Adaptive immune responses to SARS-CoV-2 infection in severe versus mild individuals

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The global Coronavirus disease 2019 (COVID-19) pandemic caused by SARS-CoV-2 has affected more than eight million people. There is an urgent need to investigate how the adaptive immunity is established in COVID-19 patients. In this study, we profiled adaptive immune cells of PBMCs from recovered COVID-19 patients with varying disease severity using single-cell RNA and TCR/BCR V(D)J sequencing. The sequencing data revealed SARS-CoV-2-specific shuffling of adaptive immune repertoires and COVID-19-induced remodeling of peripheral lymphocytes. Characterization of variations in the peripheral T and B cells from the COVID-19 patients revealed a positive correlation of humoral immune response and T-cell immune memory with disease severity. Sequencing and functional data revealed SARS-CoV-2-specific T-cell immune memory in the convalescent COVID-19 patients. Furthermore, we also identified novel antigens that are responsive in the convalescent patients. Altogether, our study reveals adaptive immune repertoires underlying pathogenesis and recovery in severe versus mild COVID-19 patients, providing valuable information for potential vaccine and therapeutic development against SARS-CoV-2 infection.

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INTRODUCTION

Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has raised a global health emergency. Worldwide studies have contributed to the characterization, diagnosis, and treatment of the disease.1–4 However, the pathogenesis of SARS-CoV-2 infection in humans remains unclear. Previous studies on severe acute respiratory syndrome (SARS),5 Middle East respiratory syndrome,6 and influenza7 demonstrated that immune changes, especially those in peripheral blood lymphocyte subsets, play a critical role in defense against coronavirus infections. Consistently, several studies of COVID-19 patients showed that both humoral and cellular immunity are involved in the pathogenesis of COVID-19.8–10 Although most COVID-19 patients presented mild-to-moderate symptoms, some infected individuals did develop severe or critical outcomes. However, the immunological features associated with the disease severity remains largely unknown. In addition, earlier studies on the recovery of SARS patients have shown that complete restoration of peripheral lymphocyte may require a longer period.11 Thus, studies of the immune system of convalescent COVID-19 patients will facilitate understanding of their recovery state and establish the relationship between adaptive immune responses and disease severity if it exists.

Here, we collected peripheral blood CD3+ T cells and CD3−CD19+CD20−CD27+ antigen-experienced B cells (AEBCs) from five severe/critical and four mild-to-moderate convalescent COVID-19 patients. These cells were analyzed with single-cell TCR sequencing (scTCR-seq), single-cell BCR sequencing (scBCR-seq), and single-cell RNA sequencing (scRNA-seq). By using these techniques, we found SARS-CoV-2-specific shuffling of adaptive immune repertoires and COVID-19-induced remodeling of peripheral lymphocytes. Characterization of variations in cell composition and functional status of the peripheral T and B cells of the recovered COVID-19 patients showed a more robust humoral immune response and T-cell immune memory in the severe patients (SPs). Data from scRNA-seq and scTCR-seq revealed concomitant responses of three major clusters of memory T cells in the adaptive immune system of the COVID-19 patients. Importantly, functional assays indicate that peptides derived from the M protein of SARS-CoV-2 are active in inducing T-cell response in most of the COVID-19 patients. Altogether, our data not only unveil the adaptive immunological features of convalescent COVID-19 patients but also provide information on the development vaccine and therapeutic agents against SARS-CoV-2 infection.

