Relationship Between Blood Immune Cell Composition and COVID-19 Susceptibility and Post-Infection Sequelae Using Full-Spectrum Flow Cytometry

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ABSTRACT

Objective: To investigate the immune mechanisms underlying susceptibility to COVID-19 and the development of sequelae.
Study Design: A cross-sectional observational study.
Place and Duration of the Study: Clinical Laboratory, Jilin Province People's Hospital, Jilin, China, from 14th to 30th December 2022.
Methodology: Seventy-one individuals were investigated, including 56 individuals who had recovered from COVID-19 infection and 15 uninfected individuals. Immunophenotypic analysis of B, T, and NK lymphocytes in peripheral blood samples was performed using full-spectrum flow cytometry. The differences in immune cell populations defined by the traditional gating strategy and the unsupervised algorithm between groups were analysed. Categorical data were compared between groups using the chi-square test. Quantitative data were compared using the t-test or Mann-Whitney U test.
Results: Compared to uninfected individuals, recovered individuals, especially those with sequelae, had significantly higher counts of lymphocytes, T cells, NKT cells, CD8+T cells, CD4+CD28+T cells, CD4+CD38+T cells, CD8+CD28+T cells, CD8+CD38+T cells, CD69+T cells, and Tregs. An increase in these lymphocytes may contribute to immune homeostasis. Meanwhile, the CD38+NKT cell count was significantly decreased in the recovered individuals. Moreover, an upregulation of T-cells, NKT cells, CD4+T cells, CD8+T cells, CD4+CD28+T cells, CD4+CD38+T cells, CD8+CD28+T cells, CD8+CD38+T cells, CD69+T cells, Treg cells, and CD38+NKT cells was observed in patients with sequelae.
Conclusion: This study showed that the lymphocyte subgroups' counts were associated with COVID-19 infection and post-infection sequelae. These findings may contribute to understanding the mechanism of COVID-19 infection and recovery.

Key Words: *COVID-19, Full-spectrum flow cytometry, Traditional gating strategy, Unsupervised algorithm, Blood immune cells, Susceptibility, Sequelae.*

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INTRODUCTION

Following the detection of SARS-CoV-2 in 2020, the virus precipitated a global health crisis as COVID-19 infections proliferated internationally.[1] Up to now, COVID-19 has infected about 765 million people and caused 7 million deaths worldwide, according to the data reported by the WHO. The median mortality rate, from asymptomatic to severe symptoms, was 0.27%.[2] After China ended its COVID-19 restrictions in late 2022, the majority of Chinese contracted COVID-19, leaving only about 15% uninfected. A previous study reported that people under the age of 20 are approximately half as susceptible to infection as those over the age of 20.[3] Blood type A has been related to a higher risk of infection.[4] In addition, host genetic background has also been associated with COVID-19 susceptibility.[5] Noticeably, post-acute sequelae were common in patients who had recovered from COVID-19. For example, post-COVID respiratory assessments conducted after 1–12 months revealed that 5–81% of patients experienced dyspnoea during hospitalisation. Cough appears to be less prevalent than dyspnoea but may persist for weeks following COVID-19 infection.[6] However, factors influencing susceptibility to COVID-19, as well as the factors related to sequelae, remain largely unknown.

Increasing evidence has demonstrated that the immune system is closely implicated in the course of COVID-19 infection.[7] One of the main pathogeneses underlying COVID-19 is its potent suppression of innate immunity.[8] Upon infection, activated B, CD4+, and CD8+ T lymphocytes react to viral entry and then produce protective immunity.[9]

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During the inflammatory response, the counts of NK and T lymphocytes are decreased, while the levels of pro-inflammatory cytokines are increased following COVID-19 infection.[10] It has been reported that CD3+CD4+ and CD+4RO+ cell counts increase as the disease progresses, resulting in lower C-reactive protein (CRP) levels, which may cause immune response homeostasis and COVID-19 recovery. Interestingly, NK cells, as innate effector lymphocytes, not only respond to acute viral infections but also contribute to immunopathology.[11] Lu *et al*. revealed that envelope (E) protein-mediated NKT cell function evasion may be an essential causative factor for the increased toxicity of coronaviruses.[8] Maucourant *et al.* found that adaptive NK cell levels were elevated in the blood of critically ill COVID-19 patients.[10] Existing studies indicate that the composition of an individual's immune cells may be a key factor influencing COVID-19 susceptibility and the occurrence of post-infection sequelae.[12] However, relevant research remains insufficient, and additional studies are urgently needed.

