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RESEARCH ARTICLE

Th1 cytokine endotype discriminates and predicts severe complications in COVID-19

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ABSTRACT. Treatment of severe and critical cases of coronavirus disease 2019 (COVID-19) is still a top priority in public health. Previously, we reported distinct Th1 cytokines related to the pathophysiology of severe COVID-19 condition. In the present study, we investigated the association of Th1 and Th2 cytokine/chemokine endotypes with cell-mediated immunity via multiplex immunophenotyping, single-cell RNA-Seq analysis of peripheral blood mononuclear cells, and analysis of the clinical features of COVID-19 patients. Based on serum cytokine and systemic inflammatory markers, COVID-19 cases were classified into four clusters of increasing (I-IV) severity. Two prominent clusters were of interest and could be used as prognostic reference for a targeted treatment of severe COVID-19 cases. Cluster III reflected severe/critical pathology and was characterized by decreased in CCL17 levels and increase in IL-6, C-reactive protein CXCL9, IL-18, and IL-10 levels. The second cluster (Cluster II) showed mild to moderate pathology and was characterized by predominated CXCL9 and IL-18 levels, levels of IL-6 and CRP were relatively low. Cluster II patients received anti-inflammatory treatment in early-stage, which may have led prevent disease prognosis which is accompanied to IL-6 and CRP induction. In Cluster III, a decrease in the proportion of effector T cells with signs of T cell exhaustion was observed. This study highlights the mechanisms of endotype clustering based on specific inflammatory markers in related the clinical outcome of COVID-19.

Key words: COVID-19, Th1 inflammation, T cell exhaustion

The coronavirus disease 2019 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), remains a serious public health problem.

Acute respiratory distress syndrome (ARDS) and subsequent multiple organ failure (MOF) are the major factors of COVID-19 indicative of poor prognosis both of which may be associated with overreacting immune systems [1]. It has been reported that circulating $CD4^+$ T helper cells play a role in the overreacted immune responses via excessive and uncontrolled secretion of pro-inflammatory cytokines, a process called "cytokine storm" (or cytokine release syndrome) [2-6]. In severe cases, the number of circulating $CD4^+$ T helper cells decreased, probably due to migration and localization in the lungs and other tissues [5, 7, 8]. As such, changes in helper T cells circulating peripherally reflect the pathophysiology after SARS-CoV-2 infection.

Inflammatory responses can be classified into endotypes that are characterized the type of immune effector cells and cytokines. For example, patterns of cytokines and inflammatory markers can be used to assess the impacts of Th1, Th2, and Th17 cells on inflammatory response [9]. Indeed, we previously reported that in patients who suffered from MOF associated with COVID-19, serum levels of Th1 cytokine/chemokines, including C-X-C motif ligand

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9 (CXCL9), C-C motif chemokine ligand 3 (CCL3) and IL-18 and the inflammatory markers IL-6 and C-reactive protein (CRP) were increased. In contrast, in these patients a decrease in the serum levels of the Th2 chemokine C-C motif chemokine ligand 17 (CCL17)/TARC (thymus and activated regulatory chemokines) was observed [1]. These findings suggest that the pathogenicity of severe COVID-19 is associated with abnormal host immune responses [10].

In CD4 T helper cell subpopulations in peripheral blood, different kinetics have been reported in patients with SARS-CoV-2 infection with respect to prognosis [11]. $CD4^+$ T helper cell subpopulations in peripheral blood produce different kinetics have been reported in patients with SARS-CoV-2 infection. In the early phase of COVID-19 infection, several inflammatory cells release pro-inflammatory cytokines such as interleukin (IL)-2, IL-6, TNF-α, and IFN-γ to eliminate viral infected target cells [12, 13]. Th1 chemokine CXCL9 is produced by IFN-γ-stimulated monocytes, macrophages, and endothelial cells, and promotes the chemotaxis of Th1 cells via CXCR3 (14-17). On the other hand, the Th2 immune response is sometime discussed in balance with Th1 inflammation. CCR4 is the marker of Th2 cells and its ligand, C-C motif chemokine ligand 17 (CCL17), represents Th2 inflammation in several diseases [18-20]. However, the kinetics and function of Th2 cells in the later disease complications of a SARS-CoV-2 infection are not fully understood.

In contrast, multiplexed immunophenotyping and single cell RNA-seq analysis demonstrated that activated T cells, Natural killer cells (NK Cell), and monocytes were increased in severe COVID-19 patients [8, 12, 13]. These cellular immunological changes are expected to relate to serum cytokines defined endotypes, and understanding these relationships is of great importance for understanding the severe inflammatory pathogenesis of COVID-19.

Our data demonstrated that a specific endotype with high levels of IL-6, CRP, CXCL9, IL-10, and IL-18 was consistently observed in severe cases along with an inefficient immune response including the depletion of effector T cells and IFN-γ signalling.

METHODS

Study design and participants

This retrospective cohort study enrolled 108 patients with COVID-19 who were admitted to Juntendo University Hospital in Tokyo, Japan, between March 21 and December 24, 2020. Cases with autoimmune diseases, cancer, and chronic infections were excluded. All patients were confirmed to be positive according to PCR-based testing for SARS-CoV-2 using the Light Mix Modular SARS-CoV-2 (COVID-19) N-gene and E-gene assay (Roche Diagnostics, Tokyo, Japan), or the 2019 Novel Coronavirus Detection Kit (Shimadzu, Kyoto, Japan). Clinical information, including complete blood counts (CBC) and blood biochemistry, was obtained from the medical records. CBCs were measured by the Sysmex XE-5000 automated haematological analyser (Sysmex, Hyogo, Japan), and biochemical tests were performed using the Labospect008 (Hitachi High-Tech, Tokyo, Japan). We classified patients into two groups according to the WHO criteria: Group M that included mild and moderate cases and Group S that included severe and critical cases (WHO. Clinical management of COVID-19 https://www.who.int/publications/i/item/clinical-management-of-covid-19).

This study complied with all relevant national regulations and institutional policies, was conducted in accordance with the tenets of the Declaration of Helsinki and was approved by the Institutional Review Board (IRB) at Juntendo University (IRB #20-089, #20-051) and Sysmex Corporation (IRB #2020-265). The donors of PBMC samples for scRNA-seq and flow cytometric analyses provided written informed consent.

