Original Article

Residual persistence of cytotoxicity lymphocytes and regulatory T cells in patients with severe coronavirus disease 2019 over a 1-year recovery process

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Aim: To clarify the immune cellular changes in critically ill patients recovering from coronavirus disease 2019 (COVID-19).

Methods: The immune response of peripheral blood mononuclear cells from patients with severe COVID-19 in different stages of recovery (3, 6, and 12 months from hospitalization) was evaluated by single-cell mass cytometry. Immunological changes in patients were compared with those in age-matched healthy donors.

Results: Three patients with severe COVID-19 were compared with four healthy donors. In the patients, there was an increase in the cell density of CD4- and CD8-positive T lymphocytes, and B cells, over the course of the recovery period. CD4- and CD8-positive T lymphocytes expressing T-bet and granzyme B (Gzm B) in patients were abundant during all recovery periods. The level of regulatory T cells remained high throughout the year. The levels of natural killer (NK) cells in patients were higher than in those in the healthy donors, and the frequency of $CD16^{+}$ NK cells expressing Gzm B increased throughout the year.

Conclusion: Patients recovering from severe COVID-19 showed persistence of cytotoxic lymphocytes, NK cells, and regulatory T cells throughout the posthospitalization year of recovery.

Key words: CD4, CD8, CyTOF, cytotoxicity, granzyme B, long COVID, NK, T-bet, Treg

Abbreviations

APACHE II: Acute Physiologic Assessment and Chronic Health Evaluation II

ARDS: acute respiratory distress syndrome

COVID-19: coronavirus disease 2019

CyTOF: cytometry by time-of-flight

Foxp3: forkhead box P3 transcription factor

GITR: glucocorticoid-induced TNFR-related receptor

Gzm B: granzyme B

HD: healthy donor

NK: natural killer

PBMC: peripheral blood mononuclear cell

SARS-CoV-2: severe acute respiratory syndrome coronavirus 2

Treg: regulatory T cells

t-SNE: t-distributed stochastic neighbor embedding

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INTRODUCTION

NOVEL CORONAVIRUS DISEASE 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection continues to remain a major global health concern. Many infected individuals do not present with symptoms or only show mild cold symptoms and heal. However, patients with severe infection develop unexplained immune abnormalities and immune dysregulation. Various immune profiling studies have revealed features such as impaired interferon induction, increased inflammatory response, and delayed adaptive immune response.[1]

As more number of patients recover from COVID-19, we have begun to find that many patients continue to suffer from a variety of symptoms after their recovery, which are termed "long COVID" or post-SARS-CoV-2 sequelae. Long COVID is characterized by long-lasting COVID-19 signs and symptoms that do not resolve within 4 weeks of infection. Long COVID occurs in 50% of patients with SARS-CoV-2 infection, with a wide range of symptoms and severity.[2,3] Su *et al*.[2] reported that long COVID is associated with autoantibodies, viremia, and diabetes using a longitudinal, multiomics study of long patients with COVID from first diagnosis to recovery (2–3 months later). However, the majority of patients in that study showed mild disease, and the immune status of the patients during different, more prolonged convalescence periods remained unclear. There is limited knowledge of the long-term effects of COVID-19 in patients with severe disease. Therefore, in this study, we evaluated the immune profile in the peripheral blood of patients with severe COVID-19 who were available for 1-year follow-up.

METHODS

Study population

THIS CLINICAL STUDY was conducted on patients with severe COVID-19 who were admitted to Osaka General Medical Center and recovered from COVID-19. This was a pilot study that was approved by the Institutional Review Board of Osaka General Medical Center (approval number C201912002). Written informed consent was obtained from all patients. The diagnosis of COVID-19 was confirmed by RNA detection of SARS-CoV-2 in the clinical laboratory of the Osaka Public Health Laboratory in accordance with the WHO interim guidelines.[4] After finishing the acute treatment of COVID-19, peripheral blood was collected 3, 6, and 12 months after admission. Data on clinical and biological parameters such as demographic characteristics, intensive care unit length of stay and hospital stay, treatment, and comorbidities were collected. Severity scores were recorded with the severity of acute respiratory distress syndrome (ARDS) assessed as mild, moderate, or severe and with the Acute Physiologic Assessment and Chronic Health Evaluation (APACHE) II score (range 0–71).[5,6] Peripheral blood was obtained from healthy volunteers who were age matched to the patients. Informed consent was obtained from the healthy donors (HDs).