RESULTS

Study design
To investigate whether immunological memory is established in the SARS-CoV-2-infected individuals, peripheral blood mononuclear cells (PBMCs) were isolated from a cohort of nine convalescent patients of COVID-19 and three age-matched healthy controls (HCs). These cells were further sorted into CD19+CD20− (AEBCs) and CD3+ T-cell populations via flow
cytometry. Listed in Supplementary Table 1 are the demographic and clinical characteristics of the nine COVID-19 donors including the four mild-to-moderate cases (M1–M4), four severe cases (S1, S2, S3, and S5), and one critical case (S4). Diagnosis of SARS-CoV-2 was based on clinical symptoms, exposure history, and chest radiography, and SARS-CoV-2 infection was confirmed by using commercial quantitative PCR with reverse transcription assay on throat swab samples from the respiratory tract. The classification of disease severity for COVID-19 is based on the Chinese Clinical Guidance for COVID-19 Pneumonia Diagnosis and Treatment. The blood samples of these patients were collected about 4 weeks post discharge. Besides the three enrolled healthy donors (H1–H3), two more HCs were acquired from the Single Cell Immune Profiling Datasets of 10x genomics and labeled as H4 and H5. To understand how SARS-CoV-2 infection perturbs B-cell and T-cell immune repertoires, we applied sTCR-seq and sBCR-seq to sequence the V(D)J region of CD3+ T cells and AECBs from 12 donors. After filtering, a total of 50,075 T cells (mean: 3070 cells for five SPs/critical patients, 3762 cells for four mild/moderate patients (MPs), 3936 for five HCs), and 29,899 AECBs (mean: 2454 for SPs, 2652 for MPs, and 1404 for HCs) were obtained. Totally, 39,775 TCR and 21,339 BCR clones were acquired from these cells (Supplementary Table 2). Nonproductive (or nonfunctional) sequences were discarded in the following analysis. Furthermore, to further assess the cellular population composition and functional status of the peripheral T cells and AECBs during the convalescing phase, we also conducted scRNA-seq on the T and AECB cells from all the 12 enrolled donors. In total, 83,816 cells were recovered including 27,216 cells from SPs (mean: 5443 cells), 28,942 cells from MPs (mean: 7236 cells), and 27,658 cells from HCs (mean: 5532 cells) (Fig. 1a). By integrative analysis of single-cell transcriptome and immune profiling, we aim to reveal the adaptive immunological features related to the disease severity in the convalescent COVID-19 patients (Fig. 1a).

T- and B-cell immune repertoires in COVID-19 patients
A direct consequence of SARS-CoV-2 infection is TCR or BCR clonal expansion and expanded memory B-cell clones have been used to screen neutralizing antibodies against SARS-CoV-2. 12 We first investigated the clonal expansion by quantifying the ratio of expanded clones, clonal abundance distributions, 13 and clonal diversity 14 in the whole TCR or BCR repertoires. We observed comparable T-cell clonal expansion in the SPs and MPs (Fig. 1b), whereas B-cell revealed increased TCR clonal abundance (Fig. 1c) and reduced diversity (Fig. 1d) were observed in the SPs. Notably, more expanded BCR clones (Fig. 1e), increased clonal abundance (Fig. 1f), and reduced clonal diversity (Fig. 1g) were present in the SPs compared with the MPs and the HCs. In contrast, no significant BCR clonal expansion was observed in most of the MPs (except for M2 (56 years), the oldest individual in the mild group) (mean: 46 years)) (Fig. 1e). Taken together, these results show the persistence of robust T-cell responses in most of the convalescent COVID-19 patients, and stronger B-cell immune responses to SARS-CoV-2 infection in the SPs than in the MPs.

To further characterize BCR clonal expansion in these patients, we illustrated BASELINYe 15 to the entire Ig sequences to estimate the selection strength in the CDR and FWR regions. The analysis revealed a clear positive selection in the CDR region of the COVID-19 patients but not in the HCs (Fig. 1h), suggesting that the patients’ Ig repertoires were modified to produce high-affinity memory and plasma cells against SARS-CoV-2 infection. An increased frequency of replacement (non-synonymous) mutations occurs in the BCR CDR region during the process of antigen-driven BCR affinity maturation. 16 Indeed, we observed higher mutation frequencies of the top expanded clones in the SPs and MPs compared with the HCs (Fig. 1i). Consistently, higher mutation frequencies were also found in the more expanded clones than in the less expanded clones (Supplementary Fig. 1). IgG and IgA isotypes were overrepresented in the convalescent patients, especially in the severe cases in comparison with the HCs (Fig. 1j). The ratio of (IgA + IgG) to (IgD + IgM) increased with disease severity in these COVID-19 patients (Fig. 1k), suggesting that SARS-CoV-2 induced a more intensive antibody response in the SPs than in the MPs.

