cells in severe, recovery and healthy stages. Bolder lines indicated predicted enriched ligand/receptor interactions between monocytes and other immune cell types.

Figure 4 | Enhanced humoral and cell-mediated immunity in severe COVID-19 patients. a, UMAP representations of B and plasma B cell clusters from the severe, recovery and healthy stages. **b**, Bar plot of the proportions of plasma B cells in B cell lineage from severe, recovery and healthy stages. **c**, UMAP representations of $CD8^+$ T cell subtypes (left) and the distribution of cells from severe, recovery and healthy stages in each subtype (right). **d**, Dot plot of the expression of *CCR7, PRDM1* and *MKI67* in all $CD8^+$ T cell subtypes. **e**, Heatmap of differentially expressed genes in effector $CD8^+$ T cells from pairwise comparison between the severe, recovery and healthy stages. **f, g,** Bar plots of GO terms enriched in effector $CD8^+$ T cells from the severe stage **(f)** or severe and recovery stages **(g)**. **h, i,** Box plots of the average expressions of genes involved in the signaling pathway "Leukocyte mediated cytotoxicity" and "Positive regulation of cytokine production" in the effector $CD8^+$ T cells from severe stage, recovery stage and healthy controls. Center line, median; box limits, upper and lower quartiles; whiskers, 1.5x interquartile range; points, outliers; **** represents *P* value < 10^{-30}.

Extended Data Figure Legends and Supplementary Tables

Extended Data Figure 1 | Identification of single-cell subpopulations. a, UMAP plots showing the expressions of selected marker genes in all identified cells. **b**, Bar chart showing the percentage of cell subpopulations in different clinical stages of patients and healthy controls.

Extended Data Figure 2 | Features of monocyte subpopulations. a-c, Bar plots of enriched GO terms of genes highly expressed in monocytes at severe stage **(a)**, severe

and recovery stages **(b)**, and healthy controls **(c)**. **d,** Severe stage specific monocyte regulatory network predicted by SCENIC. Transcription factors were shown in rectangles and their target genes in circles.

Supplementary Table 1 | Baseline characteristcs and laboratory findings of COVID-19 patients in this study.

Supplementary Table 2 | Sequencing data quality.

Supplementary Table 3 | DEGs of different stages of CD14 monocytes.

Supplementary Table 4 | GOterms of DEGs of CD14 monocytes.

Supplementary Table 5 | Gene sets of GO terms in Figure 2e and 2f.

Supplementary Table 6 | Interactions of cytokines and receptors in different stages.

Supplementary Table 7 | Drugs for targeting cytokines or their receptors.

Supplementary Table 8 | DEGs of different stages of effector CD8 T cells.

Supplementary Table 9 | GOterms of DEGs of effector CD8 T cells.

Supplementary Table 10 | Gene sets of GO terms in Figure 4h and 4i.