Sample preparation

Peripheral blood mononuclear cells (PBMCs) were prepared from each patient's blood. For cytometry by time-of-flight (CyTOF) staining of PBMC, a panel of 43 marker antibodies was used (Table S1). We analyzed samples with a Helios™, a CyTOF® System (Fluidigm Sciences Inc., South San Francisco, CA, USA). Analysis of data was performed online using Cytobank Premium (Cytobank, Mountain View, CA, USA). The protocols for PBMC and CyTOF are shown in Appendix S1.

Statistical analysis

Statistical analysis of the frequency of the immune cell subpopulations and protein expression between groups was compared by two-way analysis with Tukey's multiple comparison test or the Student test using JMP 16 Pro (SAS Institute Inc., Cary, NC, USA). A P value less than 0.05 was considered to indicate statistical significance.

RESULTS

CHARACTERISTICS OF THE patients are presented in Table 1. Patient 1 was managed on extracorporeal membrane oxygenation for severe ARDS and weaned from the ventilator after 16 days. The patient was discharged home after a 31-day hospital stay. He complained of hair loss and fatigue at the 3-month follow-up, and at 1 year later had problems with memory and concentration. Patient 2 required ventilator management for 34 days and was discharged home following rehabilitation after 92 days of hospitalization. At the 3-month follow-up, the patient had shortness of breath, cough, and decreased voice volume and at 1 year, the patient had memory problems. Patient 3 was 85 years old and required 37 days to wean off the ventilator. Because of disuse atrophy from prolonged hospitalization, this patient was discharged home with home oxygen therapy after 141 days of hospitalization. At the 3-month follow-up, the patient showed muscle weakness, joint pain, and

Table 1. Characteristics of the study participants

Characteristic	Patients			Healthy donors			
	Patient 1	Patient 2	Patient 3	Donor 1	Donor 2	Donor 3	Donor 4
Age, years	57	67	85	65	62	71	70
Sex	Male	Male	Male	Female	Female	Male	Male
BMI, kg/m^2	23	24.5	22.7	18.7	18.6	21.4	26.9
Medical history	None	DM	HT	None	None	None	None
Clinical features at admission							
	Patient 1		Patient 2			Patient 3	
Severity of ARDS	Severe		Moderate			Severe	
APACHE II score	9		10			17	
Disease course							
ECMO	+		−			−	
Tracheostomy	−		+			+	
Length of stay in ICU, days	17		27			29	
Days of mechanical ventilation, days	16		34			37	
Length of stay in hospital, days	31		92			141	
Discharge	Home		Home			Home	
Days from hospitalization to first specimen	95		124			103	
Days from hospitalization to second specimen	168		194			173	
Days from hospitalization to third specimen	368		384			361	

APACHE II, Acute Physiology and Chronic Health Evaluation II; ARDS, acute respiratory distress syndrome; BMI, body mass index; DM, diabetes mellitus; ECMO, extracorporeal membrane oxygenation; HT, hypertension; ICU, intensive care unit.

decreased vitality, and at 1 year, the muscle weakness had still not recovered. None of the participants in this study had a history of vaccination. The coronavirus lineage of the patients in the study was B.1.1, and it was not a mutant strain.

Overview of peripheral immune cells

We identified seven cell subsets and visualized the changes in the cell populations of all samples on a t-distributed stochastic neighbor embedding (t-SNE) map. For comparison of the groups (3, 6, and 12 months, and HD), data were concatenated within a group and the cell distribution was visualized on a t-SNE map (Fig. 1A). As each patient recovered from the date of hospitalization to 3, 6, and 12 months later, the cell densities of CD4-positive T lymphocytes, CD8-positive T lymphocytes, and B cells increased. Natural killer (NK) cells were more abundant in all three patients than in the HD group. Among the individual patients, in patient 1, CD4-positive T lymphocytes increased after 6 months and B cells increased after 12 months; in patient 2, CD8-positive T lymphocytes decreased and CD4-positive T lymphocytes increased over time; and in patient 3, CD8-positive T lymphocytes were still predominant and B cells had not recovered after 12 months. In the HD, CD4-positive T lymphocytes, CD8-positive T lymphocytes, and B cells showed a well-balanced distribution, with fewer monocytes and dendritic cells (Fig. 1B).