Next, to reveal the unique gene patterns and preferences for BCR or TCR in the COVID-19 patients, we compared the usage of V (D)J genes among the participants. We first evaluated the overall immune repertoire differences among them. Principle component analysis (PCA) was used to explore the inter-individual variations based on the usage properties of IGHV genes of BCRs and TRAV or TRBV genes of TCRs. PCA plot showed that clonotype composition of the healthy and the two distinct severity groups of COVID-19 patients are apart from each other (Fig. 2a), suggesting shuffling of the V gene usage preference in the adaptive immune repertoires of the patients. Importantly, the IGHV-gene repertoire was remarkably biased in the SPs (Fig. 2b, c), with IGHV3 family genes including IGHV3-23 (16.8%), IGHV7-7 (10.2%), IGHV3-48 (4.5%), IGHV3-21 (3.3%), IGHV3-11 (2.24%), and IGHV3-15 (1.64%) accounting for ~40% of the whole expanded cohort. Similarly, enhanced usage of the IGHV3 family genes was also observed in human antibodies against other viruses such as cytomegalovirus (CMV), 17 influenza virus, 18 and Ebola virus. 19 Interestingly, IGHV4-34 B-cell clones, rarely present in IgM memory B cells from healthy individuals, 20 were highly represented in one of the severe cases (S2) (Fig. 2c). Moreover, the top two pairing VJ segments IGHV3-23–IGHJ4 and IGHV5-1–IGHJ4 of BCR clones in the SPs appeared SARS-CoV-2 specific 21 (Fig. 2c). When comparing severe group with the mild group, IGHV3-23, IGHV3-48, IGHV1-2, and IGHV4-34 were dominated in severe group (Supplementary Fig. 2). We similarly discerned gene usage preference of TRAV14/DV4, TRAV20, TRAV23/DV6, TRAV25, TRBV13, TRBV14, and TRBV28 in the SPs and MPs (Fig. 2a–c), and some of them (TRAV14/DV4, TRAV20, and TRBV13) were also observed to expand in response to a range of viruses including influenza virus, 21 CMV, 22 and simian immunodeficiency virus (SIV). 23

To identify convergent antibodies for COVID-19, we pooled the BCR data from the 14 individuals together and carried out clonal grouping using Change-O toolkit. 24 based on common genes of IGHV and IGJH and nucleotide similarity of CDR3 sequences. Public antibody sequences present in more than a single donor were identified and extracted for multiple alignment analysis of their CDR3 regions (Supplementary Fig. 3). The data from the analysis revealed a repertoire of public clusters (0.786, 0.6, and 0.92% of total IgA, IgG, and IgM clusters) in the nine COVID-19 patients but not in the five HCs (0.156, 0, and 0% of total IgA, IgG, and IgM clusters) (Supplementary Fig. 4a), presumably due to the infection of SARS-CoV-2. 25 In total, we identified 19 convergent IgG and 25 IgA antibodies shared by the COVID-19 patients (Supplementary Table 3, the human antibodies sequences will be provided upon request), though their SARS-CoV-2 neutralizing activity warrants future investigations. During preparation of the manuscript, a recently published paper reported 26/uniFB01 convergent antibodies of IGHV3-30/IGKV1-39 from two COVID-19 convalescent donors. Next, we used GLIPH 27 to analyze TCRI sequences and grouped them according to the CDR3 sequence similarity. Likewise, we found more public TCR clusters in the SPs than in the MPs or HCs (1.8, 0.62, and 0.66% of total TCR clusters in SPs, MPs, and HCs, respectively) (Supplementary Fig. 4b). Taken together, these results support the notion that severe and mild COVID-19 patients experience distinct humoral and cell-mediated adaptive immune responses.

Characterization of variations in cell composition and functional status of the peripheral T and B cells in recovered COVID-19 patients
To characterize the adaptive immune system of the convalescent COVID-19 patients and understand their recovery state, we performed scRNA-seq analysis on CD3+ T cells and AECBs from
The SPs (S1–S5), MPs (M1–M4), and HCs (H1–H5) using Cell Ranger count pipeline. After quality control, a total of 83,817 cells were obtained for downstream analysis. Using a Louvain clustering algorithm\(^{28}\) and automated reference-based annotation tools (Scibet\(^{29}\) and SingleR\(^{30}\)) combined with expression of canonical genes, we identified ten distinct clusters representing different T-cell subsets and two distinct clusters representing different B-cell subsets (Supplementary Table 4 and Supplementary Fig. 5). Then t-distributed stochastic neighbor embedding (t-SNE) was performed to visualize the cells in 2D space (Fig. 3a). Mucosal
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**Fig. 2** Comparison of V gene usage of IGH, TRA, and TRB among SPs, MPs, and HCs. a Principal component analysis (PCA) for IGHV-gene usage and TRAV or TRBV-gene usage in SPs (S1–S5), MPs (M1–M4), and HCs (H1–H5). b Volcano plot representing differences of IGHV-gene usage in BCR repertoires or TRAV/TRBV-gene usage of TCR repertoires. Positive fold-change values denote more frequent IGHV genes or TRAV/TRBV genes in COVID-19 patients. Genes with a P value < 0.05 are indicated using blue or green stars, respectively. Other known biasedly used gene segments related to virus-specific antibodies are marked using relevant virus name (EBV, EBoV, Flu, CMV, HCMV, and SIV). Genes consistent with another COVID-19 recovered patient study 10 are denoted with a cross.