This study aimed to evaluate the blood immune profiles of patients who had recovered from COVID-19 and of uninfected individuals, and to explore the relationship between lymphocyte subclusters and sequelae following COVID-19 infection.

METHODOLOGY

A total of 56 patients who had recovered from COVID-19 infection were randomly recruited in the Clinical Laboratory, Jilin Province People's Hospital, Jilin, China, from 14 to 30 December 2022. Inclusion criteria were patients whose nucleic acid test results had turned from positive to negative, and who completed at least one month of follow-up monitoring for post-infection sequelae after nucleic acid test conversion to negative. Exclusion criteria were patients who had other diseases or did not sign the informed consent, and patients lost to follow-up after sampling. This study is a cross-sectional observational study. Five to ten mL of fresh peripheral blood from each patient, collected when their nucleic acid tests were negative, were placed in EDTA-containing tubes and analysed by full-spectrum flow cytometry (FSFC) on the same day in the Jilin Province People's Hospital. Infected patients who had mild symptoms (*i.e.*, cough, fever, or sore throat) within 30 days after their negative nucleic acid test result were considered to have sequelae. Participants who had been in close contact with people infected with COVID-19 for at least one month and were confirmed not to have COVID-19 at the time of this study were recruited as the Control group. Uninfected individuals were tested by nucleic acid test three times per week and had never shown a positive result. The tests were performed using throat swabs, rather than peripheral blood, in China. All individuals in this study were vaccinated with the *SARS-CoV-2* vaccine (inactivated, Vero cell) in March, April, and November 2021, respectively. This study adhered to the Declaration of Helsinki guidelines and was approved by Jilin Provincial People's Hospital, Jilin, China (No. 2022026). All patients/participants provided written informed consent to participate in this study.

Immunophenotypic analysis of B, T, and NK lymphocytes in peripheral blood samples was performed using FSFC.[13] In detail, non-brilliant violet (BV) antibodies (supplementary Table I), mixed BV antibodies (supplementary Table I), and brilliant staining buffer were added to peripheral blood samples and incubated at room temperature in the dark for 15 minutes. Lysis buffer was then added to each sample, which was centrifuged, and the supernatant was discarded. The samples were washed with phosphate-buffered saline. Finally, the supernatant was eliminated through centrifugation, and the processed samples were analysed using FSFC according to the manufacturer's instructions and default parameters. Prior to acquisition, instrument setup and Quality Control for the FSFC were performed per the manufacturer's recommendations. Samples were run at a medium flow rate (averaging 800 events/s for the sample concentration used; ~1.5 million/ml). The gating strategies for immune cells are shown in Figure 1, and the gates were set based on isotype.

Beyond conventional manual flow cytometry gating, unsupervised high-dimensional cytometry profiling was implemented using algorithmic approaches. The compensation matrix was thoroughly analysed in FlowJo by examining the N-by-N view feature, as well as the pairwise expression of all stained proteins. Samples were combined and evaluated using FlowJo plugins (available at https://flowjo.com/exchange/#/). Pheno-Graph analysis was conducted with the default number of nearest neighbours set to 45 (K = 45). Pheno-Graph clusters were visualised using t-SNE, implemented with the cytofkit package and default parameters (https://dpeerlab.github.io/dpeerlab-website/phenograph.html).

Categorical data were compared between groups using the chi-square test. Quantitative data were compared using the t-test or Mann-Whitney U test. A p-value of <0.05 was considered to be significantly different. Graphs were generated using Prism 8 (v8.2.0) and Adobe Illustrator 2021.