CD4- and CD8-positive T lymphocytes

The frequency of protein expression inside and outside the CD4-positive T lymphocytes in all samples is shown on t-SNE maps (Fig. 2A). In each patient, cell frequencies expressing T-bet and granzyme B (Gzm B) increased with recovery. Data were concatenated for each month, and the percentage of CD4-positive T lymphocytes expressing high levels of proteins is shown in a violin plot (Fig. 2B). The percentage of CD4-positive T lymphocytes expressing high levels of T-bet and Gzm B in the patient group at 12 months remained as high as in the group at 3 months. The cell frequencies of T-bet and Gzm B expressed on CD8-positive T lymphocytes are shown on t-SNE maps (Fig. 3A), and the percentages of CD8-positive T lymphocytes in which both were highly expressed are shown in violin plots (Fig. 3B). The percentage of CD8-positive T lymphocytes with high

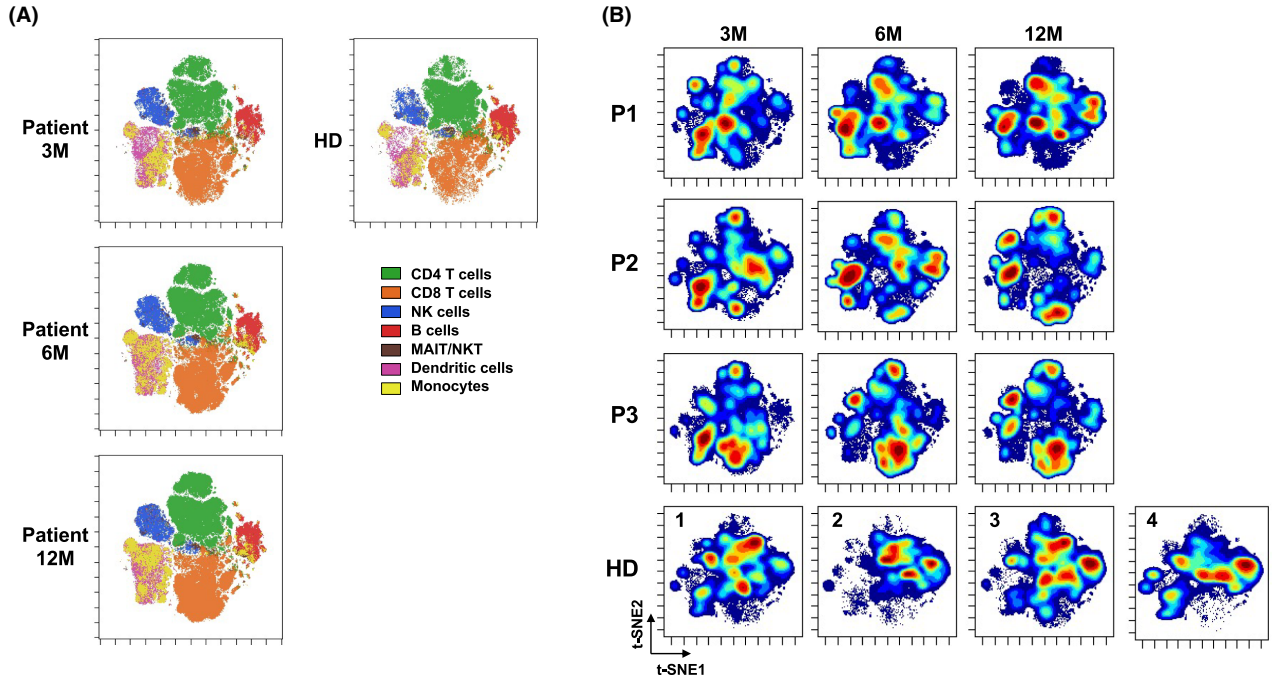

Fig. 1. Characterization of peripheral blood mononuclear cells (PBMCs) in patients with COVID-19 and HD. (A) Patient data within each group at 3, 6, and 12 months and for HD were concatenated by group, all data were merged, and cell populations defined by the manual gating strategy were projected onto a t-SNE map and assigned specific colors. (B) Cytometry by time-of-flight staining data for all patients and HD individuals from $CD45^+$ cells were analyzed by t-SNE and plotted as density contour plots. B cells, B lymphocytes; CD4 T cells, CD4 T lymphocytes; CD8 T cells, CD8 T lymphocytes; COVID-19, coronavirus disease 2019; HD, healthy donor; M, months; MAIT, mucosal-associated invariant T cells; NK, natural killer cell; NKT, natural killer T cell; P, patient; t-SNE, t-distributed stochastic neighbor embedding.

expression of T-bet and Gzm B in the patient group at 12 months remained as high as that in the group at 3 months.

