Comparison of T cell maturation profiles in the 1st and 5th wave of COVID-19 in the Polish population

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Abstract

Background. The coronavirus pandemic has become the most critical global health threat of this century and the greatest challenge to the human population. The search for simple and quick diagnostic methods enabling the identification of patients infected with the SARS-CoV-2 virus may be a valuable method to track infection.

Objectives. The aim of the study was the clinical and immunological characterization of patients by assessing the degrees of maturity of T lymphocytes from the 1st and 5th waves of coronavirus disease 2019 (COVID-19) in comparison to a healthy control group (HC).

Materials and methods. We determined leukocyte and T lymphocyte subpopulations (recent thymic emigrant (RTE), naïve, effector, central memory and effector memory) in patients from the 1st COVID-19 wave (n = 23), the 5th COVID-19 wave (n = 38) and HC (n=20) using a panel of monoclonal antibodies using multiparameter flow cytometry.

Results. We observed a lower median proportion of lymphocytes and NK cells, and elevated percentage and number of neutrophils in patients from the 5th wave compared to the 1st. We found a reduced percentage of $CD4^+$ effector memory cells in the 1st wave group compared to the 5th wave (14.1 vs 23.2, $p < 0.05$), and a higher percentage of RTE and naïve $CD8^+$ cells in the 1st wave compared to the 5th wave ($p < 0.05$). The effector memory $CD8^+$ cells were highest in the 5th wave compared to both 1st wave and HC patients (respectively, 35.1 vs 18.1 vs 19.3%, $p < 0.05$). The 5th wave group showed significantly more differences compared to HC.

Conclusions. Our results showed a clear increase of effector cells with a simultaneous decrease in virgin T cells in the 5th COVID-19 infection. Monitoring lymphocyte subsets during infection allows assessment of the patient's immune status and of readiness of lymphocytes to respond to the immune response, and may be necessary to improve clinical outcomes.

Key words: flow cytometry, effector memory T cells, SARS-CoV-2, central memory T cells, COVID-19 waves

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Background

The principal and emerging new waves of coronavirus disease 2019 (COVID-19) are primarily due to altered virus variants that are rapidly spreading worldwide.[1] They prolong the persistence of infections, causing losses in human health, life and the economy. The development of highly effective vaccines against severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) effectively reduces the risk of infection and disease development. Unfortunately, due to issues related to vaccine hesitancy, availability and distribution, COVID-19 cases cannot be entirely controlled.

The virus causing symptoms of COVID-19 is an enveloped, single-stranded RNA virus whose 5' region is rich in open reading frames and encodes proteins necessary for viral replication. The 3' region contains 5 structural proteins, namely the spike protein (S), membrane protein (M), nucleocapsid protein (N), envelope protein (E), and hemagglutinin-esterase protein (HE).[2] It is responsible for causing an infectious disease with the most common symptoms such as fever, dry cough and fatigue, shortness of breath, loss of taste or smell, and in the case of an acute course of the disease can even lead to death.[3]

The COVID-19 pandemic began in Wuhan, China, in early December 2019, then quickly spread to neighboring countries and, in the following months, appeared in most nations around the world. In this regard, the World Health Organization (WHO) on March 11, 2020 recognized the COVID-19 disease outbreak as a pandemic.[4] The first case of COVID-19 disease in Poland was detected on March 4, 2020, and as of December 2022, 6,351,408 cases of infection and 118,306 deaths have been confirmed.[5] Waves are a distinctive feature of pandemics, with seasonal variability in environmental factors affecting their duration. The start and end of COVID-19 waves were determined based on the number of identified cases of infection calculated based on the weekly incidence rate.[6,7]

The beginning of the 1st wave of COVID-19 in Poland was estimated on March 12, 2020, its duration was 109 days (until June 28) and it differed from the following waves.[8] In most people infected with the SARS-CoV-2 virus, the disease was mild, without symptoms of pneumonia and hypoxia, or in cases of moderate severity, with clinical manifestations of pneumonia, such as fever, cough and shortness of breath. Some infected patients developed severe or critical illness complicated by severe respiratory distress syndrome, sepsis or organ failure.[9] The 1st COVID-19 wave in Poland did not reveal the exact severity of the epidemic, as diagnostics were carried out only in symptomatic cases. In subsequent waves, a lower percentage of patients required hospitalization, they were younger and admitted to the ward for fewer days, with prolonged survival.[10,11] However, during the 2nd wave, twice as many cases and deaths were observed in Poland.[12] The availability of antigen and serological tests for large-scale use has contributed to this. It was found that the course of the disease in patients from the 3rd COIVID-10 wave, infected with the transformed alpha (B.1.1.7) variant, was significantly more severe than in the previous ones.[13,14] The subsequent 4th wave, comprising the next variants of SARS-CoV-2-Delta (B.1.617.2), resulted in a more severe course of the disease, being the most dangerous and having the worst results.[15,16] However, differentiation of SARS-CoV-2 viral variants was also not common in Poland.[17] Vaccination against COVID-19 was introduced at the end of December 2020, with initial availability for healthcare workers, elderly patients and persons with multiple comorbidities.[18] Despite the subsequent widespread availability of vaccines, due to high uncertainty and skepticism about the preparations, only approx. 50% vaccination coverage in Poland population was recorded.[19]

During the formation of the 5th wave, the SARS-CoV-2 transformed into BA.5 SARS-CoV-2 Omicron variant with higher infectivity but less virulence and a milder course of the disease with few clinical symptoms.[20] In Poland, the 5th COVID-19 wave began in the winter of 2021. It was the shortest of all, lasting 90 days, with the number of infected people being over 1.75 million.[8]