Sample processing

Peripheral blood mononuclear cells (PBMCs) were isolated using BD Vacutainer CPT tubes according to the manufacturer's recommendations. The collected PBMCs were suspended in 1 mL of CELLBANKER 1 plus (Nihon Zenyaku Kogyo, Koriyama, Japan), frozen at –80°C, and then used for flow cytometry analysis and 10× single-cell RNA-seq. Serum samples were stored at -80° C and used for cytokine measurements.

Serological testing

Serum levels of IL-6, IL-10, IL-18, CXCL9, CCL3, CCL17, and VEGF were measured using an automatic immune analyser HISCL-5000 and HI-1000(Sysmex Corp., Hyogo, Japan) [1].

Multiple immunophenotyping flow cytometry tests

Isolated PBMCs were utilized for flow cytometric analyses. Briefly, cell frequencies and total cell numbers in PBMC were analysed. Cells were stained for 30 min at room temperature using anti-CD8 allophycocyanin-cyanine 7 (APC-Cy7), anti-CD4 AlexaFluor 488 (AF488), anti-CD19 phycoerythrin-cyanine 7 (PE-Cy7) or AlexaFluor 647 (AF647), anti-CD56 PE-Cy7, anti-CD14 peridinin chlorophyll protein complex-cyanine 5.5 (PerCP-Cy5.5), anti-CD27 phycoerythrin (PE), anti-CD38 PE-Cy7 or APC-Cy7, anti-CD24 PerCP-Cy5.5, anti-IgD AF488, anti-IgM AF647, anti-CD16 PE or AF488, anti-CD69 PE or PerCP-Cy5.5, anti-HLA-DR PerCP-Cy5.5, anti-CD45RO APC, anti-CD94 PE, anti-NKp30 AF647, anti-CD107a APC-Cy7, anti-CCR6 PE, anti-CCR1 PerCP-Cy5.5, anti-CCR4 PE-Cy7, anti-CCR5 AF647, anti-CXCR3 APC-Cy7 monoclonal antibodies (BioLegend). After staining, the cells were measured by flow cytometry on BD FACSCanto™ II and the data analysed using the FlowJo software (TreeStar).

Single cell RNA-seq assay (10× platform)

PBMC suspensions were barcoded through the 10× Chromium Single Cell platform using Chromium Next GEM single cell 5' GEM kit v2 (10× Genomics, CA)

according to the manufacturer's protocol. The loaded cell numbers ranged from 300-500,000 aiming for 300-14,000 single cells per reaction on the chip with the Chromium controller (10× Genomics). Single-cell RNA libraries were prepared and added with unique sample indexes using the library construction kit and the Dual Index Kit TT Set A. The libraries were sequenced using BGISEQ platform.

Single-cell transcriptome analysis

CellRanger 6.0.0 was used to demultiplex raw sequence files to sample-specific fastq files. Reads were aligned to the human reference genome (GRCh38-2020-A) using STAR. Gene expression levels were quantified based on Unique Molecular Identifier (UMI) counts. The filtered gene-cell barcode matrices generated from CellRanger were used for further analysis with the R package Seurat version 4.0.5. We filtered out cells with gene counts below 200 or above 3,000, and mitochondrial gene percentages above 10% to exclude doublets and poor-quality cells. Gene counts were log transformed and scaled. The top 20 principal components were used to perform unsupervised clustering analysis and visualized using UMAP dimensionality reduction (resolution 1.0). Annotation and grouping of clusters to cell type was performed manually by inspecting differentially expressed genes. We also performed reference-based mapping using Azimuth Human PBMC. Various Seurat functions including FindMarkers, FeaturePlot, VlnPlot, and DotPlot were applied. Pathway enrichment analysis was performed using GSEA Molecular Signature Database (MSigDB) and the enrichment score was generated using the tl. score_genes tool in scanpy.

Statistical analysis

Owing to the non-normal distribution of data, data were described in terms of medians and interquartile ranges (IQR). Two-tailed P values <0.05 were considered significant. Fisher's exact test, the Steel–Dwass test, and Mann–Whitney U test were applied using R (r-project) [37]. ROC analysis was performed using the StatFlex software (Artech Co. Ltd., Osaka, Japan). The Wilcoxon test was used to analyse the biomarker changes accompanying disease progression. Unsupervised hierarchical cluster analysis was performed using Cluster 3.0 (University of Tokyo Human Genome Centre). The cluster analysis was performed using a complete linkage based on Euclidean distance.

RESULTS

Patient demographics

A total of 108 adult patients were included in the study (*table 1*). Four patients were admitted to the intensive care unit (ICU), and 104 patients were admitted to non-ICU wards. Four patients received supplemental oxygen therapies, and 4 patients received invasive mechanical ventilation. Forty-one patients received steroids.

Table 1
Demographics and clinical characteristics of COVID-19 patients.

Age, Median (1Q-3Q)		**51.5**	**(**	**33**	**-**	**67**	**)**
Sex	Male/Female, n/n	68	/	40			
Severity							
	Mild, n (%)	45	(	41.7	)		
	Moderate, n (%)	33	(	30.6	)		
	Severe, n (%)	25	(	23.1	)		
	Critical, n (%)	5	(	4.6	)		
ARDS, n (%)		4	(	3.7	)		
Treatment							
	DEX, n (%)	27	(	25.0	)		
	Favipiravir, n (%)	15	(	13.9	)		
	Remdesivir Veklury, n (%)	11	(	10.2	)		
	Remdesivir, n (%)	3	(	2.8	)		
	Ciclesonide, n (%)	15	(	13.9	)		
	PSL, n (%)	11	(	10.2	)		
	Steroid pulse, n (%)	9	(	8.3	)		
	mPSL, n (%)	2	(	1.9	)		
	Heparin, n (%)	1	(	0.9	)		
	Serum exchange, n (%)	6	(	5.6	)		
	TPPV, n (%)	4	(	3.7	)		
	ICU, n (%)	4	(	3.7	)		

Data are presented as median, (IQR), n (%), or n/N (%), where N is the total number of patients with available data.

