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Phenotypic changes in immune cells induced by granulocyte and monocyte adsorptive apheresis in patients with severe COVID-19: An ex vivo study

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Abstract

Aims: SARS-CoV-2 causes systemic immune dysfunction, leading to severe respiratory dysfunction and multiorgan dysfunction. Granulocyte and monocyte adsorptive apheresis (GMA) therapy is designed to regulate an excessive inflammatory response and has been proposed as a potential therapeutic strategy for coronavirus disease 2019 (COVID-19). We aimed to investigate a targeted subset of granulocytes and monocytes to be removed after GMA therapy in patients with severe COVID-19 infection.

Methods: We established an ex vivo experimental system to study the effects of GMA. Blood samples were collected into EDTA-treated tubes and a mixture of blood samples and cellulose acetate beads was used in GMA. After GMA, blood samples were removed, and the granulocyte and monocyte subtypes before and after GMA were determined by CyTOF mass cytometry. To analyze mass cytometry data with a self-organizing map, hierarchical clustering was used to determine the appropriate number of metaclusters from t-distributed stochastic neighbor embedding.

Results: We included seven patients with severe COVID-19 and four age- and sex-matched volunteers. Granulocyte subsets removed by GMA strongly expressed CD11b, CD16, and CD66b, and weakly expressed CD11c, consistent with mature and activated neutrophils. Monocyte subsets strongly expressed CD14, weakly expressed CD33 and CD45RO, and did not express CD16. These subsets were indicated to promote the release of inflammatory cytokines and activate T cells.

Conclusions: The identification of the granulocyte and monocyte subsets removed after GMA in patients with severe COVID-19 may help explain the potential mechanism underlying the effectiveness of GMA in COVID-19 and other inflammatory diseases.

KEYWORDS
Adacolumn, CyTOF, GMA, mass cytometry, SARS-CoV-2

INTRODUCTION

Coronavirus disease 2019 (COVID-19), an infectious disease caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) that developed at the end of 2019,[1] has spread worldwide. Recently, the severity and mortality of patients with COVID-19 have improved due to an increasing vaccination rate.[2] However, there is no definitive treatment in cases that follow a severe or fatal course. The main pathological condition is severe respiratory failure triggered by pneumonia, but it also presents systemic inflammation and multiple organ failure, and the mechanism has not been fully elucidated.[3,4] Several clinical practice guidelines for COVID-19 management have been released[5–7]; however, a

need for advanced therapies based on the pathophysiology of COVID-19 remains.

Adsorptive granulocyte and monocyte apheresis (GMA) with an Adacolumn device (JIMRO, Gunma, Japan) has been established as an extracorporeal circulating therapy designed to selectively deplete elevated and activated myeloid cells, and it has shown effectiveness in patients with ulcerative colitis (UC), Crohn's disease, and pustular psoriasis. This column is packed with cellulose acetate beads, which serve as the target of granulocytes and monocytes.[8] Granulocytes and monocytes are closely involved in the inflammation and subsequent organ failure associated with systemic inflammatory diseases. Case reports described the induction of remission with GMA treatment for patients with both active UC and COVID-19.[9,10] Additionally, Kanekura and Kawahara described the possible effectiveness of GMA for COVID-19 patients in a narrative review.[11]

We investigated whether GMA selectively removed specific subsets of peripheral blood cells in severe COVID-19 patients using an ex vivo experimental model. We identified the cell subsets rapidly eliminated from blood samples during ex vivo GMA treatment. This GMA-reactive granulocyte and monocyte population was comprehensively profiled by time-of-flight (CyTOF) mass cytometry using an antibody panel that focused on 38 surface marker proteins that can be used in phenotyping.

METHODS

Study population

Patients managed at Osaka Medical and Pharmaceutical University Hospital from March to June 2021 were enrolled. The criteria for inclusion were patients with severe COVID-19 who were older than 18 years of age. Severe COVID-19 was defined by the need for mechanical ventilation due to acute respiratory distress syndrome and intensive care unit (ICU) admission. Blood samples for the control condition were provided by healthy individuals and age- and sex-matched patients. The study, which followed the principles of the Declaration of Helsinki, was approved by the Institutional Review Board of Osaka University (approval number: T20173). Informed consent was obtained from the patients or their close relatives, and the volunteers.

Blood sampling and exposure to cellulose acetate beads

A study overview is shown in Figure 1A. Blood samples were collected from each patient within 24 h of ICU admission and from the healthy volunteers. Peripheral blood samples collected into EDTA-treated tubes were immediately used in the experiment. A mixture of heparinized peripheral blood samples and cellulose acetate beads at a 1:2 mL/g ratio was made in 5.0-mL syringes. The syringe was rotated at 15 revolutions per minute for 60 min at 37°C. Blood samples were removed from the syringes and stained for CyTOF antibodies, as described below. Additionally, unexposed sham blood was used for CyTOF staining. Cellulose acetate beads were provided by JIMRO Institute (Takasaki, Japan).