The 2 main pathways of immune response to pathogens are innate and acquired immunity. The innate immune response involves NK cells, complement and interferon components, and immunoglobulin A secreted in body fluids.[21] The acquired or adaptive immune response is triggered by viral replication. Intracellular viral antigens are presented to $CD8^+$ T cells in combination with MHC class I antigens, which in turn causes division and maturation of lymphocytes into both effector and memory cells. Contact with a foreign antigen turns lymphocytes into effector and central memory cells.[22] Effector T cells can directly kill virus-infected cells, while central memory cells can be activated after subsequent re-contact with the antigen and become memory effector cells or central memory cells.[23] The viral antigen-responsive $CD8^+$ T cells play a key role by identifying and killing virus-infected cells. These T cells with cytotoxic properties are active for a short time, and, after the elimination of the virus hidden in host cells, quickly disappear. Long-lived memory T cells, which activate very quickly after repeated contact with the virus, create a long-term immune response. Healthy people, not burdened with additional diseases, potentially destroy the virus after it enters their bodies and do not develop a targeted immune response.[23]

It is interesting to compare the immune status of patients from different waves of the epidemic. In particular, the evaluation of effector and memory cells may indicate the state of readiness of the patient's immune response to virus infection. Very little is known about the impact of different lymphocyte subsets on the immune response of COVID-19 patients or its consequences. We examined immunological parameters by assessing the expression of cell surface markers in lymphocyte subsets using a flow cytometer. The contribution of T cells to the establishment of long-lasting protective immunity against reinfection

in future epidemics is an important aspect of the T cell response that requires investigation. In addition, the results obtained from both groups of COVID-19 patients were compared to a healthy control (HC) group.

Objectives

This study aimed to examine the host cellular immune response, including memory and effector cell subsets, in COVID-19 patients admitted to the Department of Infectious Diseases and Allergology of the Military Institute of Medicine–National Research Institute in Warsaw in different waves of the pandemic in Poland. We focused on assessing T cell subpopulations that play a significant role in the antiviral response involving a specific immune reaction in people infected with the SARS-CoV-2 virus.

Materials and methods

Patients

The analyzed group was composed of Polish patients from 2 COVID-19 waves, the 1st wave of COVID-19 (tested from May 2020 to August 2020) and the 5th (December 2021 to April 2022). According to the WHO guidelines, patients with SARS-CoV-2 underwent real-time polymerase chain reaction (PCR) tests from nasopharyngeal swab samples. Patients positive for SARS-CoV-2 were admitted to the Department of Infectious Diseases and Allergology at the Military Institute of Medicine (Warsaw, Poland).

Inclusion criteria were as follows: adults over 18 years of age with laboratory-confirmed SARS-CoV-2 infection, meeting criteria for hospital admission for COVID-19, with an oxygen saturation of 94% or less. Additionally, based on oxygen demand, patients according to result on an ordinal scale were classified as: a hospitalized patient, not requiring supplemental oxygen but requiring medical attention (score 4) or hospitalized requiring normal oxygen supplementation (score 5) or non-invasive ventilation with high flow oxygen equipment (rated 6). Patients with acute respiratory distress syndrome (ARDS) at baseline were excluded. This 8-point scale is based on WHO recommendations modified to fit the specificity of the Polish healthcare system.

For the final analysis, we did not include any asymptomatic patients or those receiving corticosteroids, which may affect blood cell counts and possibly also lymphocyte subsets.

The 1st wave COVID-19 group consisted of 23 patients. There were 9 women and 14 men with a mean age of 55.9 ±18.2 years. The 5th COVID-19 wave group initially consisted of 66 patients, 37 women and 29 men, with a mean age of 68.5 ±18.3 years. From the 5th wave group, 7 vaccinated patients and 3 with previously confirmed SARS-CoV-2 infection were excluded, as well as 4 patients with an acute course of the disease, with ARDS at baseline. Fourteen patients died. Ultimately, the 5th COVID-19 wave study group consisted of 38 patients, among whom were 20 women and 18 men, aged 66.4 ±18.3 years. The exclusion of vaccinated patients and those previously infected with the SARS-Cov-2 virus allowed the generation of the optimal group from the 5th wave, which did not demonstrate many differences compared to the group from the 1st wave. Of note, patients from the 1st wave and the 5th wave were different people.

The treatment procedure was carried out according to current knowledge and recommendations of the Polish Society of Epidemiologists and Infectiologists.[24] The mean hospitalization was 21.5 ±16 days. Clinical characteristics of all COVID-19 patients from both groups are presented in Table 1. The HC group consisted of 20 volunteers, 18 women and 2 men, with an average age of 56 ±7.1 years.

The study was carried out by the Declaration of Helsinki, and approved by the Ethics Committee of the Military Institute of Medicine (approval No. 47/WIM/2020 dated September 16, 2020). Informed written consent for the study and publication of this work was obtained from all patients from whom samples were collected.

Materials

Peripheral blood (PB) samples were obtained from all COVID-19 patients within 24 h of admission and before antiviral and/or immunosuppressive treatment. Whole PB samples were incubated with monoclonal antibodies for 20 min at room temperature. The antibodies used are shown in Table 2. After 2 washes, the cells were analyzed for 2 h, and at least 20,000 events were collected for each sample. Data were interpreted with Cytexpert and Kaluza C v. 1.1 software (Beckman Coulter, Brea, USA), and an Infinicyt 1.8 Flow Cytometry (Cytognos, Salamanca, Spain).

The routine white blood cell count (WBC) analysis was performed on all patients using a Sysmex XN-1500 (Sysmex Corp., Kobe, Japan) hematological analyzer.

Flow cytometry analysis

Leukocyte and lymphocyte subpopulations were analyzed with multicolor flow cytometry with a monoclonal antibody panel using DxFLEX flow cytometry (Beckman Coulter). We reported the lymphocyte maturation for the $CD4^+$ and $CD8^+$ cells.[19] The following maturation populations among $CD4^+$ T cells and $CD8^+$ T cells were analyzed: RTE, naïve, effector, effector memory, and central memory cells. The phenotypes of the analyzed T cell subpopulations and all tested cells are presented in Table 2.