Regulatory T cells

The results of the regulatory T cells (Tregs) analysis are shown in Figure 4. Among the CD4-positive T lymphocytes, a population of cells coexpressing CD25 and forkhead box P3 transcription factor (Foxp3) was selected (Fig. 4A). The expression frequencies of Foxp3 and Helios as intracellular markers and glucocorticoid-induced TNFR-related receptor (GITR) as a cell surface marker in Tregs are shown on t-SNE maps (Fig. 4B). Among the CD4-positive T lymphocytes, the percentage of Tregs showed a tendency to be higher in the patient group than in the HD group (Fig. 4C). Helios was highly expressed in all patients. In patients 1 and 2, Helios was more highly expressed at 6 and 12 months than at 3 months after admission. In the patient group, Foxp3 was highly expressed at 6 and 12 months after admission. The expression of GITR in patients 2 and 3 at 12 months after hospitalization was higher than that in all of the other samples (Fig. 4D).

Natural killer cells

Figure 5 shows the alterations in NK cells, and the gating strategy for NK cells is shown in Figure 5A. CD 56-positive cells were selected from nonlymphocytes ($CD3^-$ and $CD20^-$) and divided into two groups according to whether they expressed CD16. Compared with the HD group, the patients had higher cell density of NK cells. The frequency of cells expressing high levels of Gzm B was elevated in all three patients (Fig. 5B). Among the NK cells, the percentage of $CD16^+$ NK cells was higher in all patients than that in the HD group (Fig. 5C).

DISCUSSION

WE HAVE PREVIOUSLY reported long-term persistence of cytotoxic T lymphocytes in the post 3-month recovery state of patients with severe COVID-19.[7] In