CyTOF mass cytometry

A 38-marker antibody panel was used for CyTOF staining (Table S1). Metal-conjugated antibodies were purchased directly from Fluidigm for available targets (Maxpar Direct Immune Profiling Assay). All CyTOF staining was performed at room temperature. For all stains, cisplatin viability staining reagent (Fluidigm Sciences) was added to plated cells for 5 min before staining. Fc-block reagent was added to the cells for 10 min before adding CyTOF antibody-staining cocktails. After incubation with/without cellulose acetate beads, 270 µL of blood was added directly to dry antibody tubes and allowed to incubate for 30 min at room temperature. Immediately after staining, erythrocytes were lysed by the addition of 250 µL of Cal-Lyse™ Lysing Solution directly to the staining tube. The tubes were gently vortexed and allowed to incubate for 10 min at room temperature, followed by the addition of 3 mL of Maxpar water and an additional 10 min of incubation in the dark. The tubes were washed two times in Maxpar Cell Staining Buffer followed by fixation in 1.6% formaldehyde for 10 min. After fixation, the cells were spun into a pellet, the fixative was removed, and the pellet was resuspended in 1 mL of 125 nm Cell-ID™ Intercalator-Ir. After a final washing step, cells were reconstituted in Milli-Q filtered distilled water (EMD Millipore, Billerica, MA, USA) at a concentration of 1×10^6 cells/mL containing EQ calibration beads (EQ Four Element Calibration Beads; Fluidigm Sciences) according to the manufacturer's protocol. Cells were analyzed using a Helios mass cytometer (Fluidigm Sciences).

Data analysis was conducted online using the Cytobank Premium software program, as shown in Figure 1B (Cytobank, Mountain View, CA, USA). For the analysis of mass cytometry data with a self-organizing map in Cytobank, hierarchical clustering was used to determine the appropriate number of metaclusters based on the median marker expression from the t-distributed stochastic neighbor-embedding (tSNE) results. The percentages of cell populations in each metacluster were exported.

Clinical data collection

We collected the following data on baseline patient characteristics: age, sex, preexisting condition, sequential organ failure assessment score, PaO_2/FiO_2 (P/F) ratio, laboratory data, and use of concomitant therapies. We also collected the following data on general outcomes: in-hospital mortality, length of ICU stay, and length of mechanical ventilation.