The relationship between the severity of COVID-19 and biomarkers

To investigate the feasibility and accuracy of inflammatory markers to predict the prognosis of COVID-19, various cytokines were measured and analysed depending on disease severity (*table 2*). Patients were divided into severe-critical (Group S) and mild-moderate (Group M) groups according to WHO classification criteria. Mild COVID-19 was defined as respiratory symptoms without evidence of pneumonia or hypoxia, while moderate or severe infection was defined as presence of clinical and radiological evidence of pneumonia. In moderate cases, SpO_2 $\geq$90% was observed on room air, while one of the following was required to identify the severe cases: respiratory rate >30 breaths/min or SpO_2 <90% on room air. (COVID-19 Clinical management: living guidance https://www.who.int/publications/i/item/clinical-management-of-covid-19).

The levels of CXCL9, IL-18, IL-6, IL-10, CRP, and blood neutrophil percentages were significantly higher in Group S than in Group M, while levels of lymphocytes, CCL17, and platelet counts were significantly lower in Group S (*table 2*, *table S1*).

To determine the proportion of lymphocyte cell subsets between the two groups, we performed multiple immunophenotyping flow cytometry tests using PBMC samples. The percentages of tissue-resident memory T cells (CD4+, CD69+), tumour necrosis factor α (TNF-α+) producing CD8 T cells (CD8+, TNF-α +), and CD56+ NK cells including mature NK cells (CD56 dim, CD16+, and CD69+) were significantly higher in Group S than in Group M. In contrast, the percentages of total CD8 T cells and CD19 transitional B cells were significantly decreased in Group S compared to Group M (*table S2*).

Classification of endotypes by various inflammatory markers

To examine if cytokine markers can predict severity, we performed model discrimination. *Figure 1A* shows the receiver operating characteristic (ROC) curves for each parameter. The area under the curve (AUC) of IL-10, IL-6, CXCL9, IL-18, CCL17, and CRP for the prediction of severe-critical outcomes ranged between 0.73 and 0.88. The levels of IL-10, IL-6, CXCL9, IL-18, and CRP were higher in Group S than in Group M, while levels of CCL17 was smaller in Group S than Group M. The AUC value of VEGF was 0.63.

To examine combinations of cytokine markers permitting to determine the inflammatory endotypes, a clustering analysis was performed using the cut-off values for IL-10, IL-6, CXCL9, IL-18, CCL17, and CRP (*figure 1A*). Based on this analysis (*figure 1B*), the patients were classified into four groups: Cluster I, negative for almost all tested cytokines and CRP; Cluster II, positive for CXCL9 and negative for IL-6 and CRP; Cluster III, positive for almost all tested cytokines and CRP; and Cluster IV, positive for IL-6 and CRP.

As shown in *figure 1C*, all patients who required ICU admission or mechanical ventilation were classified into Cluster III. Although the majority of patients were admitted within seven days of symptom onset, Cluster III patients were admitted to the hospital significantly later than Cluster I patients. Of the 24 patients in Cluster III, 18 patients (75%) were treated with steroids including steroid pulse (n=8), and 5 patients (21%) received plasmapheresis (*figure S1*).

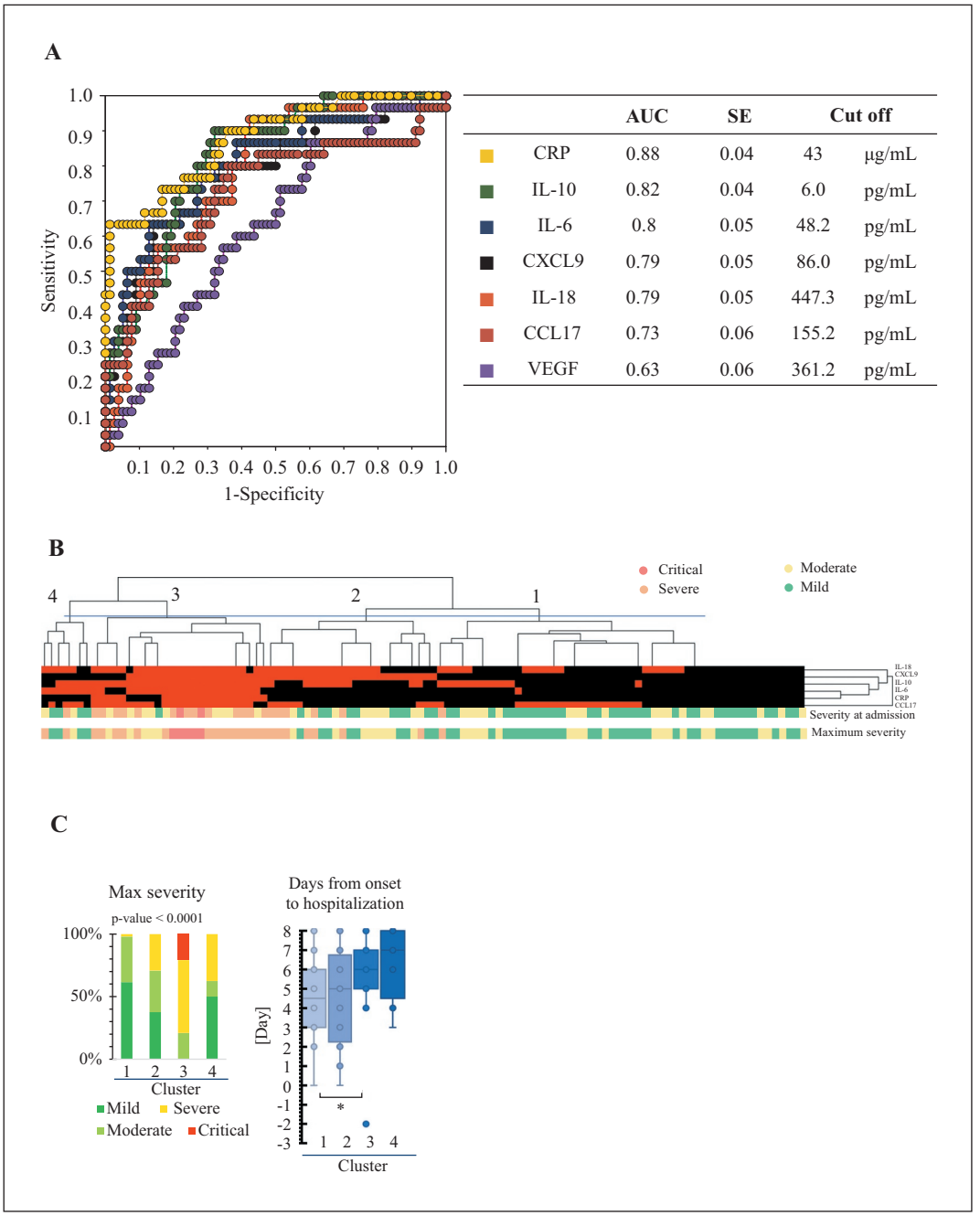
Figure 2 further demonstrates that CXCL9 and IL-18 levels in Cluster II were significantly higher than those in Cluster I. CXCL9 levels in Cluster II were also higher than in Cluster IV. Although, CRP and IL-6 levels were also statistically higher in Cluster II, that levels were not reached to the cut off levels of Figure 1a. Peak values of inflammatory markers were also evaluated among the clusters. The pattern of inflammatory markers was consistent with the pattern at hospital admission. In Cluster II, the levels of CRP, IL-6, and CXCL9 were higher than those on admission.