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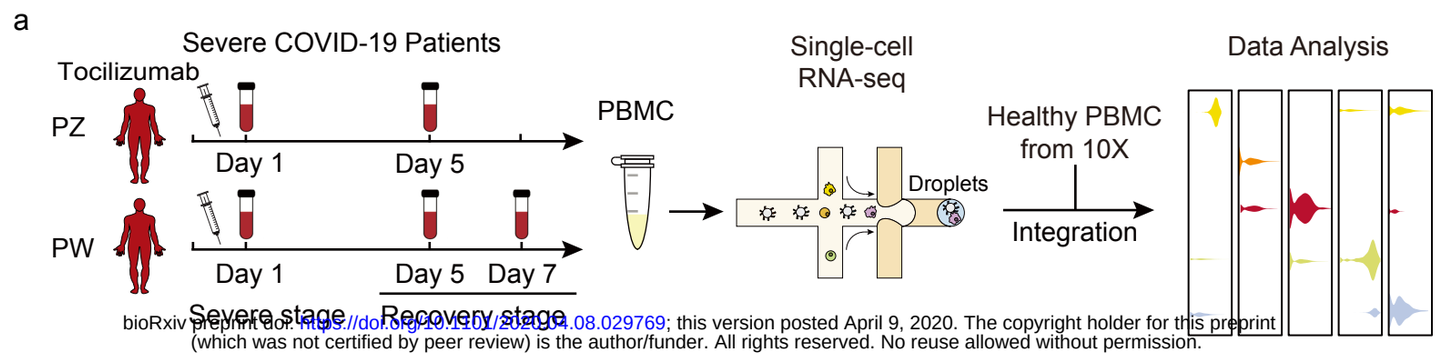