**Legend**
- **Gene Sets**
  - **Healthy**
  - **Mild**
  - **Severe**
  - **Significant difference**
  - **Gene set enrichment analysis**
  - **Pathway enrichment analysis**
  - **DEG analysis**
  - **Gene pathway enrichment analysis**

**Note:** The significance of this is still unclear, this result indicates that the immune system of COVID-19 patients has not been fully restored at early recovery stage, consistent with the study of recovery of SARS patients. Of note, depletion of MAIT cells was also observed in other viral diseases, such as HIV and HCV.31 In contrast, SARS-CoV-2-induced depletion of V9/V82+ T cells8 was recovered in the convalescent patients (Fig. 3b). In addition, plasmablasts were enriched in the SPs (P = 0.016), further supporting the conclusion that a more robust humoral immune response is stimulated in severe cases compared with the mild cases (Fig. 3b).32

To further characterize the functional status of T cells and AEBCs in the convalescent SPs and MPs, we conducted a differentially expressed gene (DEG) analysis, gene pathway enrichment analysis, and gene set enrichment analysis across the CD4+, CD8+, and memory B-cell subsets. Considering the highly heterogeneous immune responses among the COVID-19 patients,8 we detected differentially expressed immune-related and inflammation-related pathways in every patient compared with HCs in CD4+ T cells, CD8+ T cells, and AEBCs, respectively, and further investigated the upregulated canonical pathways shared by multiple COVID-19 patients (Fig. 3c). As a result, we found that the signaling pathways of "cytokine production" and "leukocyte cell-cell adhesion" are the...
Fig. 3  Characterization of variations in cell composition and functional status of the peripheral T and B cells of the recovered COVID-19 patients. a Two-dimensional t-SNE visualization of 12 major cell types identified from healthy controls (HCs, left), mild patients (MPs, middle), and severe patients (SPs, right) including four for CD8+ T cells, five for CD4+ T cells, one for γδ T cells, and two for B cells. Each dot corresponds to one single cell, and colored according to cell types. The number of total cells showed in each t-SNE map was labeled on the top. b Boxplot representing proportions of each cell type in each sample. Each dot corresponds to one sample, and colored by group-specific color scheme (severe, red gradients; mild, blue gradients; healthy, green gradients). **P < 0.01; *P < 0.05; n.s. not significant by Wilcoxon rank sum test. c Dot plot depicting upregulated canonical pathways in CD4+ T cells, CD8+ T cells, and AEBCs shared by multiple COVID-19 patients. Differentially expressed genes and their enriched pathways were calculated as each COVID-19 patient relative to all healthy controls (S# versus H or M# versus H), and each severe patient relative to all mild cases (S# versus M).
most commonly upregulated in either the CD4+ or the CD8+ T cell sets of the COVID-19 patients (Fig. 3c). The T-cell activation pathway was activated in the CD8+ T cells in three severe cases, whereas the pathways involving antiviral immunity, such as "type I interferon signaling" and "defense response to virus" were highly upregulated in the T cells of S4 and M2. Compared with the HCs and the MPs, the SPs clearly expressed more genes in the pathways of "B-cell-mediated immunity," "complement activation," "humoral immune response mediated by circulating immunoglobulin," and "phagocytosis," indicating a more extensive humoral immune response in the severe cases. This conclusion is further supported by analyses of BCR clonal expansion (Fig. 1e) and plasmablasts enrichment (Fig. 3b). The upregulation of the complement pathway in the SPs suggests a systemic pro-inflammatory response induced by SARS-CoV-2 infection as documented before. 33 Severe COVID-19 patients are normally associated with increased levels of interleukin (IL)-6, IL-10, and tumor necrosis factor-α.34,35 However, substantial expression of pro-inflammatory cytokines such as IL-6, IL-18, IL-2, IL-10, IL1A, IL-8, and CXCL-10 was not detected in most of the profiled T or B cells from all COVID-19 samples (Supplementary Fig. 6), suggesting that (IL)-6, IL-10, and probably other cytokines returned back to normal levels after recovery of these COVID-19 patients. Notably, the mitogen-activated protein kinase (MAPK) pathway (i.e., FOS, JUN, JUNB, and DUSP1) was greatly suppressed in all recovered patients compared with that in the HCs. This is in full agreement with a previous study suggesting that inhibition of the MAPK signaling pathway is a recovery sign of COVID-19 patient (Supplementary Fig. 7). 36