Haematological parameters including lymphocyte%, red blood cell (RBC) counts, and haemoglobin (HGB) were significantly decreased, and neutrophil%, immature granulocyte (IG)%, and red cell distribution width

Table 2
Biomarkers associated with severity.

	P-value	Median concentrations (Q1-Q3) (N)												
		Mild or Moderate							Severe or Critical					
Age, Year	<0.001	40	(	29	-	57	)	(78)	67.5	(	55.8	-	75.8	) (30)
CXCL9, pg/mL	<0.001	59.3	(	42.1	-	88.5	)	(78)	125	(	86.2	-	300	) (30)
CCL3, pg/mL	0.709	42.5	(	29.7	-	63.3	)	(78)	43.2	(	34.6	-	65.6	) (30)
IL-18, pg/mL	<0.001	416	(	313	-	569	)	(78)	668	(	474	-	832	) (30)
IL-6, pg/mL	<0.001	11	(	5.5	-	26.3	)	(78)	65.1	(	22.1	-	123.7	) (30)
IL-10, pg/mL	<0.001	4.7	(	3.2	-	7.9	)	(78)	10	(	7.6	-	19.8	) (30)
CRP, μg/mL	<0.001	35.0	(	12.3	-	101.5	)	(78)	585.0	(	141.3	-	1139.6	) (30)
VEGF, pg/mL	0.042	487	(	304	-	746	)	(78)	631	(	448	-	882	) (30)
CCL17, pg/mL	<0.001	173.7	(	123	-	237	)	(78)	109	(	79.2	-	148	) (30)

Levels of biomarkers are at the time of hospitalization. Data are presented as median (interquartile range). P-values were calculated using U-test.



	AUC	SE	Cut off	
CRP	0.88	0.04	43	μg/mL
IL-10	0.82	0.04	6.0	pg/mL
IL-6	0.8	0.05	48.2	pg/mL
CXCL9	0.79	0.05	86.0	pg/mL
IL-18	0.79	0.05	447.3	pg/mL
CCL17	0.73	0.06	155.2	pg/mL
VEGF	0.63	0.06	361.2	pg/mL

Figure 1

A) Receiver operating curves (ROC) of CXCL9, IL-18, IL-6 IL-10, CRP, VEGF and CCL17 in detecting Critical or Severe in COVID-19 patient; AUC, Cut-off values calculated by Yoden index are indicated in table. **B)** Unsupervised hierarchical clustering analysis of serum inflammatory marker levels at the hospital admission. The value of inflammatory markers was binarized by the cut-off value; Severe-Critical side (Red) or Mild-Modulate side (Black). The cluster analysis was performed by a complete linkage based on Euclidean distance. Severity of each patient were indicated along the bottom. **C)** maximum severalty observation periods and days from onset to hospitalization were shown. The statistical significances between the clusters were calculated using Fisher's exact test or the Steel–Dwass test.

(RDW-CV) were significantly increased in Cluster III during the observation period (*figure 3A*). Clinical laboratory data, such as lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and blood urea nitrogen (BUN), were significantly higher, and albumin (Alb) was significantly lower in Cluster III, as compared to Cluster I and II. For immune effector cells, multiplexed immunophenotyping revealed that the rate of activated CD4 memory T cells ($CD4^+$, $CD69^+$) significantly increased in Cluster III, whereas the rate of CD8 T cells and Th17 cells ($CD4^+$, $CCR6^+$) decreased (*figure 3B*). Maximum fibrosis score and D-dimer levels during the observation period showed significantly higher values in Cluster III compared to the other clusters (*figure S2*).

Chronological changes of cytokines and inflammatory markers

Next, we analysed the relationship between levels of cytokine markers and disease severity in each cluster using samples collected at various times from disease