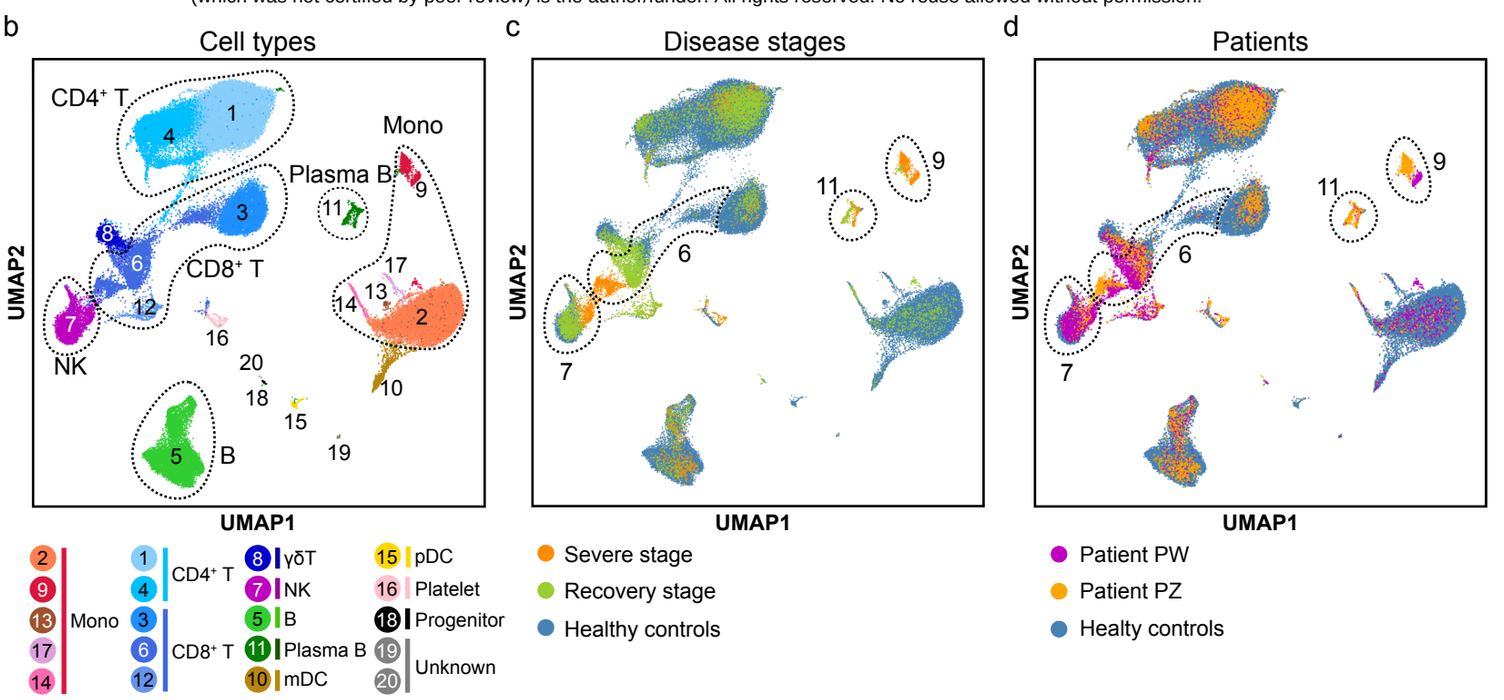
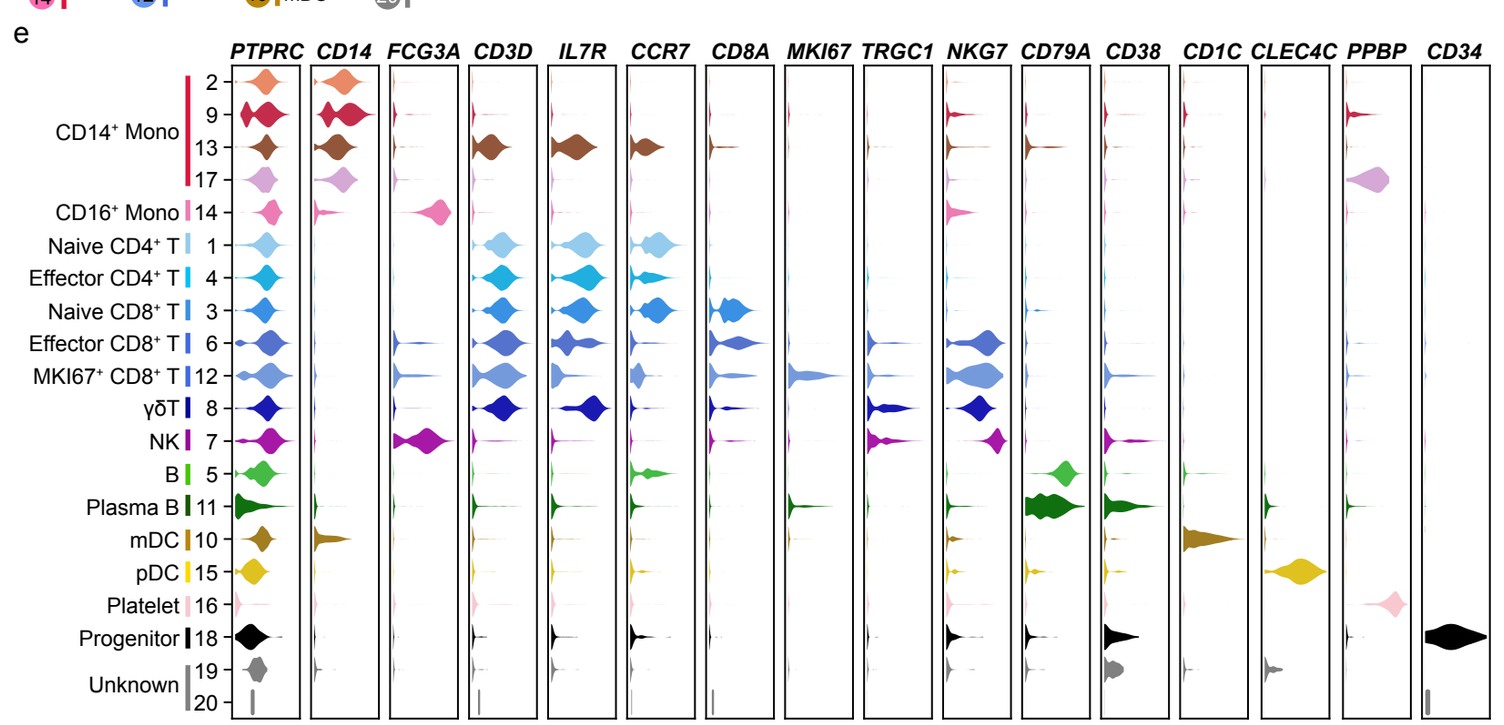