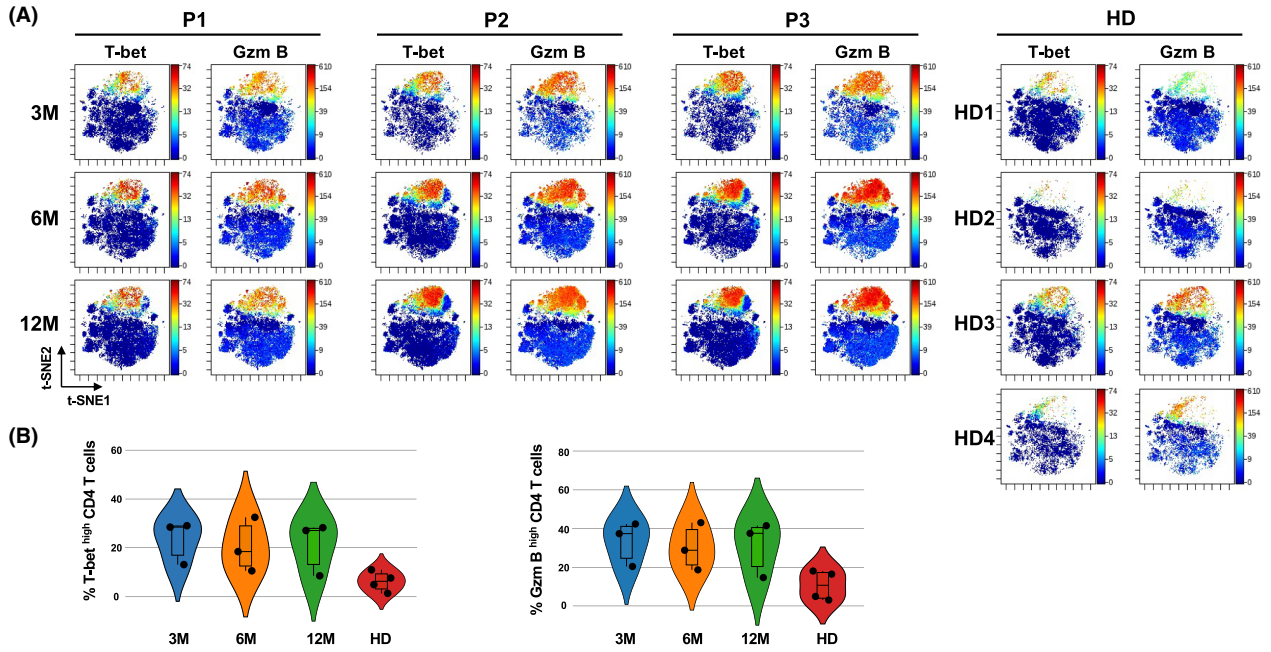

Fig. 2. Expression of T-bet and Gzm B on CD4 T cells of patients with COVID-19 and HD. (A) The t-SNE projection of CD4 T cells from each patient and HD is shown for the cell markers indicated. (B) Violin plots show the percentage of cells expressing the indicated markers in each group, with data concatenated for patients at 3, 6, and 12 months and for HDs. The black dot in the violin is the individual value, and the boxes represent the percentiles 25th and 75th with the median line. CD4 T cells, CD4 T lymphocytes; COVID-19, coronavirus disease 2019; Gzm B, granzyme B; HD, healthy donor; M, months; P, patient; tSNE, t-distributed stochastic neighbor embedding.

the present study, we extended the follow-up period to 1 year for evaluation and found the changes in the peripheral blood immune response of lymphocytes and NK cells over time in patients with severe COVID-19 1 year after the onset of the disease. The immune status of patients recovering from severe COVID-19 differed significantly from that of HD and had not been restored back to normal even after 1 year of recovery.

There have been several reports on cytotoxic lymphocytes in patients with COVID-19.[8–11] Galán *et al.*[12] reported long-term persistence of a cytotoxic population after 49 weeks in patients with COVID-19 not requiring hospitalization. In this study, high expression of T-bet and Gzm B in CD4- and CD8-positive T lymphocytes persisted in patients with severe COVID-19 for 1 year after recovery. T-bet and Gzm B are essential players in type 1 immunity and are associated with the differentiation of two major subsets of T lymphocytes, CD4-positive T helper cells and CD8-positive cytotoxic T cells, which direct inflammatory and cytotoxic responses required for the destruction of intracellular and extracellular pathogens.[13–15] Thus, high expression of T-bet and Gzm B in CD4 and CD8 T lymphocytes indicates sustained activation of type 1 immunity and increased lymphocyte cytotoxicity.

As long COVID-19 is characterized by persistent cytotoxicity against viruses, the persistence of long-term cytotoxicity in patients recovering from COVID-19 may be one of the immunologic mechanisms of long COVID-19.[3] It is known that prolonged exposure to environmental agents, such as infection, leads to prolonged activation of the immune response and loss of self-tolerance, ultimately leading to the development of autoimmune diseases.[16] It has been reported SARS-CoV-2 can remain in the airway epithelium and epithelial cells of the gastrointestinal tract for some time.[17,18] Patients with COVID-19 have markedly increased autoantibody reactivity, and autoantibodies to immunomodulatory proteins are detected at a higher frequency.[19] As coronaviruses remain in the body for long periods and lymphocyte cytotoxicity persists, autoantibodies may contribute to the progression of the disease, which may be manifested as symptoms of long COVID-19.

The behavior of Tregs in patients with COVID-19 is inconsistent.[20–22] We showed that the level of Tregs in patients with COVID-19 was increased compared with