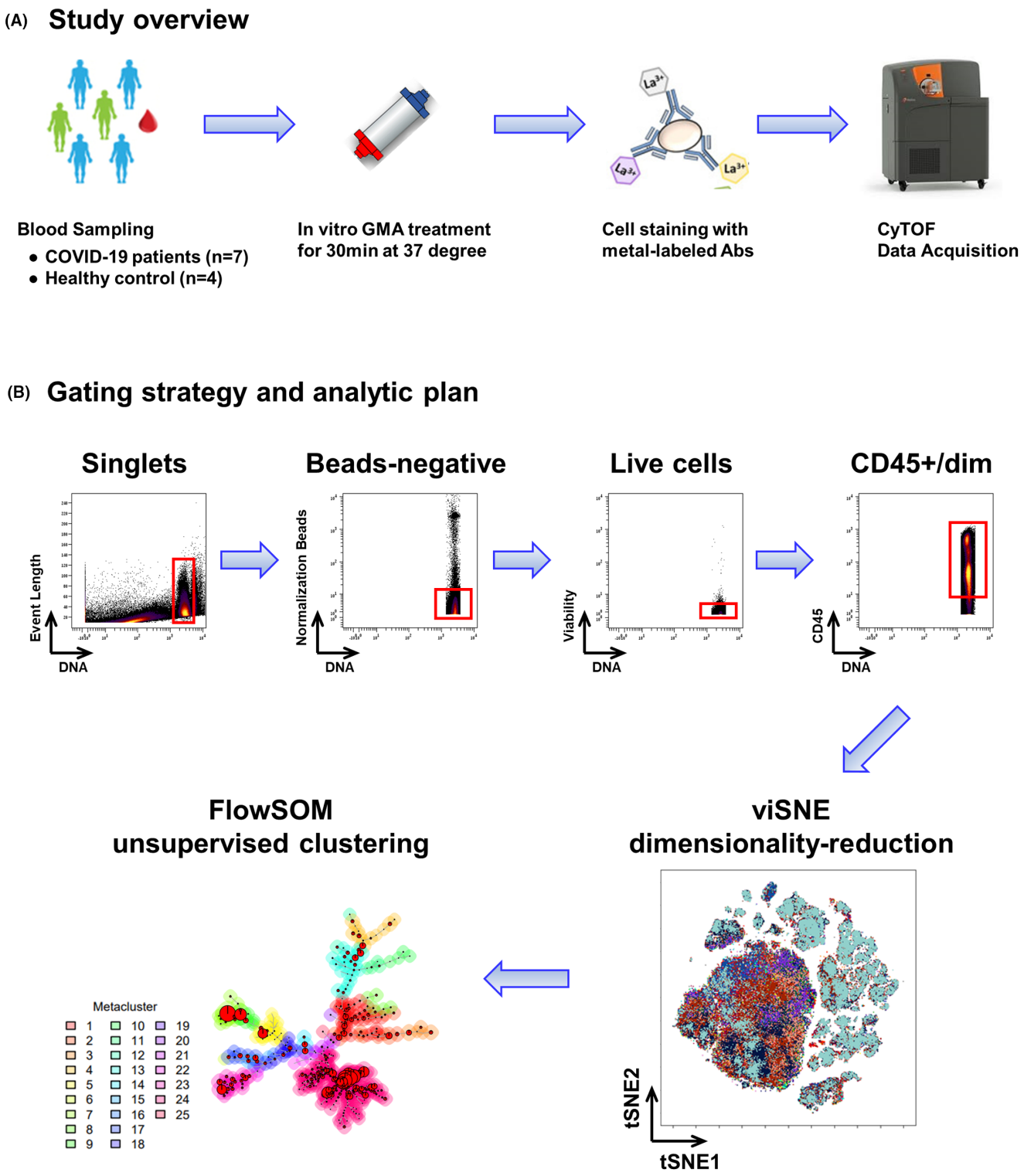


FIGURE 1 Research overview. (A) Research overview, (B) Gating strategy and plan of the analysis.

RESULTS

Patient characteristics

During the study period, seven patients (male, $n = 5$; female, $n = 2$; median age, 57 years) who met the inclusion criteria were included in this study (Table 1). The median P/F ratio was 126.8, and four patients had an FiO_2 value of >0.6. All patients had received steroid therapy before blood sampling, and six patients had received antiviral therapy. Three patients underwent prone position therapy. None of the patients underwent extracorporeal membrane oxygenation therapy. The median length of mechanical ventilation was 6 days. All patients survived during their hospitalization,

and the median ICU stay was 11 days. We also included four age- and sex-matched volunteers.

Phenotypic changes in overall hematopoietic cells after GMA treatment

Figure 2 shows a tSNE map and a heatmap indicating the fold change in the expression of multiple activations or functional markers on the cluster of differentiation (CD) 45+ cells. CD45 was expressed on whole myeloid cells. We classified each subset into appropriate myeloid cell groups using CD markers. Granulocytes and monocytes were selectively decreased after GMA in the patient and control groups. Thus, focusing on granulocytes and monocytes, we conducted a detailed investigation to determine the specific cell populations that were selectively eliminated.

TABLE 1 Patient characteristics.

Patient characteristics	COVID-19 patients ($n=7$)	Healthy controls ($n=4$)
Age, years, median (IQR)	57 (55–67)	55.5 (46.5–62)
Sex (male/female)	5/2	3/1
Pre-existing condition		
HT	3	
DM	3	
BMI, kg/m^2	28.4 (23.5–32.9)	
SOFA score	8.0 (3–8)	
Laboratory data (median)		
P/F ratio	126.8 (119.5–261.4)	
White blood cells	9470 (6215–11,805)	
Neutrophil count	7955 (5105–10,851)	
Lymphocyte count	616 (367.7–674.5)	
Monocyte count	235 (208–381)	
Concomitant therapies		
Steroid use before blood sampling	7	
Antivirus drug use	6	
MV	7	
Prone position	3	
General outcomes		
In-hospital mortality	0	
Length of ICU stay (median)	11	
Length of MV (median)	6	

Abbreviations: BMI, body mass index; COVID-19, coronavirus disease 2019; DM, diabetes mellitus; HT, hypertension; ICU, intensive care unit; IQR, interquartile range; MV, mechanical ventilation; P/F, PaO_2/FiO_2; SOFA, sequential organ failure assessment.

Neutrophil subsets selectively removed by GMA treatment

Figure 3 shows the change in granulocyte subsets in the two groups before and after GMA and the expression of the subsets. Granulocytes included neutrophils, eosinophils, and basophils. We found that metaclusters 3 and 4 were selectively decreased after GMA (Figure 3A–D). They were classified as neutrophils, which strongly expressed CD11b, CD16, and CD66b, and weakly expressed CD11c. The expression of CD11b and CD66b indicates activated and stimulated neutrophils. The percentages of metaclusters 8, 10, and 11 in the pre-GMA patient group were lower than those in the healthy controls. Metacluster 10 indicates basophils based on CD expression markers, whereas metacluster 11 indicates CD66b-negative neutrophils.