Statistical analyses

The analysis was performed using Statistica v. 12.0 software (TIBCO Software, Palo Alto, USA). The Shapiro–Wilk test was performed to evaluate assumptions regarding

Table 1. Characteristics of the study population with COVID-19 in different waves

Patients' characteristics		1st COVID-19 n = 23	5th COVID-19 n = 38	p < 0.05 Mann–Whitney U test
Sex: F/M, n		9/14	20/18	
Age (Me (Q1–Q3)) p-value (Shapiro–Wilk test) SW-W value		60.0 (39.0–72.0) p = 0.018 0.889	71 (52–78) p = 0.043 0.940	p = 0.040
Clinical symptoms n (%)	fever	19 (82.6%)	30 (78.9%)	p = 0.850
	cough	16 (69.6%)	25 (65.8%)	p = 0.876
	dyspnea	14 (60.9%)	5 (13.2%)	p = 0.239
Saturation (Me (Q1–Q3) p-value (Shapiro–Wilk test) SW–W value		91.0 ±7.5% p = 0.125 0.871	91.9 ±4.6% p = 0.043 0.940	p = 0.980
Conventional (passive) oxygen therapy		7 (30.4%)	29 (76.3%)	p = 0.023
Mechanical ventilation therapy		3 (13.0%)	2 (5.3%)	p = 0.987
Diseases comorbidities, n (%)	0 comorbidities	10 (43.5%)	5 (13.2%)	p = 0.098
	1 comorbidity	7 (30.4%)	15 (39.5%)	p = 0.138
	2 comorbidities	2 (8.7%)	10 (26.3%)	p = 0.068
	3 comorbidities	2 (8.7%)	5 (13.2%)	p = 0.654
	4 comorbidities	2 (8.7%)	3 (7.9%)	p = 0.980

Me – median; SW–W – Shapiro–Wilk test value.

normal distribution. The parameters compared did not meet the assumptions of normal distribution, so the non-parametric Mann–Whitney U test was used to compare the 2 groups (Table 1). Among the tested parameters (for comparison 3 groups) in Table 3 and Table 4, lymphocytes (%), neutrophils (%) naïve $CD4^+$, effector $CD8^+$ (%) and effector memory $CD8^+$ (%) met the assumptions of normality, and thus we checked the assumptions of homogeneity of variance (Brown–Forsyth test), which showed that the assumption of homogeneity of variance was not met. For these 2 parameters, Welch's analysis of variance (ANOVA) test (with Welch's correction) for independent variance estimation and Games–Howell post hoc tests were used. For other parameters where the assumption of normal distribution was not met, we used the non-parametric Kruskal–Wallis test and Dunn's post hoc test with Bonferroni correction. The results were expressed as means with SD or medians (Me) with interquartile range (Q1–Q3). Statistical significance was considered when $p < 0.05$. All analyses were performed in Prism v. 9 (GraphPad Software, La Jolla, USA).

Results

Clinical characteristics of the patients

The characteristics of the studied population with COVID-19 in different waves are provided in Table 1. There is a nonsignificant difference in the age of the patients, with those in the 5th COVID-19 wave being older than patients in the 1st COVID-19 wave (Mann–Whiteny U test, p = 0.040). The blood oxygen saturation value was similar in both waves (U-Mann–Whiteny test, p = 0.980). The percentage of symptoms, such as fever, cough and dyspnea, were similar in both groups. Patients in the 5th COVID-19 wave had a higher percentage of conventional (passive) oxygen therapy than patients in the 1st COVID-19 wave, and acute respiratory failure requiring mechanical ventilation was recorded in 2 patients from the 5th wave compared to 3 patients from the 1st wave. There were 14 deaths among patients from the 5th wave. However, after excluding vaccinated patients and patients with a severe course of disease, a uniform group of patients with mild disease severity was obtained. Finally, a higher percentage of comorbidities was found in patients from the 5th COVID-19 wave.

Basic leukocyte subpopulation

Differences between COVID-19 waves

We analyzed the leukocyte subset distribution in PB in different waves of COVID-19. Median values of the absolute number and percentage of leukocytes and lymphocyte types are presented in Table 3. There was a lower median proportion of lymphocytes and NK cells, and a significantly higher median proportion and absolute number of neutrophils in patients in the 5th COVID-19 wave compared to the 1st COVID-19 wave (Table 3).

Differences between COVID-19 and healthy control

Compared to the HC group, there were more significant differences with the 5th wave group compared to

Table 2. List of analyzed cell subpopulations with phenotype and list of antibodies