Generation of specific T-cell subsets in response to SARS-CoV-2 To identify SARS-CoV-2-specific T-cell subsets in the convalescent COVID-19 patients, we combined scRNA-seq and scTCR-seq to assess clonal expansion of different T-cell subsets. Full-length TCRs with both alpha and beta chains were obtained from 15,818 T cells of the 10 clusters in COVID-19 patients, including 11,805 harboring unique TCRs and 4013 (25.4%) harboring repeated TCRs, indicative of clonal expansion of T cells in COVID-19 patients. Compared with other T-cell subsets, CD8+ TEM, CD8+ TTE, and CD4+ TTE were substantially clonally expanded in the COVID-19 patients, with more expanded clonotypes containing more CD8+ TEM and CD8+/CD4+ TTE (Fig. 4a and Supplementary Fig. 8). The top 50 TCR clonotypes comprised, on average, 48.67% CD8+ TEM or 75.52% CD8+ TTE cells or 75.76% CD4+ TTE cells in the SPs, whereas a lower copy number of these clones existed in the HCs (Fig. 4b). These clonally expanded T cells may represent SARS-CoV-2-specific CD8+ or CD4+ T cells. Interestingly, the clonally expanded T cells showed an aggregative distribution on the t-SNE map (Fig. 4c), indicating transcriptional homogeneity in the COVID-19 patients and further supporting the idea that they are SARS-CoV-2 specific. Altogether, our data indicate concomitant response of CD8+ and CD4+ T cells in the adaptive immune
system of the COVID-19 patients, and the clonally expanded CD8+ effector cells in the patients may ultimately develop into long-lived memory T cells. These data are further supported by a recent study showing a robust adaptive immune response in mild COVID-19 patients. Interestingly, MAIT clones were not expanded in the COVID-19 patients but were preferentially amplified in the healthy donors, though the significance of this remains unclear.

To experimentally validate the SARS-CoV-2 specific T-cell immunity in the COVID-19 convalescent patients, we synthesized 276 potential T-cell epitope peptides from all the 29 proteins of SARS-CoV-2.37–39 With 210 corresponding to HLA class I CD8+ T-cell epitopes and 66 to HLA class II CD4+ T-cell epitopes (Fig. 5a). These peptides were divided into ten groups (as described in Supplementary Table 5) to stimulate PBMCs followed by interferon gamma (IFN-γ) ELISpot analysis. IFN-γ-secreting T cells from each donor were detected and counted. A donor was considered positive to a peptide group when there was a more than twofold increase in the numbers of IFN-γ-secreting T cells in the peptide-treated sample compared with the unstimulated control. At this cutoff point, we found that all recovered patients demonstrated specific memory T-cell responses against at least one group of the SARS-CoV-2 peptides (Fig. 5b). IFN-γ-secreting T cells in response to the S and the M peptides were detected in all SPs and most MPs (75% and 50%). Interestingly, the S peptides stimulated a markedly higher percentage of IFN-γ-secreting cells in both SPs and MPs than the other types of viral peptide. Each of the SPs demonstrated T-cell responses (mean: 12-fold increase) against at least five viral proteins, whereas most of the MPs (75%) only responded to the S protein (mean: 3.46-fold). Taken together, the ELISpot data indicate that the memory T cells specific for the S and other proteins were generated and sustained in the COVID-19 convalescent patients, which is further supported by a recent study.40 Importantly, we found that the SPs exhibited stronger T-cell immune responses than the MPs, suggesting that SPs may retain a more robust T-cell memory against SARS-CoV-2 than MPs.