Monocyte subsets selectively removed by GMA treatment

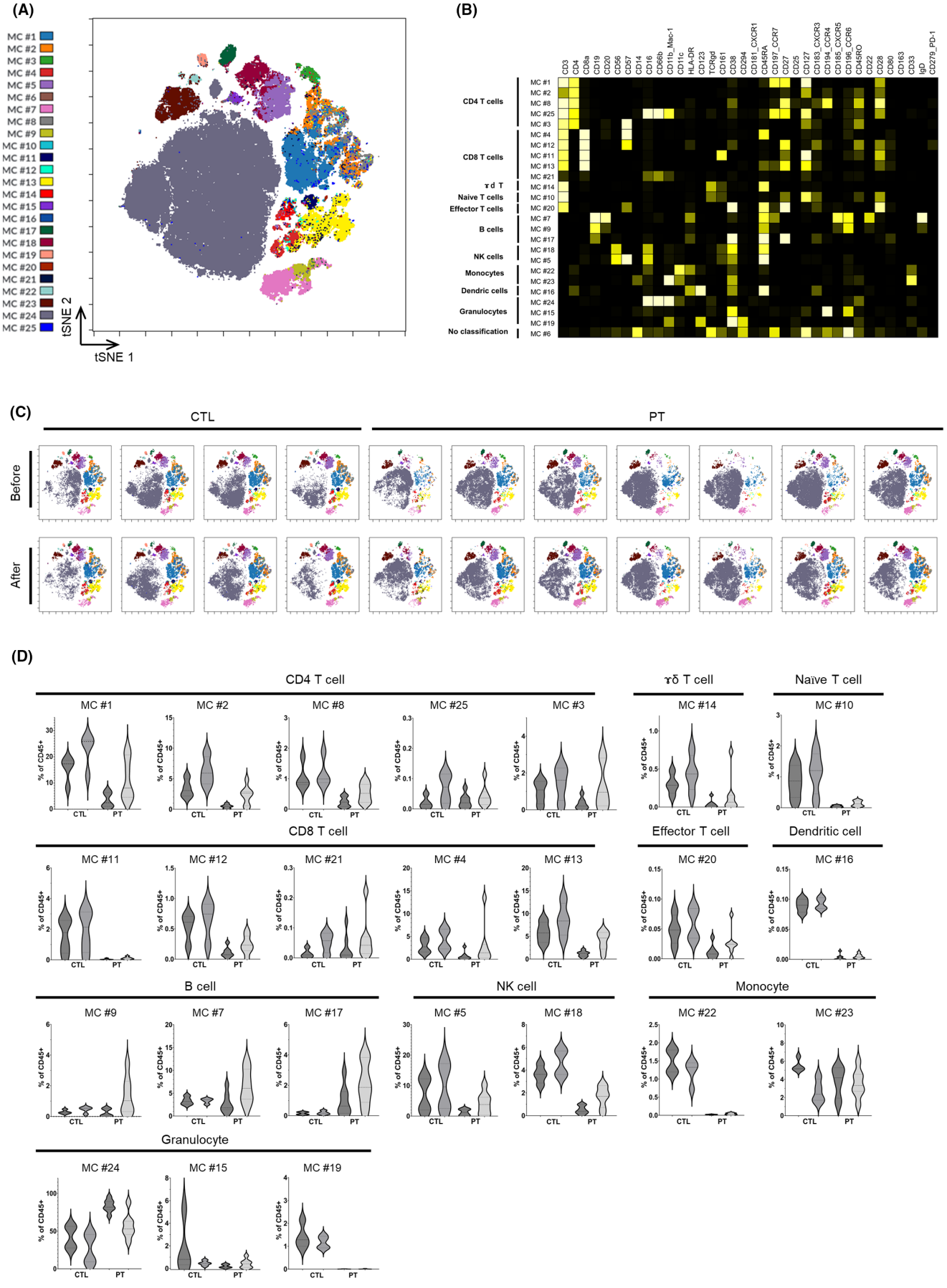
Figure 4 shows the change in the monocyte subsets in the two groups before and after GMA and the expression of the subsets. Monocytes included classical monocytes (CD14+ CD16-), intermediate monocytes (CD14+ CD16+), and nonclassical monocytes (CD14dim CD16+). We found that metacluster 2 was decreased after GMA (Figure 4A–D). They expressed CD14 and did not express CD16, and were therefore classified as classical monocytes. In addition, they expressed CD33 and CD45RO. When CD33 was expressed, CD66b was also expressed, which displayed high phagocytic activity, matrix adhesion, and migration. The expression of CD45RO was associated with T-cell activation.

DISCUSSION

Changes in immune cells in the peripheral blood of patients with severe COVID-19

The pandemic of SARS-CoV-2-induced respiratory disease has caused many deaths around the world. The general immune response against SARS-CoV-2 infection was gradually