Analyzed population	Phenotype	Antibody list	Catalog No.	Clone No.
Lymphocytes	CD45$^+$bright SSC-A$^+$dim	CD45-V500	655873	2D1
Lymphocytes T	CD45$^+$bright CD3$^+$	CD45-V500 CD3-PerCP-Cy5.5	655873 332771	2D1 SK7
Lymphocytes T CD4$^+$	CD45$^+$bright CD3$^+$ CD4$^+$	CD45-V500 CD3-PerCP-Cy5.5 CD4-FITC	655873 332771 345768	2D1 SK7 SK3
Lymphocytes T CD8$^+$	CD45$^+$bright CD3$^+$ CD8$^+$	CD45-V500 CD3-PerCP-Cy5.5 CD8-APC	655873 332771 345775	2D1 SK7 SK1
Lymphocytes B	CD45$^+$bright CD19$^+$	CD45-V500 CD19-PE-Cy7	655873 341113	2D1 SJ25C1
Lymphocytes NK	CD45$^+$bright CD16$^+$ CD3$^-$	CD45-V500 CD16-APC-H7	655873 560195	2D1 3G8
Neutrophils	CD45$^+$ CD16$^+$ SSC-A$^+$	CD45-V500 CD16-APC-H7	655873 560195	2D1 3G8
Eosinophils	CD45$^+$bright SSC-A$^+$	CD45-V500	655873	2D1
Basophils	CD45$^+$dim SSC-A$^+$dim	CD45-V500	655873	2D1
Monocytes	CD45$^+$ HLA-DR$^+$	CD45-V500 HLA-DR-V450	655873 655874	2D1 L243
RTE	CD45RA$^+$ CD62L$^+$ CD31$^+$ CD3$^+$ CD45$^+$	CD45RA-APC CD62L-PE CD31-PerCP-Cy5.5 CD3-APC-H7 CD45-V500	550855 555544 566563 641415 655873	– – WM59 SK7 2D1
Naïve T cells	CD45RA$^+$ CD197$^+$ CD3$^+$ CD45$^+$	CD45RA-APC CD197-PerCP-Cy5.5 CD3-APC-H7 CD45-V500	550855 353220 641415 655873	– G043H7 SK7 2D1
Effector T cells	CD45RA$^+$ CD197$^-$ CD3$^+$ CD45$^+$	CD45RA-APC CD197-PerCP-Cy5.5 CD3-APC-H7 CD45-V500	550855 353220 641415 655873	– G043H7 SK7 2D1
Central memory T cells	CD45RO$^+$ CD197$^+$ CD3$^+$ CD45$^+$	CD45RO-PE-Cy7 CD197-PerCP-Cy5.5 CD3-APC-H7 CD45-V500	560608 353220 641415 655873	UCHL1 G043H7 SK7 2D1
Effector memory T cells	CD45RO$^+$ CD197$^-$ CD3$^+$ CD45$^+$	CD45RO-PE-Cy7 CD197-PerCP-Cy5.5 CD3-APC-H7 CD45-V500	560608 353220 641415 655873	UCHL1 G043H7 SK7 2D1
Th17	CD45RO$^+$ CD196$^+$ CD3$^+$ CD4$^+$ CD45$^+$	CD45RO-PE-Cy7 CD197-PerCP-Cy5.5 CD3-APC-H7 CD-4 FITC CD45-V500	560508 353220 641415 345768 655873	UVHL1 G043H7 SK7 SK3 2D1

RTE – recent thymic emigrants T cells.

the 1st wave group. Lymphopenia, including reduced absolute numbers relative to healthy controls, was demonstrated for both COVID-19 groups for T cells, CD4$^+$ and CD8$^+$ cells, and B cells and NK cells. A similar relationship was found for neutrophil and eosinophil numbers (Table 3). The HC group showed significantly higher percentages of lymphocytes, CD3$^+$, both CD4$^+$ and CD8$^+$ T lymphocytes, B lymphocytes and basophils compared to patients from the 5th wave group (Table 3).

T cell maturation subpopulation

Differences between COVID-19 waves

There was a significantly higher median proportion of effector memory CD4$^+$ cells in the 5th COVID-19 wave compared to the 1st (Table 4). We also observed a significantly lower median proportion of RTE CD8$^+$ cells in the 5th COVID-19 wave than in the 1st COVID-19 wave

Table 3. The median proportion of leukocytes subpopulation in peripheral blood (PB): lymphocytes, lymphocytes T ($CD4^+$, $CD8^+$), natural killer cells, granulocytes, eosinophils, basophils and monocytes in the 1st COVID-19 wave, the 5th COVID-19 wave and in healthy control

Leukocytes subpopulations	1st COVID-19 wave Me (Q1–Q3) or mean (SD)[1] A (n = 23)	5th COVID-19 wave Me (Q1–Q3) or mean (SD)[1] B (n = 38)	HC Me (Q1–Q3) or mean (SD)[1] C (n = 20)	*p < 0.050 [1]Welch's ANOVA test (with Welch's correction) for independent variance estimation [2]nonparametric Kruskal–Wallis	* p < 0.050 [1]Games–Howell post hoc [2]Dunn's post hoc test with Bonfferoni correction
Lymhocytes [%]	[1]33.6 (18.8)	[1]21.6 (12.8)	[1]39.7 (10.6)	p < 0.001[1]	A–B, B–C[1] A–B; p = 0.015 A–C; p = 0.271 B–C; p < 0.001
Lymhocytes [k/μL]	1087 (817–2420)	1154 (905–1799)	2037 (1838–2934)	p < 0.001[2]	A–C, B–C[2] A–B; p = 1.000 A–C; p = 0.004 B–C; p < 0.001
Lymphocytes T $CD3^+$ [%]	21.9 (13.8–37.5)	17.5 (10.3–22.5)	29.3 (24.0–37.2)	p < 0.001[2]	B–C[2] A–B; p = 0.154 A–C; p = 0.089 B–C; p < 0.001
Lymphocytes T $CD3^+$ [k/μL]	805 (572–1891)	897 (729– 1369)	1659 (1409–2292)	p < 0.001[2]	A–C, B–C [2] A–B; p = 1.000 A–C; p = 0.001 B–C; p < 0.001
Lymphocytes T $CD3^+$ $CD4^+$ [%]	12.3 (5.3–23.1)	9.3 (5.3–13.9)	18.6 (13.6–22.0)	p < 0.001[2]	B–C[2] A–B; p = 0.439 A–C; p = 0.073 B–C; p < 0.001
Lymphocytes T $CD3^+$ $CD4^+$ [k/μL]	526 (261–1035)	557 (450–796)	977 (756–1559)	p < 0.001[2]	A–C, B–C[2] A–B; p = 1.000 A–C; p = 0.003 B–C; p = 0.001
Lymphocytes T $CD3^+$ $CD8^+$ [%]	9.3 (3.6–12.6)	5.7 (3.1–8.1)	10.5 (7.8–13.2)	p = 0.002[2]	B–C[2] A–B; p = 0.125 A–C; p = 0.413 B–C; p < 0.001
Lymphocytes T $CD3^+$ $CD8^+$ [k/μL]	313 (160–847)	399 (206–552)	624 (456–790)	p = 0.003[2]	A–C, B–C[2] A–B; p = 1.00 A–C; p = 0.028 B–C; p = 0.003
Ratio CD4/CD8	1.6 (1.0–2.7)	1.7 (0.9–2.4)	1.8 (1.5–2.2)	p = 0.863[2]	–
Lymphocytes B $CD19^+$ [%]	2.2 (1.4–5.1	2.1 (0.8–3.7)	3.9 (3.0–5.0)	p = 0.004[2]	B–C[2] A–B; p = 0.861 A–C; p = 0.116 B–C; p = 0.003
Lymphocytes B $CD19^+$ [k/μL]	141 (77–191)	132 (58–257)	216 (190–284)	p = 0.004[2]	A–C, B–C[2] A–B; p = 1.000 A–C; p = 0.013 B–C; p = 0.007
Natural killer (NK) cells [%]	4.5 (1.5–9.1)	1.8 (0.4–3.5)	4.2 (2.8–7.0)	p < 0.001[2]	A–B, A–C, B–C[2] A–B; p = 0.003 A–C; p = 0.002 B–C; p = 0.002
Natural killer (NK) cells [k/μL]	184 (101–400)	116 (35–241)	245 (204–447)	p = 0.001[2]	B–C[2] A–B; p = 0.093 A–C; p = 0.531 B–C; p = 0.001
Neutrophils [%]	[1]55.3 (22.0)	[1]64.8 (15.8)	[1]59.4 (21.2)	*p < 0.001[1]	A–B[1] A–B; p < 0.001 A–C; p = 0.756 B–C; p = 0.817
Neutrophils [k/μL]	2704 (1556–3937)	4203 (2581–6373)	3310 (2139–4338)	p = 0.001[2]	A–B, A–C, B–C[2] A–B; p < 0.001 A–C; p < 0.001 B–C; p < 0.001
Eosinophils [%]	1.1 (0.2–2.5)	1.1 (0.6–2.9)	1.8 (1.0–3.2)	p = 0.210[2]	–
Eosinophils [k/μL]	62 (8–109)	79 (29–171)	108 (66–197)	p = 0.074[2]	–