To further determine the subpopulations of cytokine-producing memory T cells, totally 31 ELISpot-positive PBMCs from recovered donors were stained with antibodies against CD4/CD69 and CD8/CD69 for FACS analysis. SARS-CoV-2 spike-specific CD8+/CD69+ T cells were detected in all tested cases (P = 0.005 versus DMSO control, paired t-test, Fig. 5c). CD8+/CD69+ T cells were detected in 71, 75, 83, and 80% tested cases after stimulation with M, E, NSP, and ORFs (ORF3, ORF3a, ORF6, ORF7a, ORF7b, ORF8, and ORF10), respectively. These results show that the convalescent COVID-19 patients generated a substantial CD8+ memory T-cell response against SARS-CoV-2 antigens. CD4+ T-cell activation in response to S, M, E, NSP, and other ORFs was also detected in most tested cases (≥75%) (Fig. 5d), which is well correlated with CD8+ T responses (R = 0.7, P = 1.2e−05, Pearson’s correlation test, Fig. 5e).

**DISCUSSION**

Differential adaptive immune responses between severe and mild COVID-19 patients remain largely unknown. This study aims to investigate the dynamics of antibody levels and T-cell responses against SARS-CoV-2 according to disease severity. In this respect, we observed multiple differences between the SPs and the MPs including the SPs and the MPs only responded to the S protein (mean: 3.46-fold). Taken together, the ELISpot data indicate that the memory T cells specific for the S and other proteins were generated and sustained in the COVID-19 convalescent patients, which is further supported by a recent study.40 Importantly, we found that the SPs exhibited stronger T-cell immune responses than the MPs, suggesting that SPs may retain a more robust T-cell memory against SARS-CoV-2 than MPs.

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**DISCUSSION**

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Research Ethics Committee of Heilongjiang Provincial Hospital (2020-112). All participants provided written informed consent for sample collection and subsequent analyses.

Patients and clinical sample collection
All nine patients with COVID-19 in this study were enrolled from the Heilongjiang provincial Hospital from March to April, 2020. The clinical classifications was defined as mild to moderate, severe, and critical, according to the “Diagnosis and Treatment Protocol of COVID-19 (the 7th Tentative Version)” by the National Health Commission of China issued on 3 March 2020 (http://www.nhc.gov.cn/yzygj/s7653p/202003/46c9294a7dfde4cef80d7f5912eb1989.shtml).

Briefly, moderate cases have fever, respiratory symptoms, and pneumonia evidenced by computed tomography (CT) imaging. This study enrolled two patients with moderate infection with bilateral pneumonia, labeled M1 and M2, two patients with mild infection, labeled M3 and M4. Patients with severe pneumonia were diagnosed on the basis of one of the following criteria: (1) respiratory distress with respiratory rate ≥ 30 times min⁻¹; (2) fingertip oxygen saturation ≤ 93% at room air; (3) ratio of partial pressure of arterial oxygen to fraction of inspired oxygen (PaO₂/FIO₂) ≤ 300 mmHg (1 mmHg = 0.133 kPa); and (4) obvious progression of lesions in 24–48 h shown by pulmonary CT imaging > 50%. Two patients with severe pneumonia were enrolled, labeled as S1, S2, S3, and S5. These four patients showed PaO₂/FIO₂ ≤ 300 mmHg. The patients with critical pneumonia (S4) were diagnosed on the basis of one of the following criteria: (1) respiratory failure and an artificial airway required for invasive mechanical ventilation; (2) shock; and (3) combined failure of other organs that required intensive care unit monitoring. S4 is the patient with critical pneumonia received invasive mechanical ventilation.

Isolation of PBMCs
PBMCs were isolated from anticoagulant blood using Ficoll under the biosafety level 2 facility. To isolate PBMCs, blood was diluted with the same volume of PBS, then gently put on the equal volume of Ficoll in a 15 ml centrifuge tube and centrifuged for 35 min at 700 × g without brake. Discard the first layer that contains serum and platelet, carefully transfer thebuffy coat to a new centrifuge tube, wash with 10 ml PBS, and centrifuge at 350 × g, room temperature, 5 min. The supernatant was discarded and the pellet was washed again. After that, recollect these PBMCs, count the cell number, and store in the liquid nitrogen.