FIGURE 2 Characterization of CD45+ hematopoietic cell subsets. (A) tSNE map according to the metacluster group. The tSNE map of CD45+ hematopoietic cells from concatenated data of individuals is shown. (B) Heatmap of each metacluster of CD45+ hematopoietic cells. This heatmap shows the expression intensities of the indicated markers on metaclusters of CD45+ hematopoietic cells. The color bar legend indicates differences in the marker expression levels on metaclusters: yellow (increased) or black (decreased). (C) Changes in metaclusters in the tSNE map by individual. (D) Violin plots for the changes in the percentages in each metacluster in the COVID-19 and control groups. The black dot in the violin is the individual value, and the boxes represent the 25th and 75th percentiles with the median line. The left graph shows the results of both groups before GMA. The right graph shows the results of both groups after GMA treatment. CD, cluster of differentiation; COVID-19, coronavirus disease 2019; CyTOF, cytometry by time of flight; Flow SOM, flow mass cytometry data with a self-organizing map; GMA, granulocyte and monocyte adsorptive apheresis, MC, metacluster; tSNE, t-distributed stochastic neighbor embedding.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CD194_CCR4
CD185_CXCR5
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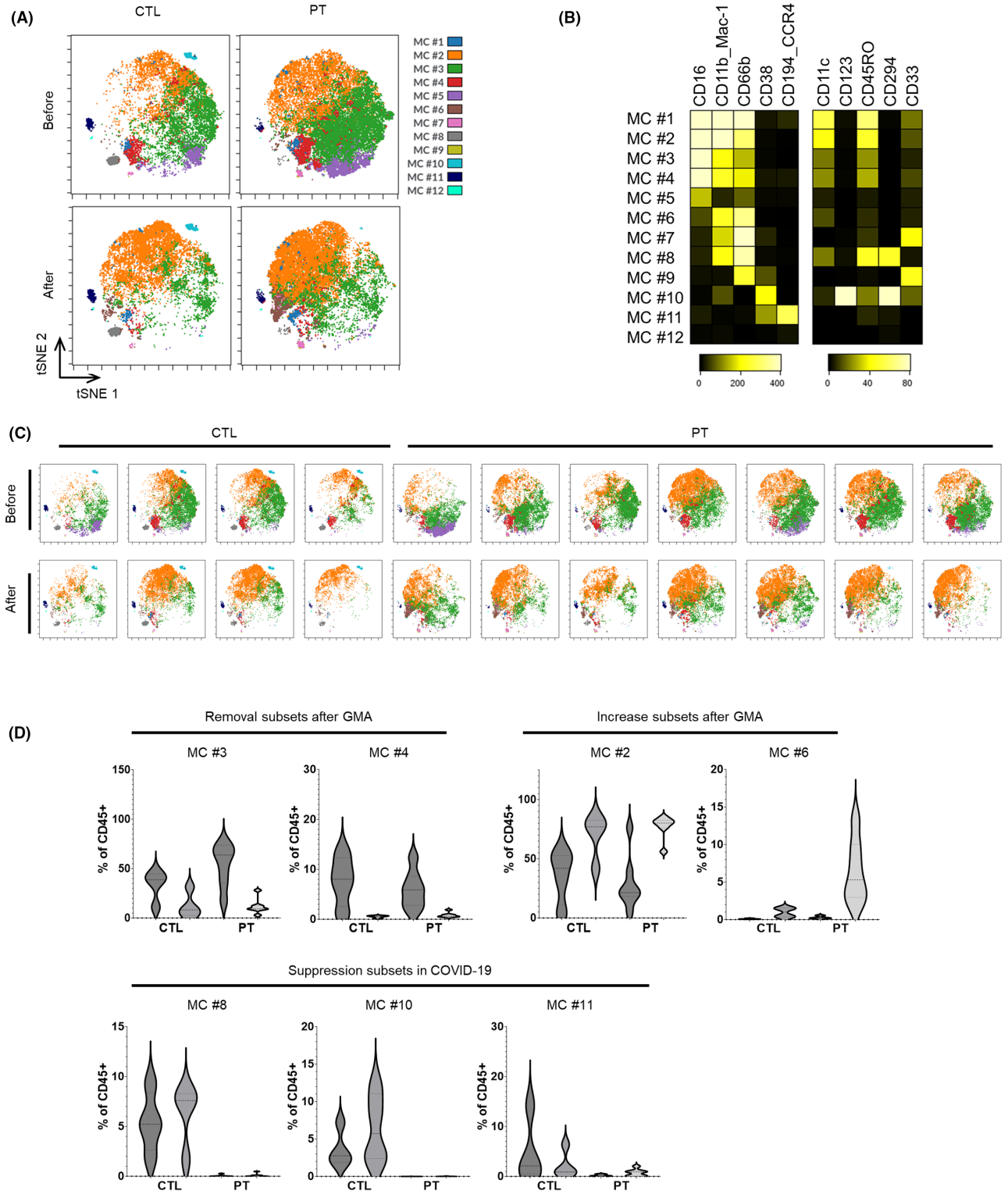