RESULTS

This study recruited 71 participants, comprising 25 males and 46 females, with ages ranging from 21 to 57 years (median: 32). According to infection status, 56 patients were recovered from *SARS-CoV-2* (Recovered group), and 15 individuals were uninfected at the time of this study (Uninfected group). Among the 56 COVID-19-infected cases, the median age was 32 years (ranged: 21-56), of which 15 were male. Comprised to uninfected individuals until this study, a significantly higher proportion of females (73.2% *vs*. 33.3%) was observed in the *SARS-CoV-2* infected patients. No significant disparity in age was observed between *SARS-CoV-2*-positive cases and uninfected individuals until this study (Table I).

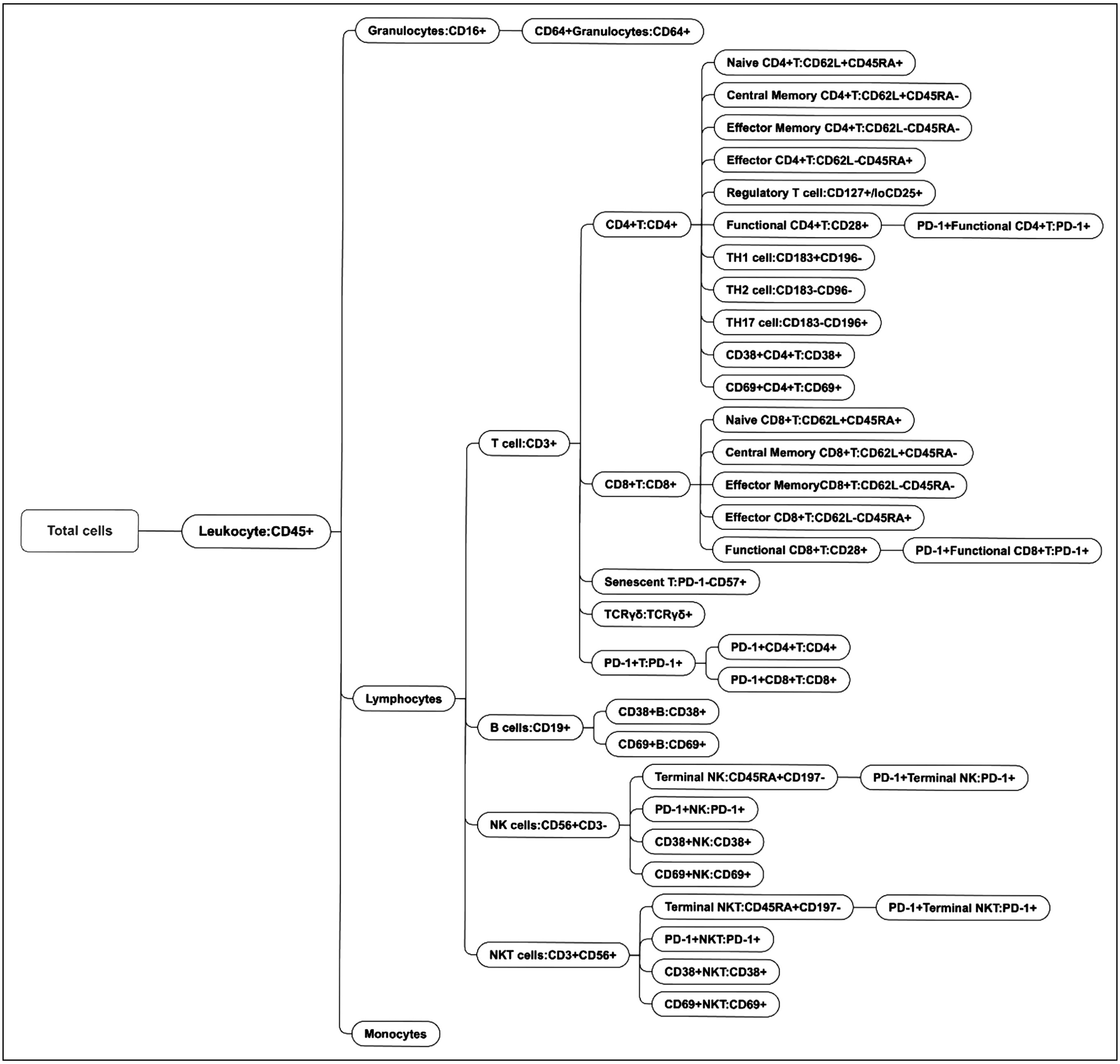

Figure 1: Gating strategies for immune cells.