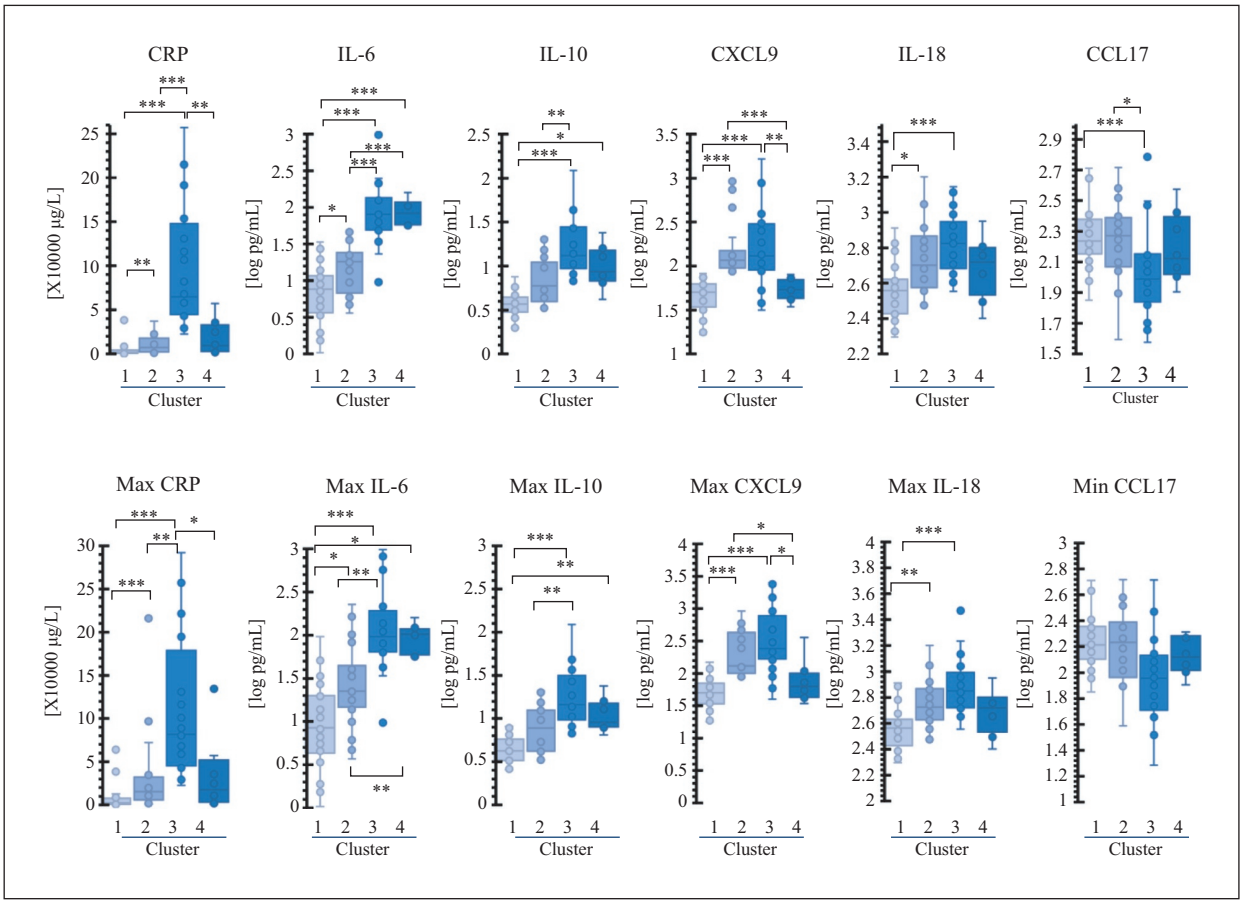


Figure 2

A comparison of patient characteristics in the four identified clusters. Each cluster is indicated on the x-axis. Inflammatory markers, that were used for cluster analysis, at the hospital admission and maximum or minimum value among observation periods are shown. The statistical significances between the clusters were calculated using Fisher's exact test or the Steel–Dwass test. * P<0.05, ** P<0.01, *** P<0.001. .

onset. *Figure 4* shows that, in Cluster III, IL-6 and CRP showed the highest levels upon admission with severe/critical conditions and decreased with time as severity changed to moderate/mild. On the other hand, the levels of Th1 chemotaxis promoting CXCL9 in Cluster II and III remained higher than those in Cluster I and IV throughout all stages.

Figure S3 shows that neutrophil %, AST, and BUN levels were highest upon admission and decreased with time in Cluster III. RBC levels did not recover during the observation period, and increased RDW-CV levels were observed 20 days from disease onset.

Differences in cell-mediated immune response of different severity of COVID-19

To assess more detailed responses of various immune cells in different severities of COVID-19 patient observed in immunophenotyping flow cytometry tests, we then performed single-cell transcriptome analysis with PBMCs obtained from a moderate and a severe patient of COVID-19, using a healthy case as a control (*table S5*). The severe case was characterized by a T cell depletion and a decreased ratio of CD8 effector memory T cells (CD8 TEMs) to central memory T cells (CD8 TCMs), indicating the exhaustion of cytotoxic T cells (*figure 5A, B* and *table S4*). We further observed downregulation of the interferon-γ (IFN-γ) transcriptional pathway and upregulation of hypoxia and TNF-α pathways in the severe case compared to the moderate case (*figure 5C*). IFN-γ gene expression was increased in CD4 cytotoxic T helper cells (CD4 CTLs), effector memory T cells (CD4 TEMs), and NK cells in the moderate case, but this was not observed in the severe case (*figure 5D*). On the other hand, simultaneously increased gene expression of IL-6, IL-10, and IL-18 was observed in CD16 monocytes in the severe case (*figure S4A*). In the severe case, induction of the cell exhaustion markers PD-1 and TIM3 was further observed in CD8 TCMs, CD4, and NK cells (*figure S4B*). The relative proportion of CD4 central memory T cells (CD4 TCMs), known to work on immune tolerance [21], was also increased in the severe case (*figure 5A, B* and *table S4*). Of note, the gene expression of CXCR3, a receptor of CXCL9 that increased in severe and critical patients, decreased in the severe case, prominently in CD4 TEMs (*figure 5E*). This indicates the dysfunctional CXCL9-CXCR3 response in the severe condition.

DISCUSSION

Previous reports indicate that SARS-CoV-2 virulence hinges upon its high infectivity and transmissibility, an aberrant host immune response, and induction of immunosuppressive states such as T-cell exhaustion