FIGURE 3 Characterization of granulocyte subsets and trends of subsets after GMA. (A) tSNE map by metacluster group of granulocytes in each COVID-19 patient and non-COVID-19 control before and after GMA. Each tSNE map of granulocytes from concatenated data of each COVID-19 patient and non-COVID-19 control before and after GMA is shown. (B) Heatmap of each metacluster of granulocytes. This heatmap shows the expression intensities of the indicated markers on metaclusters of granulocytes. The color bar legend indicates differences in marker expression levels on metaclusters: yellow (increased) or black (decreased). (C) Changes in metaclusters in the tSNE map by individual. (D) Violin plots for changes in percentages in each metacluster in the COVID-19 and control groups in granulocytes. The black dot in the violin is the individual value, and the boxes represent the 25th and 75th percentiles with the median line. The left graph displays both groups before GMA treatment, while the right graph displays both groups after GMA treatment. CD, cluster of differentiation; COVID-19, coronavirus disease 2019; CyTOF, cytometry by time of flight; Flow SOM, flow mass cytometry data with a self-organizing map; GMA, granulocyte and monocyte adsorptive apheresis; MC, metacluster; tSNE, t-distributed stochastic neighbor embedding.

elucidated by several studies.[12,13] During the acute phase of SARS-CoV-2 infection, immune cells are activated and generated due to the secretion of inflammatory cytokines.[13] Systemic inflammation and dysregulation of the immune system response occur as a consequence of the overproduction of inflammatory mediators.[14] This immune response induces the expansion of neutrophils, monocytes, and Th1 T cells and the suppression of Tregs and M2 macrophages.[13] Additionally, CD8+ T cells and natural killer cells are exhausted, which causes immune pararesis.[13,15] Our results were almost identical to these previously reported studies. Almost all metacluster subsets of T cells in the COVID-19 group were decreased in comparison to the control group.

Granulocytes account for >50% of the total leukocytes in COVID-19 patients' peripheral blood. In neutrophils, SARS-CoV-2 stimulates a hyperactive state to promote hyperinflammation.[16] The hyperactive neutrophils seemingly expressed an immature phenotype.[17] Our results showed that the proportion of cells, which are considered to be composed of activated immature neutrophils, was particularly elevated in the COVID-19 group.

In monocytes, the proportion of nonclassical monocytes is increased in the peripheral blood of patients with severe COVID-19 infection.[18] A higher fraction of circulatory nonclassical monocytes was associated with increased polarization toward Th1 cells with procytolytic activity.[18] Our results showed an increased proportion of classical subsets. Additionally, all subsets of nonclassical monocytes, which are considered to be anti-inflammatory cells, were suppressed in the COVID-19 group.

In the COVID-19 group, the proportions of hyperactivated neutrophils and monocytes were increased, whereas the proportion of anti-inflammatory monocytes was suppressed. This inflammatory network was thought to play an important role in immune dysfunction.

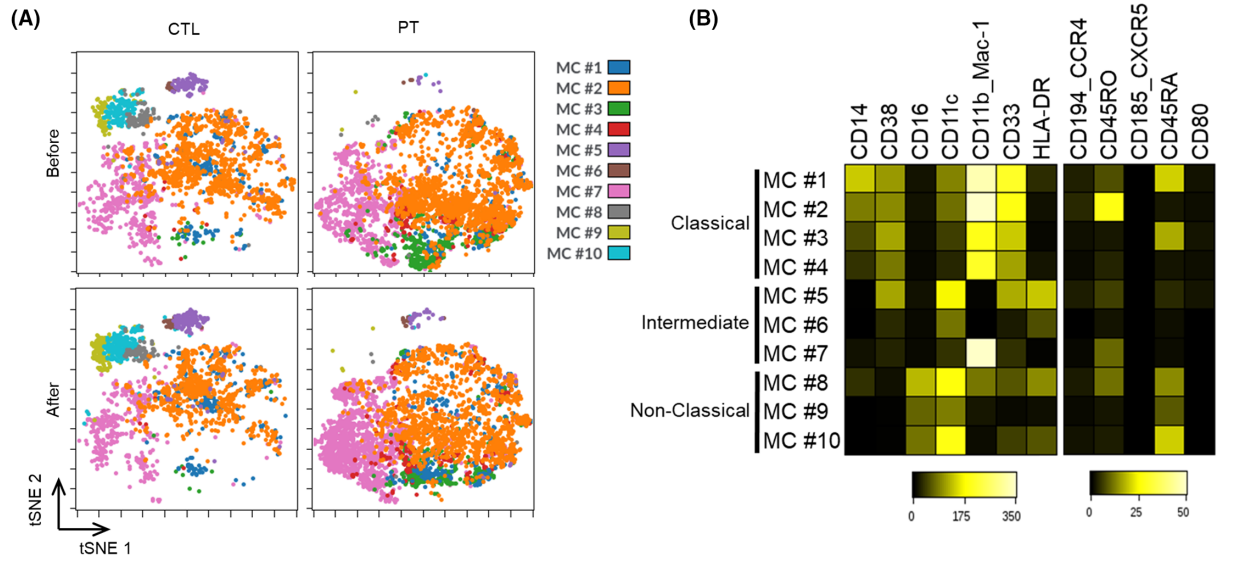
Predicted effectiveness of GMA for COVID-19 patients