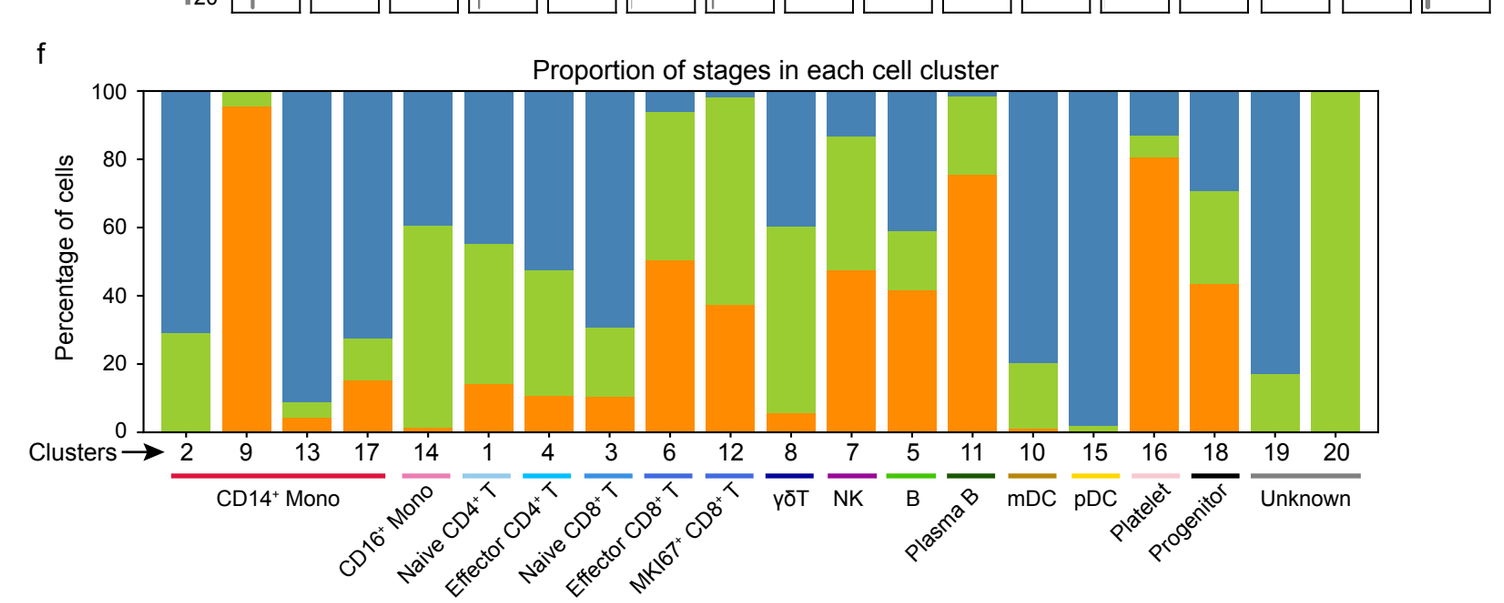
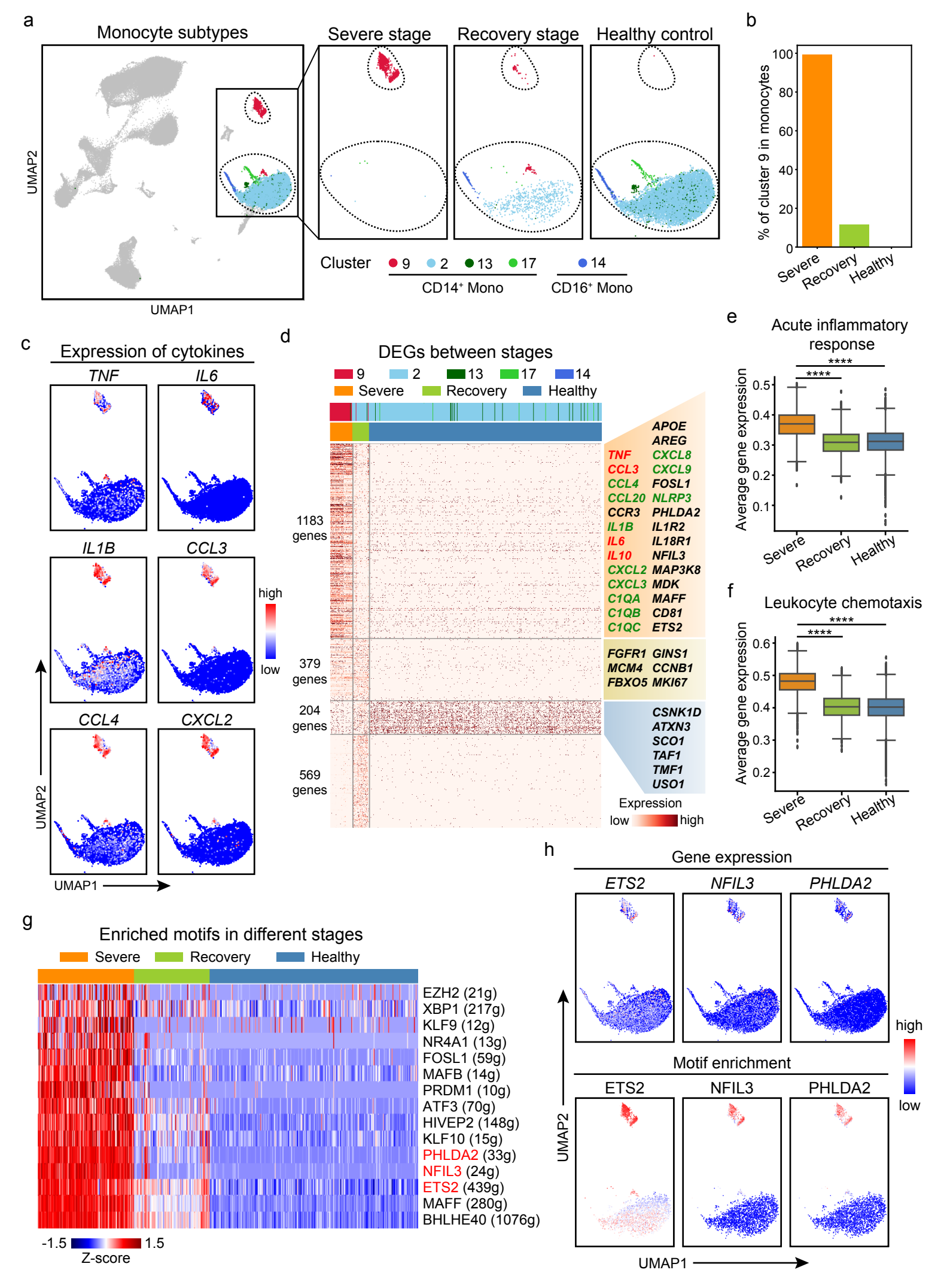
Figure1