Isolation of T and B cells
Approximately 20 ml of human peripheral blood was obtained and placed on ice, and returned to room temperature before use. PBMCs isolation was processed within 2 h in a BSL-2 laboratory. PBMCs were isolated from whole blood samples by Ficoll density gradient centrifugation. Then, cells were counted in 0.4% Trypan blue, 1 × 10⁶ PBMCs was prepared for flow cytometry and cell sorting, and residual cells were centrifuged and resuspended at a concentration of 2 × 10⁶/ml for further use.

Flow cytometry and cell sorting
A FACS Aria cell sorter (BD Biosciences) was used to isolate T cells (CD3+), and CD3−CD19+CD20+CD27+ AEBCs (comprising of mostly memory B cells with a small number of peripheral plasmablasts). Surface marker staining and cell sorting was performed by pelleting and resuspending cells in FACS buffer (2% FBS in PBS) with antibodies at indicated concentrations for 20 min at room temperature in the dark. Cells were washed once in the FACS buffer before resuspension. PBMCs were filtered with a 100-µm nylon cell strainer to remove clumps and debris before flow cytometry.

Materials and Methods
Ethics statement
This study was conducted according to the principles expressed in the Declaration of Helsinki. Ethical approval was obtained from the
IFN-γ ELISPOT
IFN-γ-secreting T cells were detected by Human IFN-γ ELISPOT pro 3 kits (MACBTECH AB, Sweden) according to the manufacture’s protocol. Briefly, frozen PBMCs in cryotubes were placed in a 37 °C water bath till completely thawed, then transferred to a new 15-ml centrifuge tubes containing 10 ml complete RPMI medium (90% RPMI-1640, 10% heat-inactivated FBS, 1% penicillin–streptomycin), and centrifuged at 1000 rpm, room temperature for 5 min. The cell pellet was resuspended in 2–4 ml complete RPMI and balanced at 37 °C with 5% CO2 for 6 h. Cell concentrations were adjusted to ~2 × 10⁶/ml and plated at 100 K per well in duplicate and incubated for 36 h with 10 μg/ml of peptide pools (the concentration of each peptide is 10 μg/ml). Spots were then counted using an Elispot Reader System (AT-Spot2100, atyx).

The number of spots was converted into the number of spots-forming cells (SFCs) per million cells and the mean of duplicate wells was plotted.

We collected 276 peptides identified as potential T-cell epitopes for SARS-CoV-2 by at least two previous studies. This pool of 9–15-mer peptides was derived from 29 proteins spanning the whole proteome of SARS-CoV-2, including 76 epitopes from spike envelope (E), 157 from Orf1ab (Nsp1-Nsp16), and 20 from 7 other proteins. After 24 h culture, PBMCs from each well were counted using an Elispot Reader System (AT-Spot2100, atyx). The number of spots was converted into the number of spots-forming cells (SFCs) per million cells and the mean of duplicate wells was plotted.

Cells were activated and surface marker staining
Cells activation and surface marker staining were performed as described in Supplementary Table 5.

Batch-effect correction. Though many genes show significant differences in expression, these differences may come from experimental batch effect. Therefore, Harmony was utilized for batch-effect correction based on the top 50 principal components of PCA.

Cluster identification. After removing the batch effect, dimension reduction was performed using t-SNE algorithm, and cell clusters were identified at resolution 3.0 by FindClusters function of Seurat (v.3.1.4) utilizing a shared nearest neighbor graph constructed by FindNeighbors function. Finally, 59 clusters were obtained.

Cell type annotation. The corresponding cell types of the clusters were annotated using SingleR with the reference datasets of human immune cells.

Differential gene expression analysis
Differential gene expression analysis was performed using Seurat v.3 (FindMarkers function) with default parameters. We identified differentially expressed genes of each COVID-19 sample by comparing CD4⁺, CD8⁺, or B cells of each COVID-19 sample to those cells of all HCs. Genes with adjusted P < 0.05 and fold change > 1.5 were considered as significantly upregulated in patients and were used for further GO enrichment analysis.