Then, this study measured the immune cell populations between recovered patients and uninfected individuals. Immune cells were analysed using a traditional gating strategy (Figure 1). The results showed that the counts of lymphocytes, T cells, NKT cells, CD8+T cells, CD4+CD28+T cells, CD4+CD38+T cells, CD8+CD28+T cells, CD8+CD38+T cells, CD69+T cells, and regulatory T (Treg) cells were significantly higher in recovered patients compared with uninfected individuals, while the CD38+NKT cell count was significantly decreased (Figure 2A). Additionally, this study compared the ratios of Tregs to CD4 effector cells and CD4 to CD8 cells between uninfected patients and recovered patients. The results showed that, compared with uninfected patients, recovered patients demonstrated a significantly higher Tregs/CD4 effector cells ratio. Albeit not statistically significant, the recovered patients demonstrated a relatively low ratio of CD4/CD8 compared to that of uninfected patients (Figure 2B). These results suggested that recovered patients may have higher percentages of CD8+T cells and Treg cells compared to uninfected patients.

To gain a deeper understanding of the immunophenotypes of different groups, this study combined fluorescence data from 10 samples of recovered patients and 10 samples of uninfected individuals. This combined dataset was then analysed using PhenoGraph, a method capable of accurately identifying distinct cell subpopulations. PhenoGraph revealed the presence of 45 putative subpopulations (Pop0-Pop44) in both recovered patients and uninfected individuals.