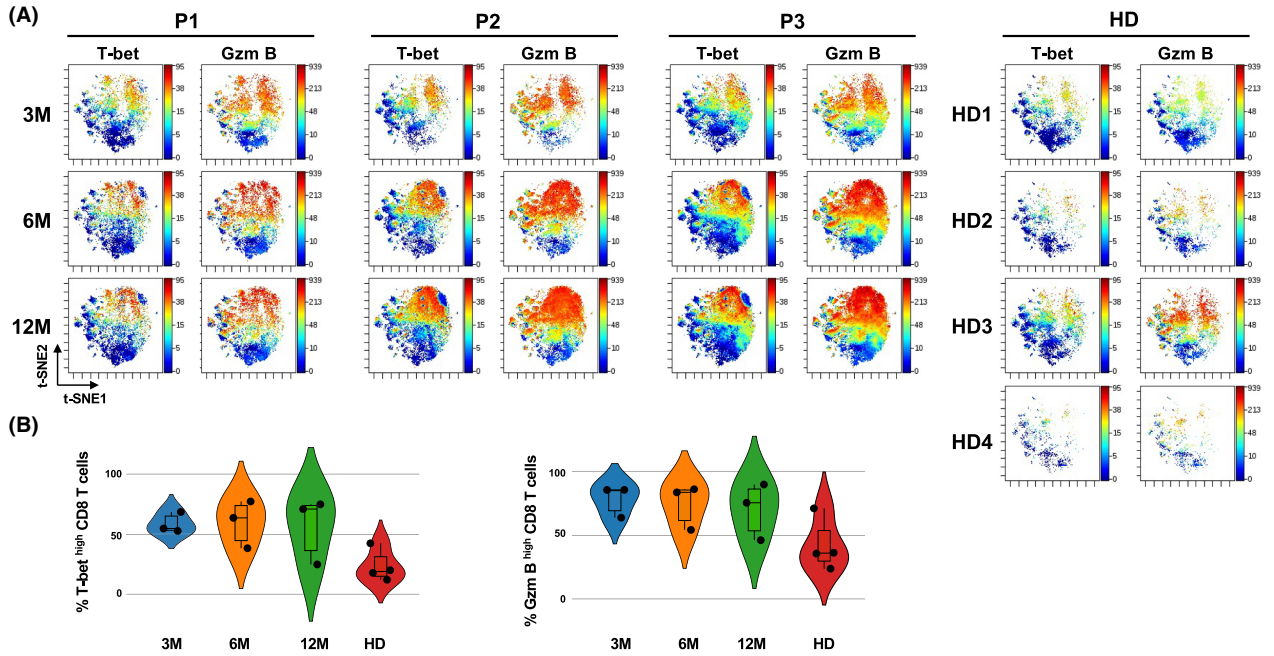

Fig. 3. Expression of T-bet and Gzm B on CD8 T cells of patients with COVID-19 and HD. (A) The t-SNE projection of CD8 T cells from each patient and HD is shown for the cell markers indicated. (B) Violin plots show the percentage of cells expressing the indicated markers in each group, with data concatenated for patients at 3, 6, and 12 months and for HD. The black dot in the violin is the individual value, and the boxes represent the percentiles 25th and 75th with the median line. CD8 T cells, CD8 T lymphocytes; COVID-19, coronavirus disease 2019; Gzm B, granzyme B; HD, healthy donor; M, months; P, patient; tSNE, t-distributed stochastic neighbor embedding.

that in the HD at 3 months to 1 year of recovery, and Foxp3 was increased from 6 months. During infection, Tregs play a dual role, benefiting the host by suppressing immune-mediated pathogenesis and promoting chronic persistence of infectious pathogens by decreasing effector immunity and inflammatory clearance.[23] In patients with sepsis, Tregs contribute to good outcomes in the early stage of sepsis, but prolonged dysfunction of Tregs leads to immunosuppression and increased susceptibility to secondary infections.[24] Therefore, persistence of high levels of Tregs in patients after recovery from severe COVID-19 may imply immunosuppression and promote chronic persistence of SARS-CoV-2.

This study shows that there is a long-term abnormality in the distribution of NK cell subsets and the level of $CD16^{+}$ NK cell remained elevated in patients with severe COVID-19, even at 1 year after infection. In patients with acute COVID-19, the activation state of circulating NK cells was high, and the number of NK cells with high levels of cytotoxic proteins was increased and restored to normal after 1 month.[25,26] However, in patients with long COVID-19, the population of $CD16^{+}$ NK cells was increased and their cytotoxic potential was enhanced.[12] The Fc receptor CD16, present on $CD56^{dim}$NK cells, recognizes antibody-bound cells and sends a strong signal to NK cells, which then eliminate their targets by direct killing and cytokine production.[27,28] If the long-lasting abnormal peripheral blood immune response in patients with severe COVID-19 is attributable to chronic viral infection, as in chronic viral infections such as human immunodeficiency virus type 1 and hepatitis C virus, an expansion of a subset of NK cells with impaired cytotoxicity and exhaustion would be expected.[29]

The persistence of cytotoxic lymphocytes, NK cells, and Tregs in patients with severe COVID-19 shown in this study may have an underlying immune mechanism different from that of so-called chronic viral infections. These immune abnormalities occurring after recovery from COVID-19 may lead to prolonged inflammation and systemic organ damage, including the lungs, kidneys, muscles, and brain. In an animal model of recovery from coronavirus infection, Frere *et al.*[30] reported that persistent inflammation leads to organ damage such as lung, kidney, and brain damage and ultimately to behavioral changes. Further studies are needed to elucidate the relationship between the symptoms of long COVID-19 and these immune abnormalities.