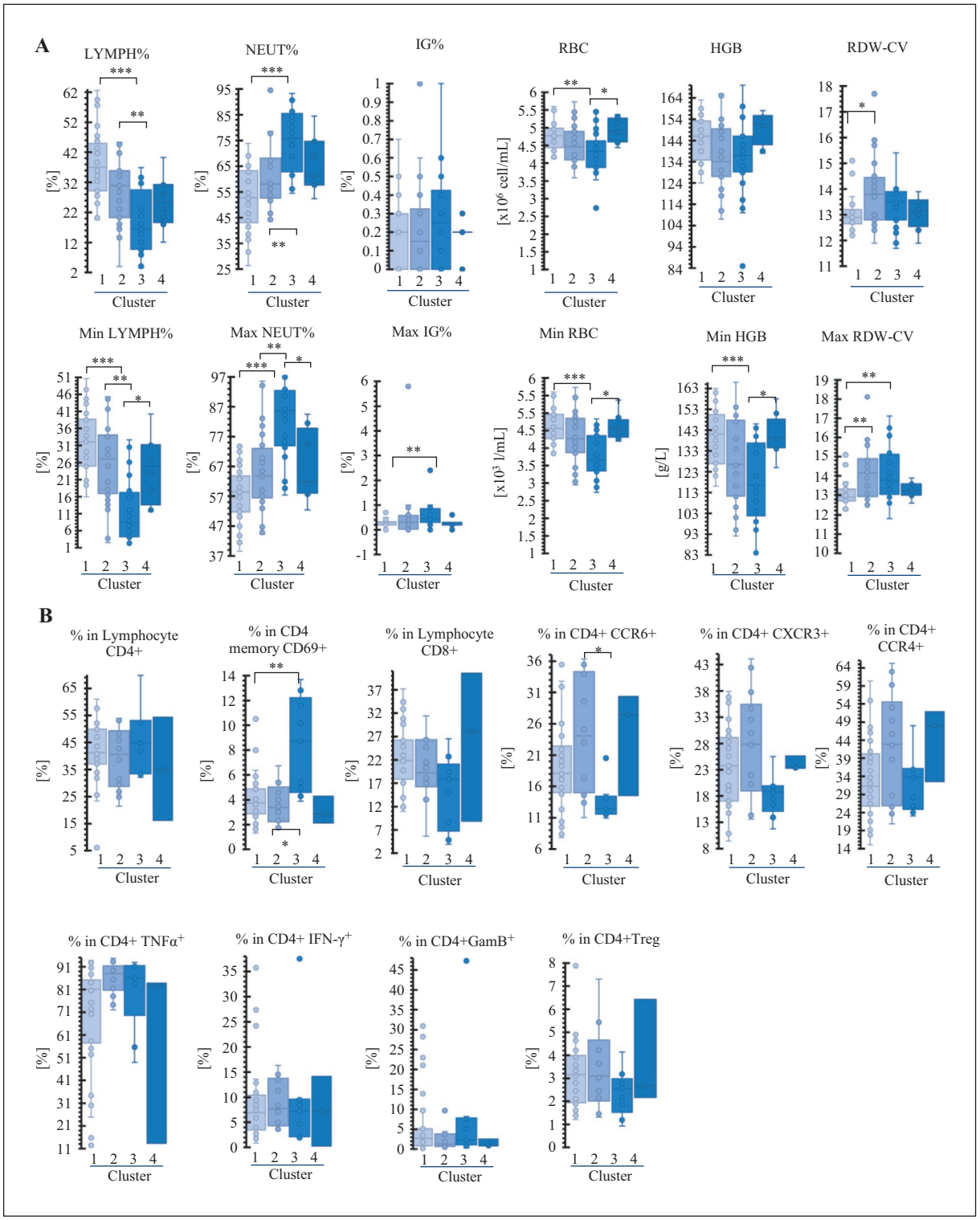


Figure 3

A comparison of patient characteristics in the four identified clusters; Each cluster is indicated on the x-axis. Levels of biomarkers and maximum or minimum value among observation periods were shown. Haematology parameters **(A)**. Lymphocyte subsets **(B)**. The statistical significances between the clusters were calculated using the Steel–Dwass test. * P< 0.05, ** P<0.01, *** P<0.001.

[22]. In this study, we classified the patients with various severity of COVID-19 patients into four endotypes based on the profiles of serum cytokines inflammation markers and compared the prognosis among the groups. Our data demonstrated that the endotype (Cluster III) in which there was significantly high values in CRP, IL-6, and CXCL9 showed the most severe disease.

Clustering

The patients were classified into four clusters based on the profiles of serum cytokines and an acute inflammatory protein CRP. Patients who required ICU admission or mechanical ventilation were classified as Cluster III (i.e., high CXCL9, CRP, and IL-6), which is consistent with our previous findings; the