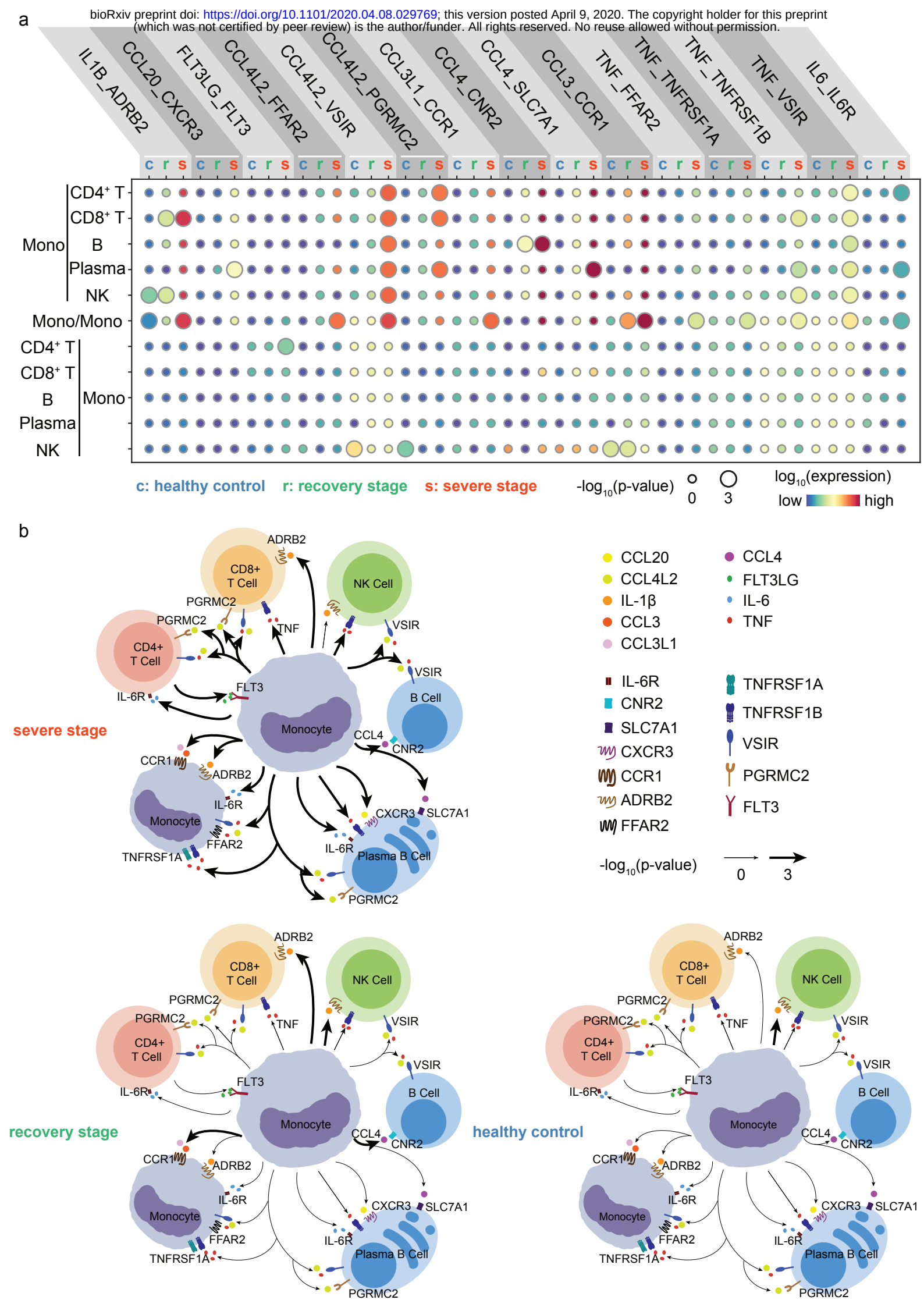


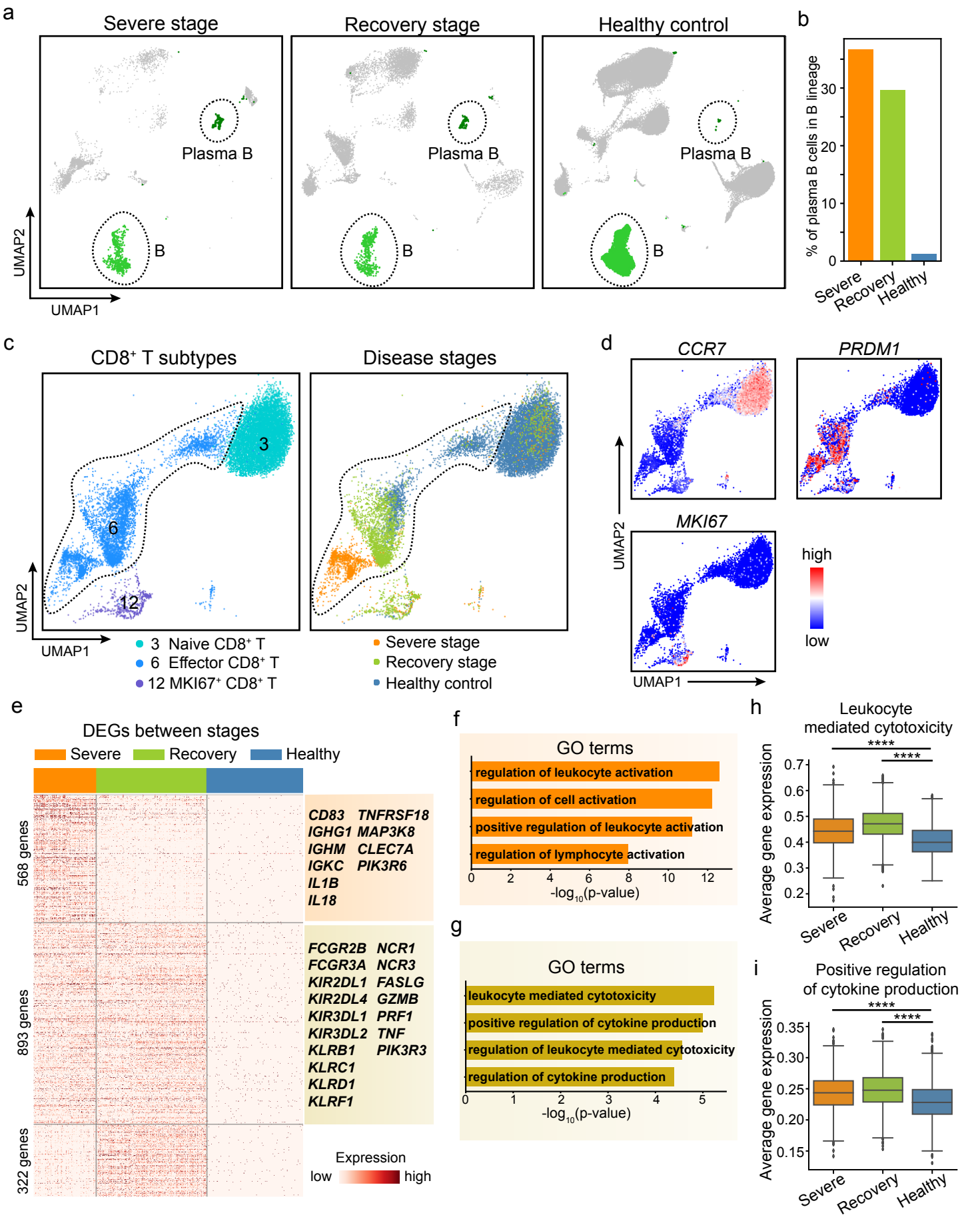







a
Monocyte subtypes
Severe stage
Recovery stage
Healthy control
UMAP2
UMAP1
Cluster 9 2 13 17 14
CD14+ Mono
CD16+ Mono
b
% of cluster 9 in monocytes
100
80
60
40
20
0
Severe
Recovery
Healthy
c
Expression of cytokines
TNF
IL6
IL1B
CCL3
CCL4
CXCL2
high
low
UMAP2
UMAP1
d
DEGs between stages
9 2 13 17 14
Severe Recovery Healthy
1183 genes
379 genes
204 genes
569 genes
APOE
AREG
TNF CXCL8
CCL3 CXCL9
CCL4 FOSL1
CCL20 NLRP3
CCR3 PHLDA2
IL1B IL1R2
IL6 IL18R1
IL10 NFIL3
CXCL2 MAP3K8
CXCL3 MDK
C1QA MAFF
C1QB CD81
C1QC ETS2
FGFR1 GINS1
MCM4 CCNB1
FBXO5 MKI67
CSNK1D
ATXN3
SCO1
TAF1
TMF1
USO1
Expression
low high
e
Acute inflammatory response
****
****
Average gene expression
0.5
0.4
0.3
0.2
0.1
Severe
Recovery