Table 3. The median proportion of leukocytes subpopulation in peripheral blood (PB): lymphocytes, lymphocytes T ($CD4^+$, $CD8^+$), natural killer cells, granulocytes, eosinophils, basophils and monocytes in the 1st COVID-19 wave, the 5th COVID-19 wave and in healthy control – cont.

Leukocytes subpopulations	1st COVID-19 wave Me (Q1–Q3) or mean (SD)[1] A (n = 23)	5th COVID-19 wave Me (Q1–Q3) or mean (SD)[1] B (n = 38)	HC Me (Q1–Q3) or mean (SD)[1] C (n = 20)	*p < 0.050 [1]Welch's ANOVA test (with Welch's correction) for independent variance estimation [2]nonparametric Kruskal–Wallis	* p < 0.050 [1]Games–Howell post hoc [2]Dunn's post hoc test with Bonfferoni correction
Basophils [%]	0.3 (0.0–0.7)	0.3 (0.0–0.5)	0.5 (0.4–0.7)	p = 0.035[2]	B–C[2] A–B; p = 0.980 A–C; p = 0.233 B–C; p = 0.032
Basophils [k/μL]	14 (0–27)	16 (0–32)	31 (25–45)	p = 0.011[2]	A–B, A–C, B–C[2] A–B; p = 0.027 A– C; p = 0.027 B–C; p = 0.021
Monocytes [%]	7.2 (5.8–10.9)	9.5 (6.7–13.2)	8.2 (6.7–9.6)	p = 0.173[2]	–
Monocytes [k/μL]	388 (249–615)	615 (417–831)	449 (395–562)	p = 0.003[2]	A–B[2] A–B; p = 0.002 A–C; p = 0.382 B–C; p = 0.355

HC – healthy control; Me – median. Data expressed as median (Q1–Q3). A * marked p < 0.05 statistically significant.

Table 4. Differences in the median proportion of T lymphocyte cells in peripheral blood: recent thymic emigrants (RTE), naïve, effector, effector memory, central memory and Th17 cells between the 1st COVID-19 wave, the 5th COVID-19 wave and healthy control