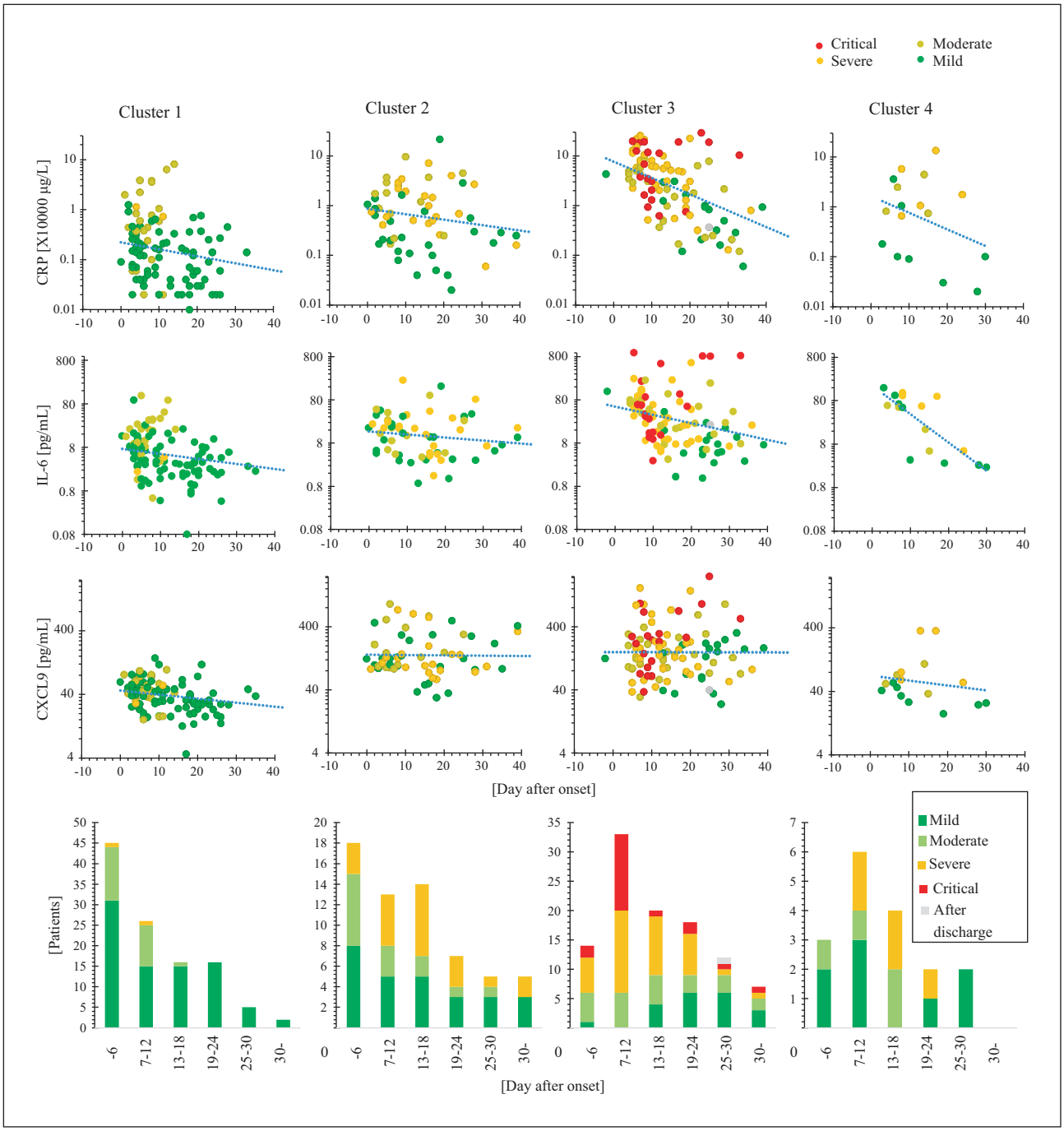


Figure 4

Time course of CPR, IL-6, and CXCL9 after disease onset. Each dot indicates inflammatory marker levels at each sampling point of each patient. Disease severity at each sampling point is indicated by colour. The number of patients by severity are indicated for each period. (bar graph) .

patients with MOF showed high levels of Th1 inflammatory markers along with IL-6 and CRP [1]. In this cluster, CD69-positive Th1 resident memory T (TRM) cells were increased. These cells are known to play an important role in producing IFN-γ, IL-17, IL-18, TNF-α and CXCL9 in viral respiratory infections, such as influenza [23-26].

Multi-cytokine-induced severe pathogenic Cluster III

Blood neutrophil levels were high at the time of hospital admission, and immature granulocyte number increased as the disease progressed. It has been shown that, in severe COVID-19 cases, expansion of peripheral neutrophils potentially suppresses Th1 cells differentiation and triggers Th17 cells polarization [27]. However, we observed that Th17 levels in Cluster III were also significantly lower than in other clusters. These findings suggest the migration of effector cells to the site of inflammation due to a strong inflammatory response.

In severe inflammatory conditions, such as SIRS and myocardial infarction, RDW is known to be increased since reticulocytes increases as a consequence of erythrocyte damage and oxidative stress [28, 29]. Elevated RDW can be associated with an increased mortality in patients with COVID-19 [30]. We observed a RDW increase during the recovery stage,