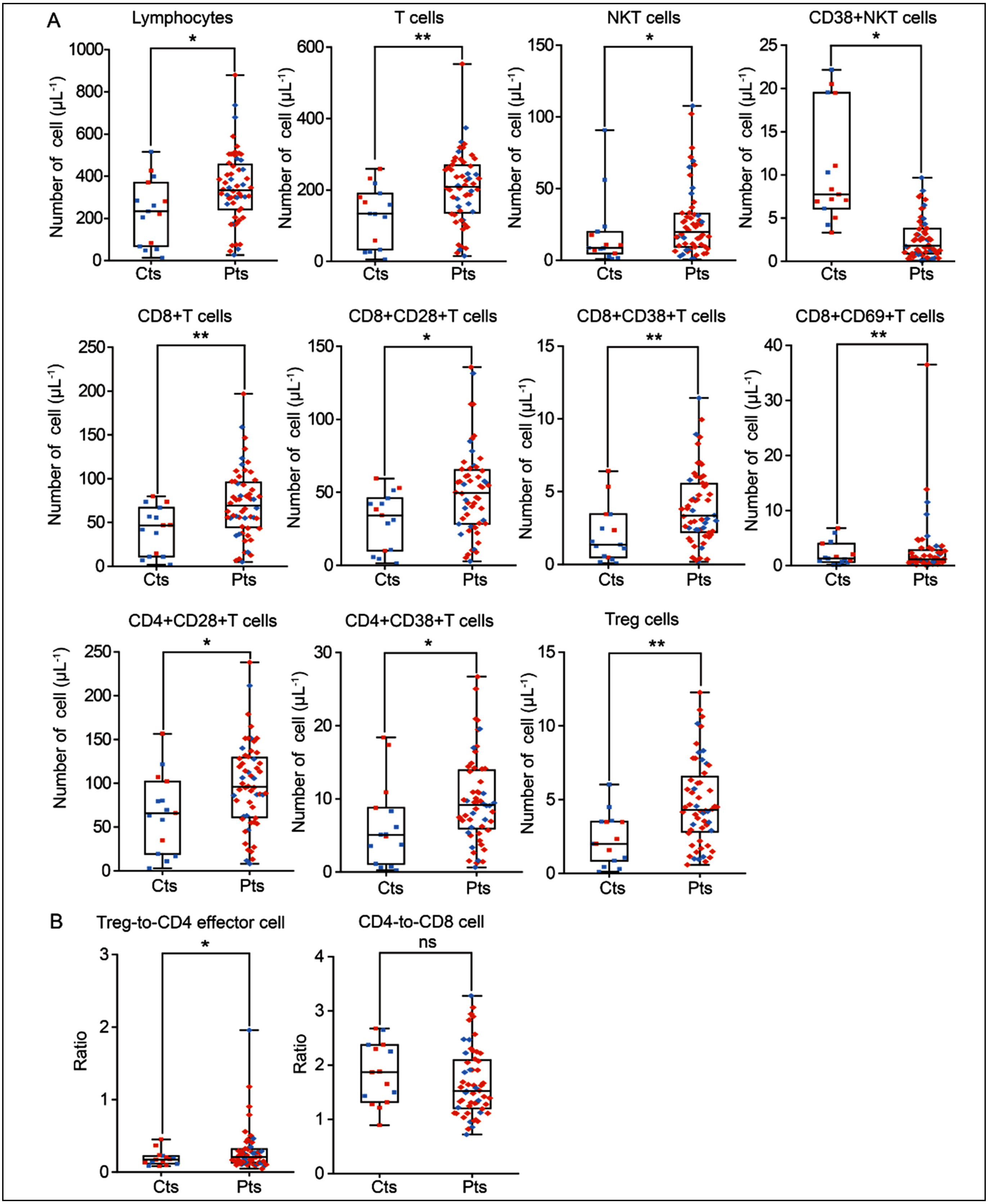

Figure 2: Numbers of immune cells in recovered patients and uninfected individuals until this study.

Samples from 15 uninfected controls and 56 recovered COVID-19 patients were collected, and FSFC was performed to measure immune cells numbers in blood. Box plots show the medians (middle line) and first and third quartiles (boxes). Blue represents males, and red represents females. Immune cell count were compared using t-test or either the Mann-Whitney U test, as appropriate. A p-value <0.05 was considered statistically significant.