Lymphocytes T subpopulations	1st COVID-19 wave Me (Q1–Q3) or mean (SD)[1] A (n = 23)	5th COVID-19 wave Me (Q1–Q3) or mean (SD) or mean (SD)[1] B (n = 38)	HC Me (Q1–Q3) or mean (SD)[1] C (n = 20)	* p < 0.050 [1]Welch's ANOVA test (with Welch's correction) for independent variance estimation [2]nonparametric Kruskal–Wallis	* p < 0.050 [1]Games–Howell post hoc [2]Dunn's post hoc test with Bonfferoni correction
RTE $CD4^+$	19.9 (5.8–30.3)	14.2 (9.4–23.6)	31.2 (26.3–37.6)	p < 0.001[2]	A–C, B–C[2] A–B; p = 0.792 A–C; p = 0.014 B–C; p < 0.001
Naïve $CD4^+$	41.4 (20.3)[1]	33.4 (16.9)[1]	50.0 (10.9)[1]	p < 0.001[1]	B–C[1] A–B; p = 0.365 A–C; p = 0.125 B–C; p = 0.005
Effector $CD4^+$	2.8 (1.2–6.4)	2.6 (1.0–4.4)	1.8 (1.1–3.4)	p = 0.647[2]	–
Effector memory $CD4^+$	14.1 (9.2–22.3)	23.2 (18.0–35.9)	12.5 (9.2–15.0)	p < 0.001[2]	A–B, B–C[2] A–B; p < 0.001 A–C; p = 1.000 B–C; p < 0.001
Central memory $CD4^+$	35.2 (26.3–46.5)	33.4 (28.1–43.1)	33.2 (27.2–40.3)	p = 0.757[2]	–
Th17 (among $CD4^+$)	22.5 (15.5–29.1)	21.8 (16.2–31.3)	28.8 (25.0–34.9)	p = 0.054[2]	–
RTE $CD8^+$	28.1 (13.4–47.3)	11.7 (7.2–24.7)	39.5 (34.4–52.9)	p < 0.001[(2]	A–B, B–C[2] A–B; p = 0.026 A–C; p = 0.073 B–C; p < 0.001
Naïve $CD8^+$	22.1 (10.5–40.5)	11.8 (7.0–21.3)	42.4 (35.5–59.7)	p < 0.001[2]	A–B, A–C, B–C[2] A–B; p = 0.027 A–C; p = 0.019 B–C; p < 0.001
Effector $CD8^+$	36.5 (23.2)[1]	39.8 (20.1)[1]	28.4 (11.8)[1]	p = 0.145[1]	–
Effector memory $CD8^+$	20.8 (11.9)[1]	36.3 (12.9)[1]	19.6 (6.9)[1]	p < 0.00[1]	A–B, B–C[1] A–B; p < 0.001 A–C; p = 1.000 B–C; p < 0.001
Central memory $CD8^+$	9.5 (6.6–14.5)	6.1 (3.5–12.0)	7.8 (4.1–11.4)	p = 0.242[2]	–

RTE – recent thymic emigrants; HC – healthy control; Me – median. Data expressed as median (Q1–Q3). A * marked p < 0.05 statistically significant

and naïve CD8+ cells in the 5th COVID-19 wave than in the 1st COVID-19 wave (Table 4). When we analyzed the median proportion of effector memory CD8+ cells, we noticed a significantly higher proportion in the 5th COVID-19 wave than in the 1st COVID-19 wave (Table 4). Moreover, there was a lower median proportion of central memory CD8+ cells in the 5th COVID-19 wave than in the 1st COVID-19 wave (Fig. 1, Table 4). Sample flow cytometry graphs from a selected patient from the 1st COVID-19 wave to a patient from the 5th COVID-19 wave for T cells maturation population: lymphocytes, lymphocytes T, CD4+, CD8+, naïve, effector, effector memory and central memory, Th17 and RTE cells are presented in Fig. 2 and Fig. 3.

Differences between COVID-19 and healthy control

Compared to the HC group, we found a significantly lower percentage of CD4+ RTE cells and CD8+ naïve cells in both groups of patients with COVID-19. Lower percentages of CD4+ naïve cells, CD8+ RTE cells, and higher percentages of memory effector cells of both CD4+ and CD8+ were also found in 5th-wave patients relative to the HC group (Table 4, Fig. 1).

Discussion

Despite developed immunity and vaccinations showing significant activity against various viral variants,