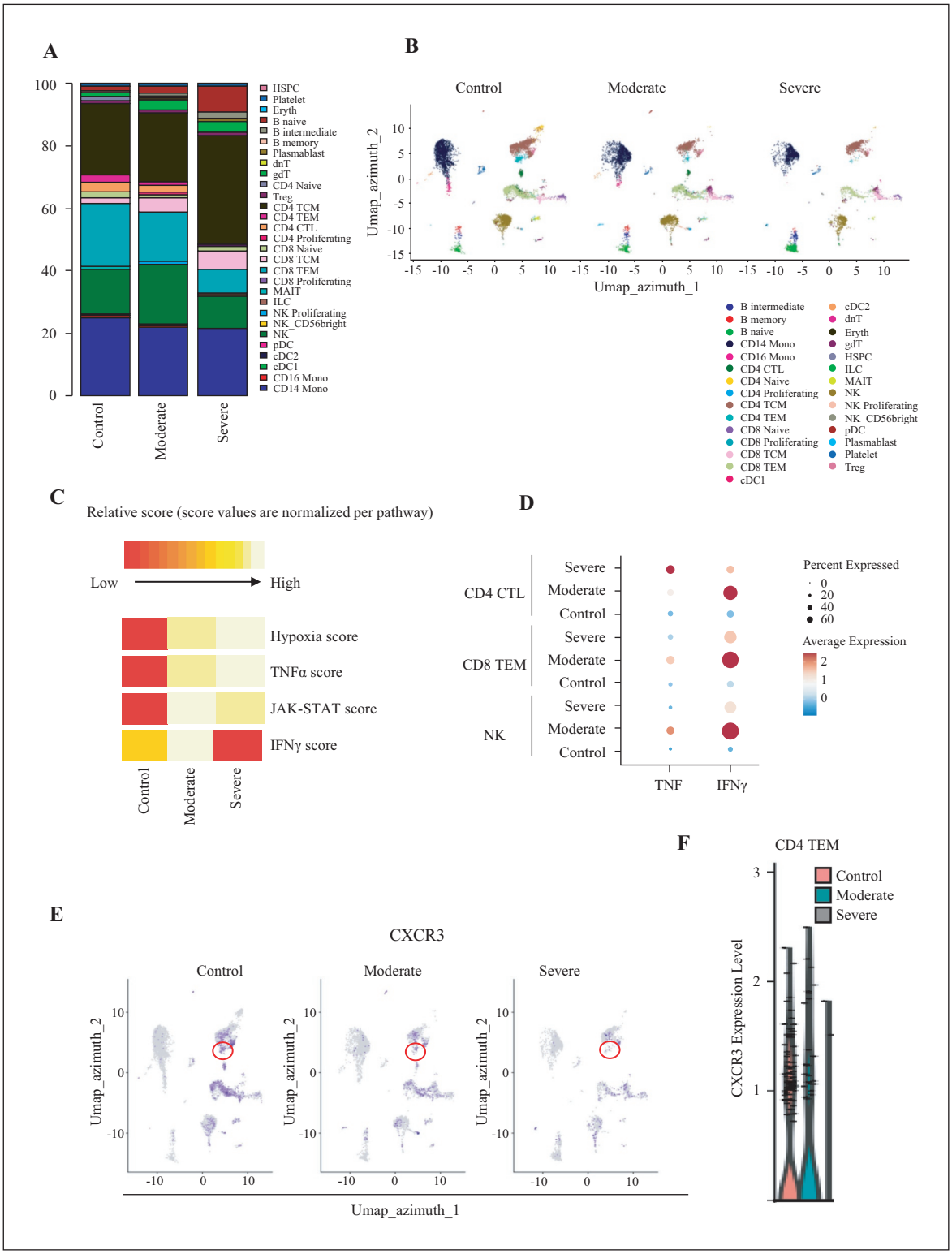


Figure 5

Single cell analysis of PBMCs from individuals with COVID-19 and healthy control.
(A) Bar plot of the proportion of cell types in healthy control and COVID-19 moderate and severe cases. **(B)** UMAP visualization after QC. Leiden clusters based on 5′gene expression shown and coloured by cell types shown in **(A)**. HSPC, hematopoietic stem and progenitor cell; Eryth, Erythrocyte; dnT, double-negative T cell; gdT, γδ T cell; Treg, regulatory T cell; TCM, central memory T cell; TEM, effector memory T cell; CTL, cytotoxic T lymphocyte; MAIT, mucosal-associated invariant T; ILC, innate lymphoid cell; NK, natural killer cell; pDC, plasmacytoid dendritic cell; cDC, classical dendritic cell; mono, monocyte. **(C)** Heat map showing enrichment patterns of Hypoxia, TNF-α, JAK-STAT, and IFN-γ response. **(D)** Dot blots displaying normalized values of TNF-α and IFN-γ in designated clusters in healthy control, COVID-19 moderate and severe cases. **(E)** UMAP visualization shown mRNA expression of CXCR3 (coloured purple) with red circle in the same embedding as UMAP of CD4+ TEM cells; (left). **(F)** Violin plots of CXCR3 mRNA expression (log2) in CD4+ TEM cells;(right).

but RBC remained low even after recovery, suggesting that reticulocytes are released from the bone marrow to the peripheral circulation but with insufficient hematopoietic function.

The chronic exposure to type I IFN could arise a depletion or loss of function of hematopoietic progenitors. Especially, recent work suggests that chronic exposure to IFNα impair the generation of B, NK, myeloid cells, erythrocytes, and platelets [31]. It is possible to understand that our Cluster III patients may be suffered equivalent catastrophic changes due to strong cytokine inflammation of COVID-19.

IFN-γ, a regulator of efficient antigen presentation and stimulator of cytotoxic T-lymphocytes, is mainly produced by CD4 helper T cells (particularly Th1 cells), CD8 killer T cells (CTL), and activated NK cells [32]. Low IFN-γ levels are a risk factor for lung fibrosis in COVID-19 patients, and recombinant IFN-γ therapy has shown favourable response in COVID-19 patients [33, 34]. Indeed, the multiple immunophenotyping in this study showed that the IFN-γ-producing CD4 lymphocytes were decreased in the severe cases predominant in Cluster III compared to Cluster II. The attenuation of IFN-γ production in severe cases was also demonstrated by single cell RNA-Seq analysis with increased expression of T cell exhaustion marker of PD-1 and TIM3 in CD8 TCL and proliferation CD4 and NK cells [35]. On the other hand, the moderate case showed increased IFN-γ score, particularly in CD4 TCL, CD8 TEM and NK cells, along with induction of IFN-γ associated IL-6, IL-10, and IL-18 gene expressions in CD16 monocytes. These results indicate that the pathogenicity of SARS-CoV-2 depends on an ineffective and disproportionate immune response, such as IFN-γ suppression and exhaustion of cytotoxic T cells. [36, 37].

We further demonstrated that CXCL9 is one of the most prominently elevated cytokines in severe and critical COVID-19. Simultaneously, reduction and exhaustion of cytotoxic effector T cell subsets with abrogation of a CXCL9 receptor, CXCR3 expression, and decrease of IFN-γ production were observed in critical disease under hypoxic stress. T cell exhaustion, a dysfunctional state of over-stimulated T cells due to persistent inflammation [12, 35, 36, 38-40], might be an indicator of COVID-19 disease severity.

CXCL9 predominant Cluster II

The high CXCL9 and low IL-6 pattern seen in Cluster II patients was not observed in our previous study. Cluster II patients suffered certainly had more advanced disease than the patients in Cluster I with more severe levels of SpO2, BUN, RDW-CV, and D-dimer, but Cluster II did not included patients who eventually required mechanical ventilation or ICU admission. The ratio of tissue-resident memory T cells ($CD4^+$, $CD69^+$) % and Th17 cells ($CD4^+$, $CCR6^+$) % in Cluster II patients showed no significant difference from Cluster I, while the ratio was significantly higher in Cluster II than Cluster III. These findings indicate that the critical pathological conditions with T cell exhaustion were not fully established in Cluster II.

Most cases in Cluster II were hospitalized by day 6 of onset, and more than 55% received steroid or steroid pulse treatment. In these patients, CRP and % neutrophils peaked shortly after the start of treatment and then recovered. This suggests that these patients were treated before the inflammatory pathology with increased IL-6 was established [41]. We did not find the IFN-γ-producing Th1 predominant cluster such as Cluster II in our previous study performed during of the first wave of March and April 2020, when anti-inflammatory treatments were uncommon [5]. These findings indicate the possible association of anti-inflammatory treatment with the severity-definitive endotype of COVID-19, although the evidence is insufficient to reach definitive conclusions.

In severe cases, high levels of IL-6, CRP, CXCL9, IL-10, and IL-18 were continuously observed along with an inefficient immune response including the depletion of effector T cells and IFN-γ signalling.

Limitations

This study has several limitations. This is a retrospective study from a single hospital using a relatively small number of samples. The samples were not collected at identical time points for all patients, and sampling points of mild cases were fewer than those of severe cases due to shorter hospital admission periods.

In conclusion, we demonstrated that endotype clustering based on specific Th1/Th2 markers is a useful indicator of the severity of COVID-19 complications and subsequent clinical interventions. Endotypes based on cellular and molecular mechanisms may predict the early prognosis permitting the use of more targeted immunomodulatory therapies.

AUTHOR CONTRIBUTIONS

THS established assay system, performed data analysis, and wrote the manuscript. THT, TO, and KI performed data analysis and wrote the manuscript. YM, NI, and KS established assay system and performed data acquisition and analysis. MY established FCM system, performed data acquisition and wrote the manuscript. KY, SY, ET, KN established assay system. AK, FJP, and RO performed data acquisition and wrote the manuscript. TN contributed to sample collection and data interpretation. YT performed data analysis and wrote the manuscript.

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