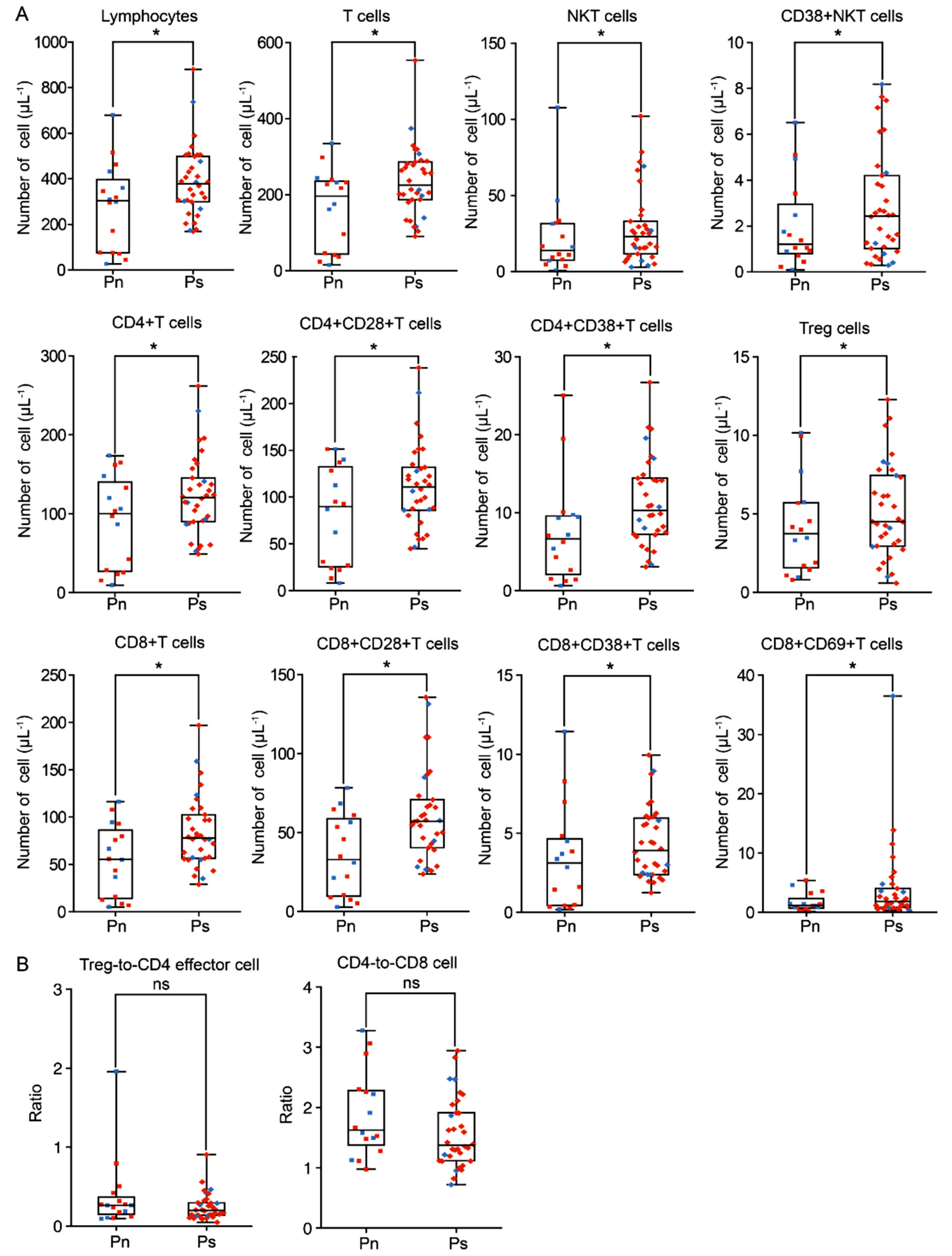

Figure 3: Immune cell numbers in recovered patients with or without sequelae.

(A) Samples from 16 recovered patients with no sequelae and 34 recovered patients with mild sequelae were collected, and assays were performed to measure immune cell numbers in blood. Box plots show the medians (middle line) and first and third quartiles (boxes). Blue represents males, and red represents females. Immune cell numbers were compared using either the t-test or the Mann-Whitney U test, as appropriate. A p-value <0.05 was considered statistically significant.

Supplementary Table I: Non-brilliant violet (BV) antibodies and mixed BV antibodies.

Markers	Antibodies
CD45	Percp-if680
CD14	mFV450
CD16	IF430
CD3	FITC
CD19	iFluorR662/713
CD4	PE-IF710
CD8	SB645
CD56	BV750
TCRγδ	Percp-ef710
CD25	AF700
CD127	PE-cy5
CD45RA	mFV580
CD62L	PE-cy7
CD196	SB436
CD183	BV785
CD28	PE-IF594
CD38	APC-cy7
CD69	SB600
PD-1	SB702

Table I: Baseline characteristics of study participants.

Features	Uninfected individuals (n = 15)	Recovered patients (n = 56)	p-values
Gender, n (%)			0.0063
Female	5 (33.3)	41 (73.2)	
Male	10 (66.7)	15 (26.8)	
Age (years), median (IQR)	33 (27, 38)	32 (24.75, 36)	0.4229

Categorical variables are presented as n (%), and the chi-square test was applied.

Table II: Clinical features of recovered patients with and without sequelae.

Features	Recovered patients without sequelae (n = 16)	Recovered patients with sequelae (n = 34)	p-values
Gender, n (%)			0.1627
Female	10 (62.5)	28 (82.4)	
Male	6 (37.5)	6 (17.6)	
Age (years), median (IQR)	35 (29.5, 39.5)	29 (24, 34.75)	0.0321
Days from diagnosis to recovery (days)	7.00 ± 4.46	7.00 ± 3.86	0.9568
Days from recovery to sampling (days)	19.00 ± 4.17	17.00 ± 3.51	0.1466

Continuous variables are presented as mean (SD) ± median (IQR), while categorical variables are presented as n (%). Independent t-test, Mann-Whitney U test, and chi-square tests were applied.