Healthy
f
Leukocyte chemotaxis
****
****
Average gene expression
0.6
0.5
0.4
0.3
0.2
Severe
Recovery
Healthy
g
Enriched motifs in different stages
Severe Recovery Healthy
EZH2 (21g)
XBP1 (217g)
KLF9 (12g)
NR4A1 (13g)
FOSL1 (59g)
MAFB (14g)
PRDM1 (10g)
ATF3 (70g)
HIVEP2 (148g)
KLF10 (15g)
PHLDA2 (33g)
NFIL3 (24g)
ETS2 (439g)
MAFF (280g)
BHLHE40 (1076g)
-1.5
1.5
Z-score
h
Gene expression
ETS2
NFIL3
PHLDA2
high
low
Motif enrichment
ETS2
NFIL3
PHLDA2
UMAP2
UMAP1




a
Severe stage
Recovery stage
Healthy control
Plasma B
B
UMAP2
UMAP1
b
% of plasma B cells in B lineage
0
10
20
30
Severe
Recovery
Healthy
c
CD8⁺ T subtypes
Disease stages
3
6
12
3 Naive CD8⁺ T
6 Effector CD8⁺ T
12 MKI67⁺ CD8⁺ T
Severe stage
Recovery stage
Healthy control
d
CCR7
PRDM1
MKI67
high
low
UMAP2
UMAP1
e
DEGs between stages
Severe
Recovery
Healthy
568 genes
893 genes
322 genes
CD83 TNFRSF18
IGHG1 MAP3K8
IGHM CLEC7A
IGKC PIK3R6
IL1B
IL18
FCGR2B NCR1
FCGR3A NCR3
KIR2DL1 FASLG
KIR2DL4 GZMB
KIR3DL1 PRF1
KIR3DL2 TNF
KLRB1 PIK3R3
KLRC1
KLRD1
KLRF1
Expression
low
high
f
GO terms
regulation of leukocyte activation
regulation of cell activation
positive regulation of leukocyte activation
regulation of lymphocyte activation
-log10(p-value)
g
GO terms
leukocyte mediated cytotoxicity
positive regulation of cytokine production
regulation of leukocyte mediated cytotoxicity
regulation of cytokine production
-log10(p-value)
h
Leukocyte mediated cytotoxicity
****
****
Average gene expression
Severe
Recovery
Healthy
i
Positive regulation of cytokine production
****
****
Average gene expression
Severe
Recovery
Healthy