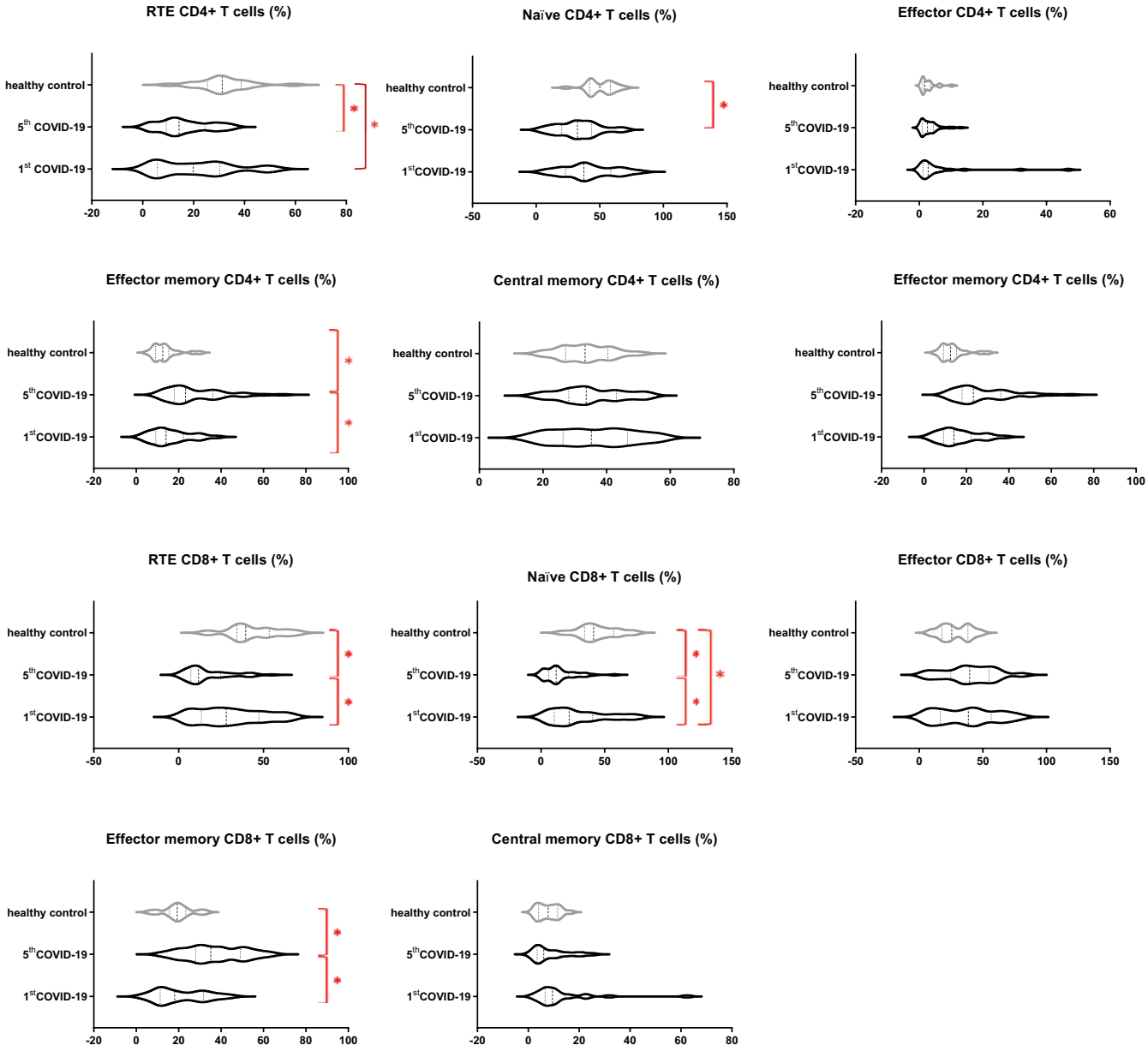

Fig. 1. The differences in the median values of T CD4+ and CD8+ lymphocytes types: Recent thymic emigrants T cells (RTE), naïve T cells, effector T cells, central memory T cells, and effector memory T cells between the 1st COVID-19 wave, 5th COVID-19 wave and healthy control. Graphs show the median values (A * marked $p < 0.05$ statistically significant)

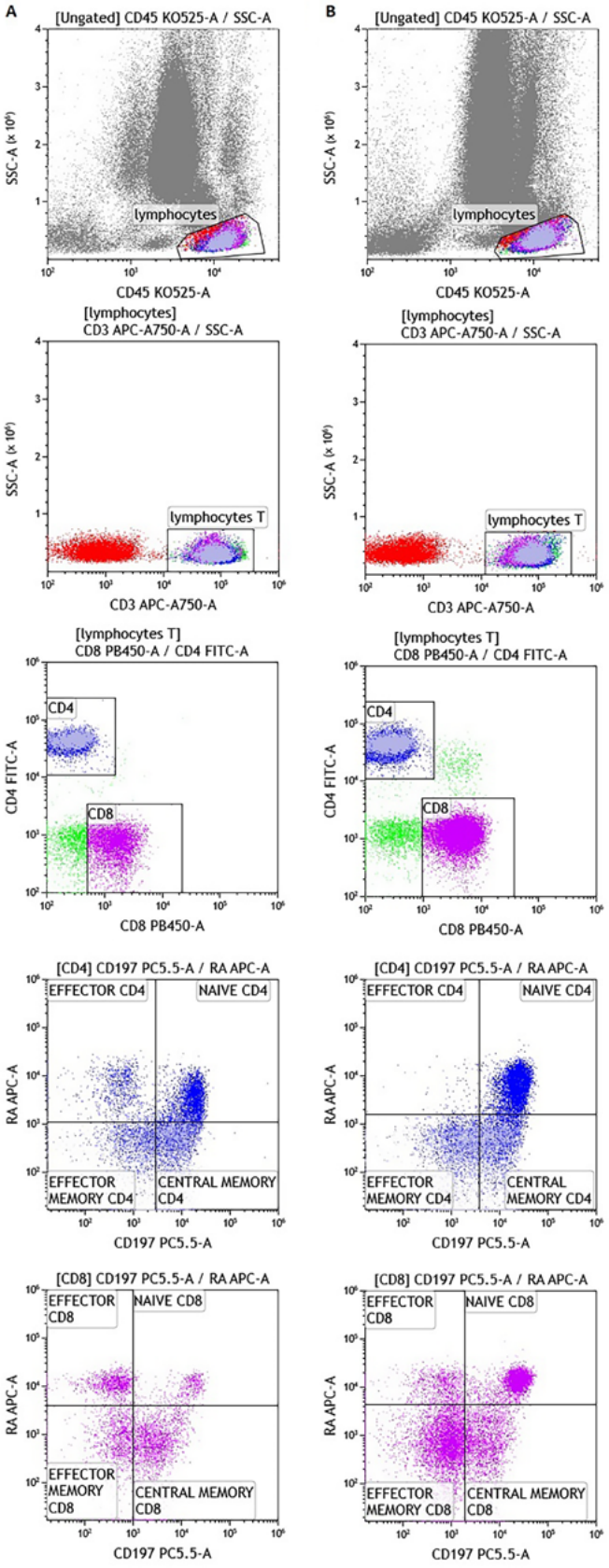

Fig. 2. Sample flow cytometry graphs from a selected patient from the 1st COVID-19 wave (A) and patient from the 5th COVID-19 wave (B) for lymphocytes, lymphocytes T, $CD4^+$, $CD8^+$ and T cells maturation population: naïve, effector, effector memory and central memory

SSC-A – side scatter area; RTE – recent thymic emigrants.

SARS-COV-2 still causes significantly high mortality, especially in patients with many comorbidities.[25] In our study, we presented new results comparing the clinical and immunological features of the 2 extreme waves of COVID-19 cases in Poland. We showed in our work, for the first time, the full maturation profile of T lymphocytes, from naïve cells to memory cells of patients from 2 distant waves of the COVID-19 pandemic in Poland. Our study provides characteristics of COVID-19 patients from the pandemic's 1st and 5th waves through clinical description and evaluation of leukocyte and main T-cell subpopulations. Patients from both groups showed typical symptoms of COVID-19. The group of patients from the 5th wave was associated with an elevated number of comorbidities and the amount of oxygen therapy used. The differences between the waves in the clinical picture could be due to the development of other virus variants, large-scale vaccination and greater population immunity. In the 5th wave of COVID-19, the positive group consisted mainly of elderly, unvaccinated patients with comorbidities, due to the younger infected patients not requiring hospitalization. Only a few studies have conducted comprehensive comparisons of hospitalized patients from different waves of COVID-19. According to some researchers, COVID-19 patients in the 1st wave had a more severe course of the disease than patients admitted in the 2nd wave, in which fewer patients received mechanical ventilation and experienced symptoms such as fever, cough and shortness of breath.[26] Similarly, the results of studies conducted in Spain, Japan and Iran showed a milder course of the disease during the 2nd wave.[10,27,28] There are many plausible explanations for the milder course of the disease during subsequent waves of COVID-19. The risk of infection was higher at the beginning of the pandemic, improved diagnostics and treatment could translate into the condition of hospitalized patients, and potential changes in the SARS-CoV-2 genome in subsequent waves could have an impact on the severity of the disease.[26]