To define the phenotypic characteristics associated with PhenoGraph clustering, the integrated median fluorescence intensity (iMFI) values for individual markers were determined. Based on quantitative results, this study identified distinct immune cell populations. The results showed that the proportion of Pop4 (effector CD8+T cells), Pop6 [terminally differentiated effector memory (Temra) CD4+T cells], Pop20 [NK cells (CD16-CD38+CD25-)], Pop27 [NK cells (CD16-CD25+CD38-)], and Pop43 [effector memory CD4+T cells] was significantly higher in recovered patients.

Among 56 recovered patients, the median time to COVID-19 turning negative was 6 days (range, 1-21 days). The median interval between COVID-19 turning negative and blood collection was 18 days (range, 9-25 days). Furthermore, 34 of 56 recovered patients had mild sequelae, including cough, fever, and sore throat, with a median age of 29, while 16 patients had no sequelae (Table II). Sequelae information was unavailable for the remaining six patients. No significant differences were found in age, time to COVID-19 turning negative, or the interval between COVID-19 turning negative and blood collection between recovered patients with or without sequelae. The counts of lymphocytes, T cells, NKT cells, CD4+T cells, CD8+T cells, CD4+CD28+T cells, CD4+CD38+T cells, CD8+CD28+T cells, CD8+CD38+T cells, CD69+T cells, Treg cells, and CD38+NKT cells were significantly higher in recovered patients with sequelae compared to those without sequelae (Figure 3). Additionally, significant differences were not found in the Treg-to-CD4 effector cell ratio. Patients with sequelae demonstrated a trend toward a lower CD4/CD8 ratio compared with those without sequelae, indicating that patients with sequelae may have a higher percentage of CD8+T cells. Also, the unsupervised clustering results showed that the proportion of Pop6 (Temra CD4+T cells) was significantly higher in recovered patients with sequelae.

DISCUSSION

Most individuals are susceptible to COVID-19 and present pauci-symptomatic (*i.e.*, mild symptoms); however, few studies have characterised the immunological profiles of individuals who have recovered from COVID-19 or those with low susceptibility. This study described the blood immune cell profile in patients recovered from COVID-19 using both a traditional gating strategy and an unsupervised algorithm. This study also contrasted the immune profiles of individuals not susceptible to COVID-19 (uninfected) and patients recovered from infection, as well as recovered patients with or without sequelae. These findings may contribute to understanding of the mechanism of COVID-19 infection and recovery.

First, the clinical and immunological factors influencing the susceptibility to COVID-19 were explored. The results demonstrated a significantly higher proportion of females (73.2% *vs*. 33.3%) among *SARS-CoV-2*-infected patients; however, this finding was inconsistent with previous studies.[14] This inconsistency may be attributable to the small sample size and therefore cannot be excluded; future multicenter cohort studies are recommended. Importantly, a full understanding of the specific immune responses associated with COVID-19 infection is quite important for disease surveillance and targeted therapy.[15] Several studies have identified a unique immune signature in patients with COVID-19.[7] Notably, T cell immunity plays a dominant role in maintaining immune control of chronic infections. CD4+ and CD8+ cells may protect COVID-19 patients from severe illness and support remission.[16] This study showed that the numbers of certain T cell subclusters, such as activated CD4+ T cells (CD4+CD38+) and cytotoxic T cells (CD8+CD38+), were significantly higher in 56 recovered patients. Previous studies have shown that most recovered COVID-19 patients develop broad and durable immunity after infection,[17] which may partly explain the current study's findings. However, several studies have reported contrasting findings. For example, Gil-Manso *et al*. revealed that recovered patients exhibited a lower count of activated CD4+HLA-DR+CD38+T-cell subsets.[18] This discrepancy may be attributable to heterogeneity within the cohort of recovered patients.