GMA is an extracorporeal apheresis technique that selectively removes approximately 65% of activated granulocytes and 55% of monocytes/macrophages along with a small number of platelets. Indeed, inflammatory cytokines, such as interleukin (IL)-6, IL-8, IL-23, and tumor necrosis factor α (TNF-α), are decreased after GMA.[19] Our study identified the subsets of granulocytes and monocytes that are removed after GMA. In granulocytes, the expression of CD66b and CD11b was associated with neutrophil activation. CD66b contributes to emergency granulopoiesis.[20] Neutrophils that acquire the high expression of CD11b exhibit adhesion, chemotaxis, and extravascular migration.[21] These results suggested that the removed subsets of granulocytes were activated neutrophils. In particular, these subsets weakly expressed CD11c. As CD11c on neutrophils was recently discovered, its function on neutrophils is not completely understood. Hou et al. reported that CD11c regulated the maturation and effector function of neutrophils.[22] Additionally, they reported that neutrophil maturation was impaired in the absence of CD11c, causing a significant increase in the proliferation and apoptosis of pre-neutrophils. Our results may indicate the heterogeneity of mature and activated neutrophils based on the difference in the expression of CD11c.

CD14+ CD33+ CD66b + classical monocytes are reported to have higher phagocytosis, matrix adhesion, and migration abilities.[23] Our results suggested that there was heterogeneity in classical monocytes. Classical monocytes have a strong expression of chemokine receptors, which have enhanced adherence to damaged and inflamed tissues and secrete inflammatory substances such as IL-6 and IL-8.[23] In particular, our results found that the CD45RO-expressing subset of classical monocytes was removed after GMA. CD45RO is a low-molecular-weight isoform of the receptor.[24] CD45RA/RO is generally expressed on T cells; therefore, their function has gradually been clarified in T-cell studies. The main findings explain that the CD45RA expression on naive T cells is replaced by CD45RO when they mature into memory T cells.[24] CD45RO supports efficient signal transduction and activation of T cells.[13] In the activated monocytes, CD45RO enhances both T-cell receptor and B-cell receptor signaling-mediated activation. However, the function of the CD45RO/RA isoform expression on monocytes is not yet understood. The CD45 isoform on monocytes will be a subject of future study.