GO and pathway enrichment analysis
GO and pathway enrichment analysis were performed using clusterProfiler (PvalueCutoff = 0.01 and qvalueCutoff = 0.05). For each comparison, the top ten enriched pathways containing at least five upregulated genes were kept. Significantly upregulated pathways shared by at least two patients were selected to display in bubble charts.

Single-cell BCR/TCR V(D)J sequencing and analysis
Chromium single cell V(D)J enriched libraries were quantified, normalized, and sequenced according to the User Guide for Chromium Single Cell V(D)J Reagent kits. The V(D)J enriched libraries were pooled and sequenced with an Illumina NovaSeq 6000 S2 Reagent Kit (300 cycles) (Illumina). BCR/TCR sequences for each single B/T cell were assembled by Cell Ranger vdj pipeline (v.3.1.0), with CDR3 sequences and rearranged full-length BCR/TCR V(D)J segments as well as clonotype frequency obtained. Only productive BCR/TCR sequences were kept for subsequent analysis. The assembled FASTA sequences of IGH chains were collected and assigned germline sequences using Change-O to perform clonal grouping and mutation analysis. V(D)J segment usage counts were calculated for all cohorts and PCA analysis was performed to compare the V(D)J segment usage among SPs, MPs, and HCs.

BCR/TCR clonal grouping
All BCR/TCR clones were identified by Cell Ranger vdj pipeline (v.3.1.0). The BCR/TCR clonotypes from SPs, MPs, and HCs were pooled together before carrying out the clonal grouping step. TCR clusters were predicted by GLIPH to bind the same MHC-restricted peptide antigen. Public TCR clusters were detected for patients and HCs, respectively. BCR clusters were identified using the single-cell transcriptome data analysis.
Change-O based on hierarchical clustering approach. First, IGH sequences with the same V and J segment calls and CDR3s of the same length were grouped. Second, IGH sequences within each group were clustered into BCR clusters based on the hamming distances of CDR3 nucleotide sequences. Public BCR clusters of each isotype (IgM, IgA, and IgG) were detected for patients and HCs, separately.

Clonal abundance and diversity

Clonal abundance distributions were inferred using resampling strategies to correct for variations in sequencing depth. Clonal diversity distributions were calculated by plotting diversity index (\( qD \)) for the colored bands, the difference between the donor groups is distribution; thus, when three lines are separated and fall outside HC s using BASELIN e b a s e d o n u n d e r l y i n g S H M a r g e t i n g and more on common ones as range of de. The colored bands abundances for the set of clones undetected in the group but inferred to be present in the assemblage. The colored bands indicate the middle 95% percentiles of the clone distribution with 200 bootstrap estimates in each donor group.

The Hill diversity index (\( qD \)), which measures diversity in a population, was calculated for the set of BCR IGH or TCR clones to determine whether the diversity in the repertoires differed between the donor groups. For each donor group, the repertoire was subsampled to the number of sequences in the smallest sample and the Hill diversity index was calculated independently in 200 equally spaced \( q \) values between 0 and 4. Because \( q \) varies from 0 to infinity, the diversity (\( qD \)) depends less on rare species and more on common ones as \( q \) increases, thus encompassing a range of definitions that can be visualized as a single curve. For \( q = 0 \), the diversity is defined as the total number of clones. As \( q \) approaches infinity, the diversity is given by one over the frequency of the largest clone. At a given value of \( q \) (x axis), lower values of \( qD \) (y axis) indicate lower diversity. The colored bands indicate the middle 95% percentiles of the sampled distribution; thus, when three lines are separated and fall outside of the colored band, the difference between the donor groups is significant.

Mutation and selection analysis

Replacement (R) mutations within subregions of the V segment were identified by comparing the input BCR sequences with the germline sequences using Change-O. In addition, the quantification of selection pressure was performed for SPs, MPs, and HCs using BASELINE based on an underlying SHM targeting model. The probability density function (PDF) for selection strength in CDR and FWR regions was calculated using a Bayesian estimation model.

Statistics

DESEQ2 was used to find differential segment usages in BCR and TCR. The \( P \) value and fold change were plotted as volcano map using ggplot2. Wilcoxon signed rank test and t-test was used pairwise comparisons.

DATA AVAILABILITY

The datasets used for the current study are available to download via http://www.microbiome-bigdata.com/project/SARS-CoV-2/.
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