Next, NKT cells (CD3+CD56+) play an intriguing role in predicting COVID19 severity. This study revealed that recovered patients had a significantly elevated count of NKT cells compared to individuals who had not been infected, suggesting a change in T-cell composition due to past infection. Kreutmair *et al*. found that a drop in peripheral blood NKT cell ratio below 2.3% within the first 1-2 days of hospitalisation was a strong predictor for severe disease development.[19] This finding strongly supports that the NKT cell population can serve as a potent biomarker for predicting the severity of COVID-19. Additionally, this finding aligned with a previous report, which demonstrated a decrease in NKT cell levels among patients suffering from severe diseases.[20] However, Zhang *et al*. found that severe patients exhibited a higher percentage of NKT cells.[21] Therefore, a deeper understanding of this specific group may yield valuable insight into the immunological mechanisms linked to severe COVID-19.

Moreover, this study revealed the differences in the number of immune cells between patients with and without sequelae. Remarkably, compared to recovered patients without sequelae, those with sequelae exhibited significantly higher numbers of multiple T cell and NK cell types. LaVergne *et al*. reported that participants with persistent symptoms had significantly higher levels of inflammation at multiple time points during convalescence compared with those who fully recovered from COVID-19.[22] This suggests that persistent inflammation may contribute to the development of sequelae and could drive sustained immune activation in recovered patients. Tregs, a subset of CD4+ T cells, exhibit characteristics such as increased CD25 expression and decreased CD127 expression. Galaan *et al*. observed a 2.5-fold higher frequency of Tregs in COVID-19 patients with sequelae compared with those who fully recovered.[23] This study also reported an increase in Treg numbers in recovered patients with sequelae. On the contrary, another study found a decrease in Treg numbers in COVID-19 patients with sequelae compared with those who recovered completely. Further research is required to elucidate the role of Tregs in the development of COVID-19 sequelae.

In addition, an unsupervised analysis of flow cytometry data was applied to explore new immune cell subpopulations with significant disparities between COVID-19 patients. Using high--dimensional flow cytometry data, this study found that 5 cell subpopulations were more abundant in recovered patients than in uninfected individuals. This study observed that both Pop20 and Pop27 expressed high levels of CD56, which corresponded to NK cells. Significantly fewer NK cells have been reported in patients with severe COVID-19 compared with mildly infected individuals and healthy controls, indicating that NK cells may serve as an indicator for predicting susceptibility to and severity of COVID-19 infection. Additionally, this study identified a specific cell subpopulation (Pop6) that was more abundant in recovered patients compared with uninfected individuals, as well as in recovered patients with sequelae compared with those without sequelae. The phenotype of this subpopulation was CD3 + CD4 + CD28 + CD45 + CD45RA + CD57 + CD127 +, corresponding to Temra CD4+T cells. Vazquez-Alejo *et al*. found that Temra levels were increased in COVID-19 patients and remained elevated after recovery.[24] Similarly, Roldan-Santiago *et al*. reported a strong expansion of Temra cells following COVID-19 infection.[25] These findings confirmed that Temra cell levels increase following COVID-19 infection, and these cells may be potentially associated with sequelae. One major limitation of this study is the small sample size; therefore, future multicentre cohort studies with large sample sizes are recommended.

CONCLUSION

This study found that patients who had experienced post-COVID-19 infections showed dysregulation in various immune subsets, including CD4+T cells and CD8+T cells. Moreover, there was an upregulation of T cells, NKT cells, CD4+T cells, CD8+T cells, CD4+CD28+T cells, CD4+CD38+T cells, CD8+CD28+T cells, CD8+CD38+T cells, CD69+T cells, Treg cells, and CD38+NKT cells in patients with sequelae compared to those without sequelae. These findings may contribute to the understanding of the pathogenesis of COVID-19 infection and recovery.

ETHICAL APPROVAL:

Ethical approval was obtained from the Ethics Committee of the Jilin Province People's Hospital, China (No. 2022026).

PATIENTS' CONSENT:

Written informed consent was obtained from the participants.

COMPETING INTEREST:

The authors declared no conflict of interest.

AUTHORS' CONTRIBUTION:

RX: Investigation, formal analysis, methodology, and writing of the original draft.
XX: Investigation, formal analysis, writing, review, and editing.
XJ: Investigation.
GY: Conceptualisation, methodology, and supervision.
YY: Conceptualisation, methodology, project administration, and funding acquisition.
All authors approved the final version of the manuscript to be published.

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