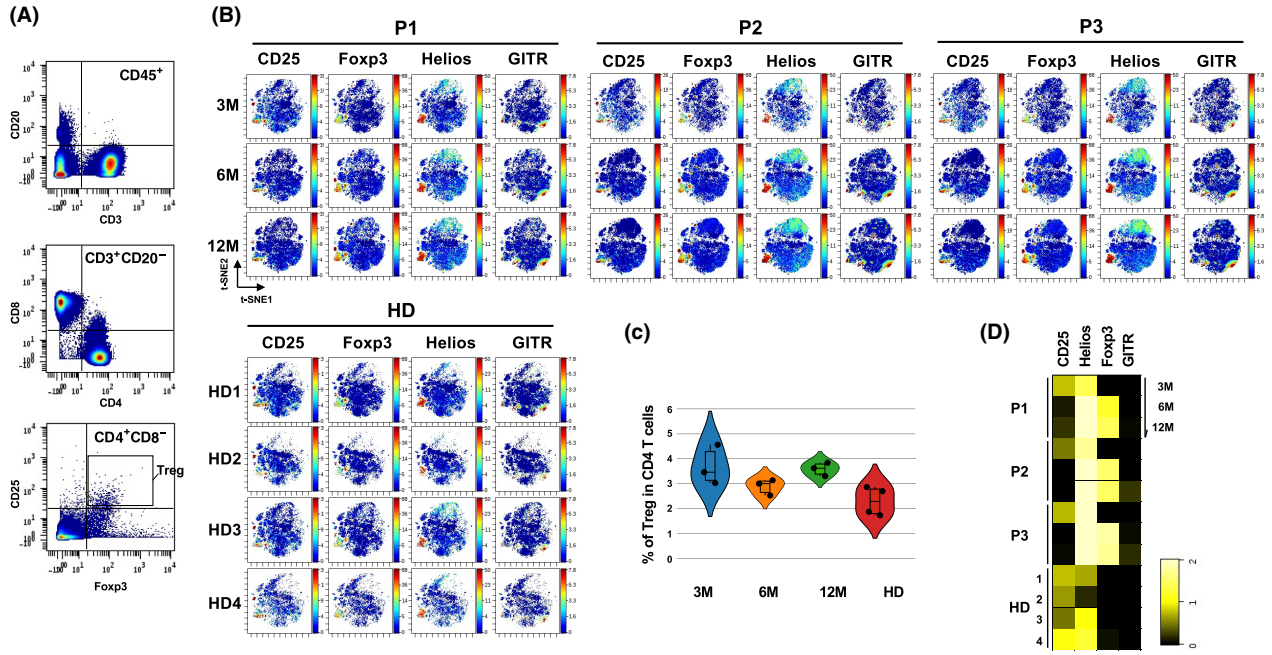

Fig. 4. Expression of intracellular and extracellular markers indicative of Tregs in CD4 T cells from patients with COVID-19 and HD. (A) Gating strategy to select Treg. (B) The t-SNE projection of CD4 T cells from each patient and HD is shown for the cell markers indicated. (C) Violin plots show the percentage of Tregs among CD4 T cells. The black dot in the violin is the individual value, and the boxes represent the percentiles 25th and 75th with the median line. (D) Heat map showing expression levels of indicated intracellular and extracellular markers in patients and HD. CD4 T cells, CD4 T lymphocytes; COVID-19, coronavirus disease 2019; Foxp3, forkhead box P3 transcription factor; GITR, glucocorticoid-induced TNF-related receptor; HD, healthy donor; M, months; Treg, regulatory T cell; tSNE, t-distributed stochastic neighbor embedding.

There are several limitations of this study. First, the number of patients included in the study was small. This is due to the small number of critically ill patients who were discharged alive and could be followed up after discharge. Second, we did not evaluate the immune response of the peripheral blood of the patients in the early stages of their infection. It is unclear whether the series of immunological changes revealed in the study developed during the course of recovery or were established in the early stages of infection. Third, it was not possible to fully evaluate the relationship between immune status after recovery from COVID-19 and vaccination or virus mutant strain type.

CONCLUSION

WE FOLLOWED PATIENTS with severe COVID-19 for 1 year after their recovery and revealed some of the immunological mechanisms of long COVID-19, which included the persistence of cytotoxic lymphocytes, NK cells, and Tregs.

DISCLOSURE

APPROVAL OF THE research protocol: The Institutional Review Board of Osaka General Medical Center approved this study [approval number: C201912002].