Influence of steroid therapy on the immune system in COVID-19 patients

Severe COVID-19 patients requiring oxygen typically exhibit pathophysiological features such as diffuse alveolar damage, inflammatory infiltrates, and microvascular thrombosis.[25] These pathophysiological effects have been associated with the response of the immune system, including that of neutrophils.[26] Based on the results from the RECOVERY trial,[27] dexamethasone is widely used as a standard treatment to control inflammation in COVID-19. Recently reported findings regarding the effects of dexamethasone on immune cells in COVID-19 show that in neutrophils, dexamethasone can suppress interferon-stimulated genes and activate IL-1R2+ while also stimulating the expansion of immunosuppressive immature neutrophils.[28] In monocytes, despite the significant expansion of CD14+ CD16+ populations producing IL-6 observed in the peripheral blood in COVID-19 infections,[29] corticosteroid administration was associated with an increased proportion of CD14++CD16 + CD163+ monocyte cells. They could represent a long-lived functional phenotype that may be important in terminating the inflammatory response.[30] However, when patients were transported to our hospitals, almost all of them were already receiving steroid treatment, making it difficult to explore the association between steroid administration and GMA treatment.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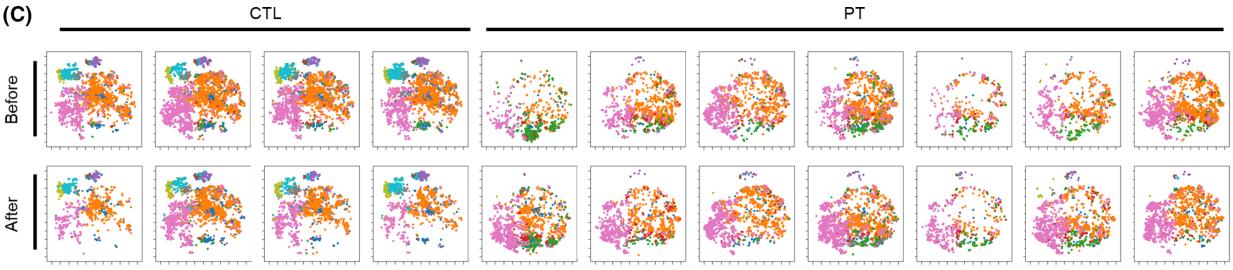
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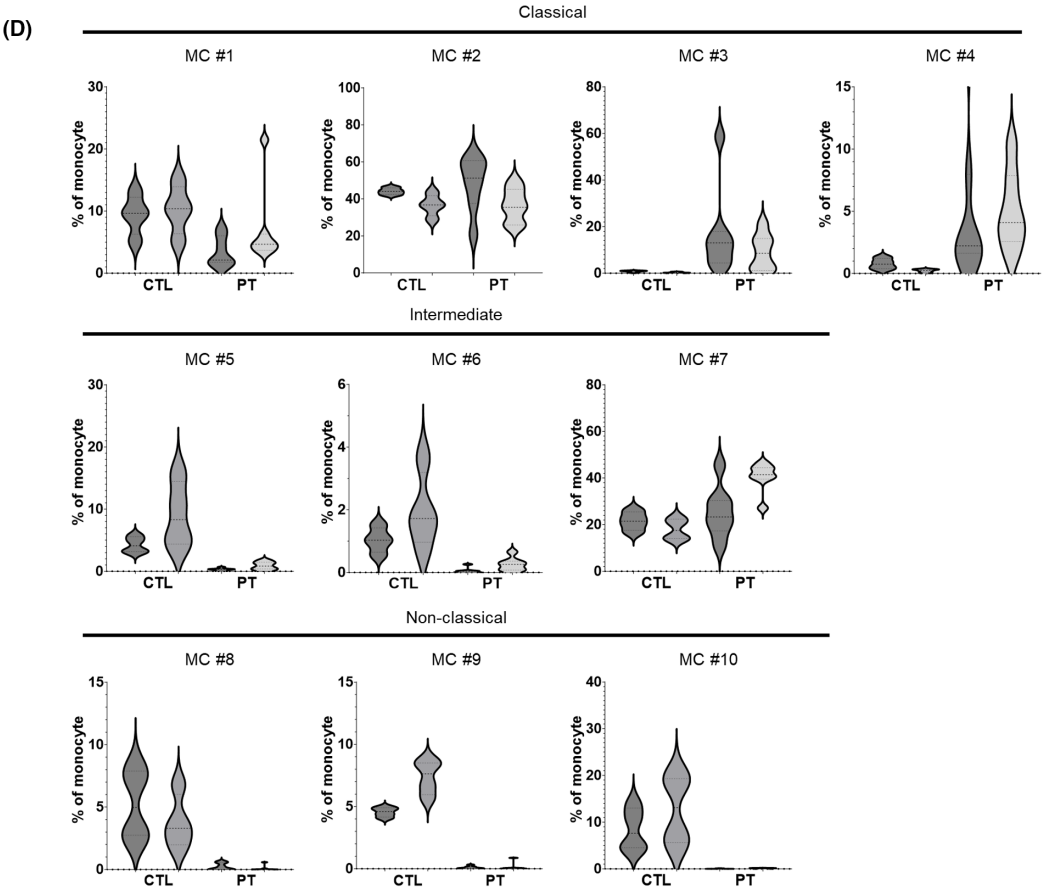
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% of monocyte
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FIGURE 4 Characterization of the monocyte subsets and trends of the subsets after GMA. (A) A tSNE map by metacluster group of monocytes in each COVID-19 patient and non-COVID-19 control before and after GMA. Each tSNE map of monocytes from concatenated data of each COVID-19 patient and non-COVID-19 control before and after GMA is shown. (B) Heatmap of each metacluster of monocytes. This heatmap shows the expression intensities of the indicated markers on metaclusters of monocytes. The color bar legend indicates differences in marker expression levels on metaclusters: yellow (increased) or black (decreased). (C) Changes in metaclusters in the tSNE map by individual. (D) Violin plots for changes in the percentages of monocytes in each metacluster in the COVID-19 and control groups. The black dot in the violin is the individual value, and the boxes represent the 25th and 75th percentiles with the median line. The left graph displays both groups before GMA treatment, while the right graph displays both groups after GMA treatment. CD, cluster of differentiation; COVID-19, coronavirus disease 2019; CyTOF, cytometry by time of flight; Flow SOM, flow mass cytometry data with a self-organizing map; GMA, granulocyte and monocyte adsorptive apheresis; MC, metacluster; tSNE, t-distributed stochastic neighbor embedding.

CONCLUSIONS

This was the first report to identify the subsets of granulocytes and monocytes that are removed after GMA in patients with severe COVID-19. Elimination by GMA of the CD45RO/RA isoform expression in monocytes may be associated with the maturation of monocytes. The removal of these cell populations may be one of the potential mechanisms underlying the effectiveness of GMA treatment for COVID-19 and other inflammatory diseases.

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CONFLICT OF INTEREST STATEMENT

KY reported receiving grants from JIMRO Co. Ltd. No other disclosures are reported. AT is an Editorial Board Member of AMS Journal and a co-author of this article. To minimize bias, they were excluded from all editorial decision-making related to the acceptance of this article for publication.

DATA AVAILABILITY STATEMENT

The datasets analyzed during the current study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

Approval of the research protocol: The study protocol was approved by the Institutional Review Board of Osaka University (approval number: T20173).
Informed Consent: Written informed consent was obtained from the patients or their close relatives and the volunteers.
Registry and the Registration No. of the study/trial: N/A.
Animal studies: N/A.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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