Informed Consent: Written informed consent was obtained from all patients.

Registry and the Registration No. of the study/trial: N/A.

Animal Studies: N/A.

Conflict of Interest: None declared.

ACKNOWLEDGMENTS

NONE.

AUTHOR CONTRIBUTIONS

Y.M. AND K.Y. designed the study; Y.M. enrolled patients and acquired data; K.K. and M.M. processed patient samples. K.K. performed CyTOF. Y.M., K.Y., Y.U., T.W., and S.F. drafted the manuscript and revised it

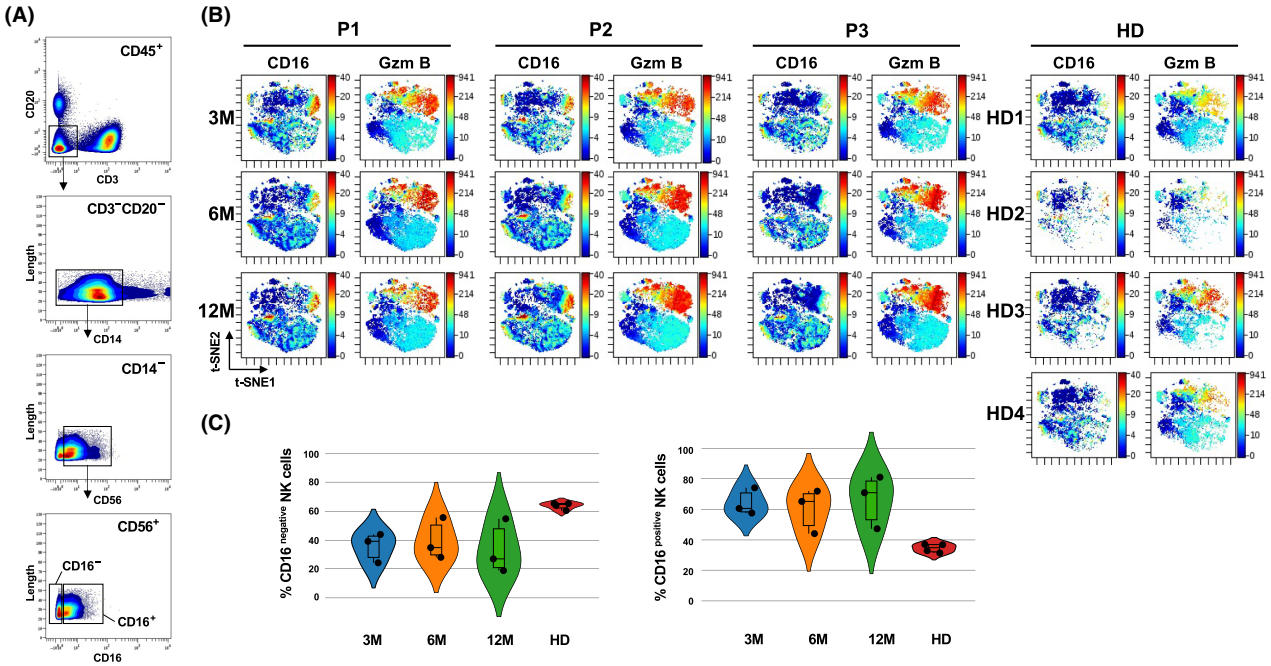

Fig. 5. Expression of indicated markers in NK cells from patients with COVID-19 and HD. (A) Gating strategy to select NK cells. (B) The t-SNE projection of NK cells from each patient and HD is shown for the cell markers indicated. (C) Violin plots show the percentage of NK cells that do not (negative) or do (positive) express CD16. The black dot in the violin is the individual value, and the boxes represent the percentiles 25th and 75th with the median line. COVID-19, coronavirus disease 2019; Gzm B, granzyme B; HD, healthy donor; M, months; NK, natural killer cell; tSNE t-distributed stochastic neighbor embedding.

critically. K.Y. organized and supervised the conduction of the study. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

THE STUDY WAS approved by the Institutional Review Board of Osaka General Medical Center [approval number: C201912002]. Written informed consent was obtained from all patients. The study was conducted in accordance with the Declaration of Helsinki.

DATA AVAILABILITY STATEMENT

THE DATA SETS used and analyzed during this study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1

Table S1 CyTOF antibody panel. CyTOF, cytometry by time-of-flight.