It is known that lymphopenia is a characteristic feature in patients with COVID-19 and may be a basic, useful prognostic factor.[29,30] Neutrophilia is also a characteristic symptom of SARS-CoV-2 infection.[31] In our research, lymphopenia and neutrophilia were significantly higher in the 5th wave, comparing both patients from the 1st wave group and the HC group. It is known that lymphopenia, elevated neutrophil-to-lymphocyte ratio, monocyte-to-lymphocyte ratio, and elevated cytokine levels are correlated with disease severity and poor prognosis.[32,33] Charostad et al., comparing 5 waves of COVID-19, noticed the greatest increase in the number of leukocytes and the highest neutrophilia and lymphopenia in the 3rd wave, while the 1st wave had the least impact on these parameters.[34] Our data indicate that hematological parameters can serve as valuable predictive biomarkers for assessing disease status and clinical outcomes in each wave of the COVID-19

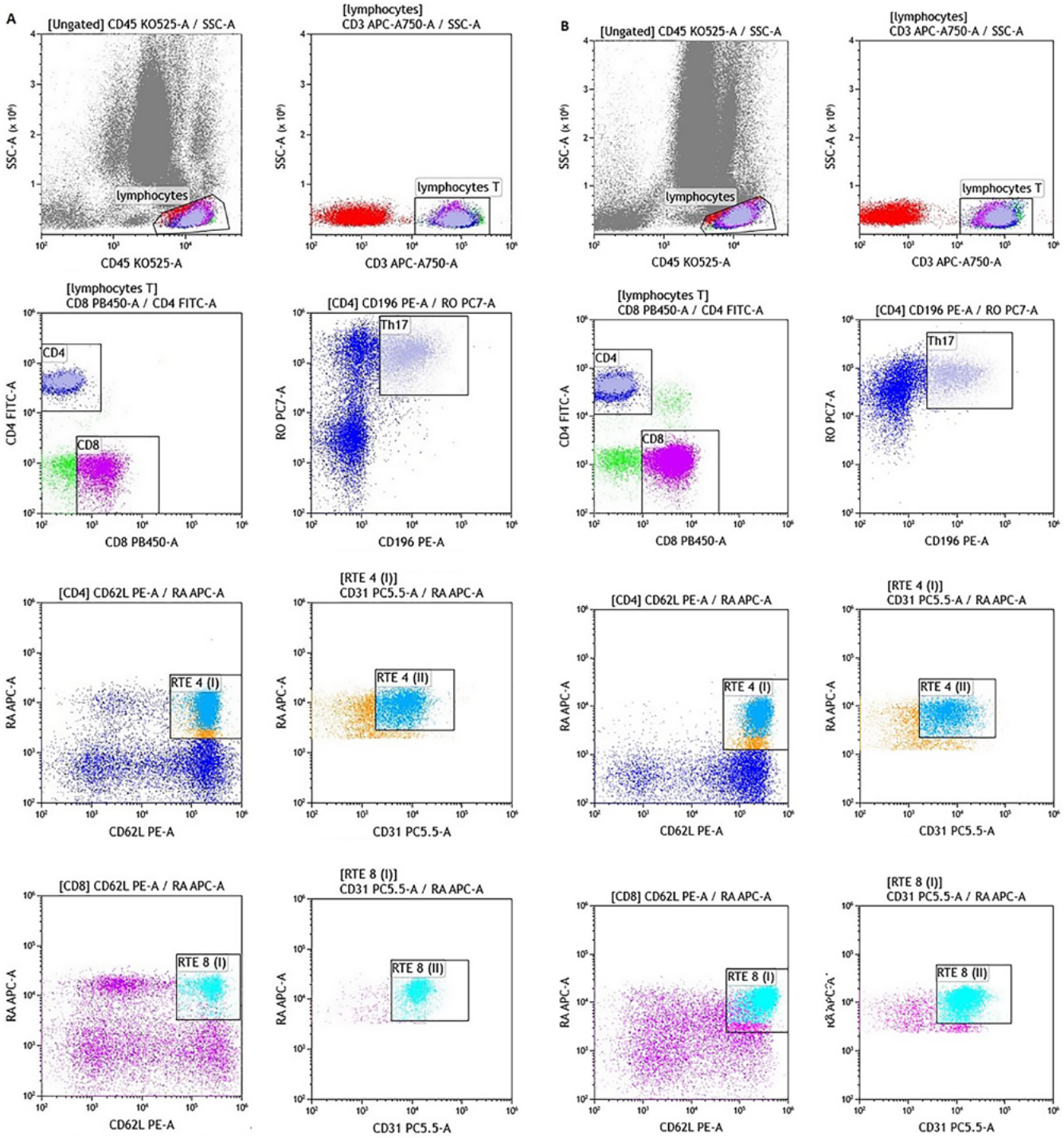

Fig. 3. Sample flow cytometry graphs from a selected patient from the 1st COVID-19 wave and a patient from the 5th COVID-19 wave for lymphocytes, lymphocytes T, $CD4^+$, $CD8^+$, Th17 cells and recent thymic emigrant T cells (RTE)

SSC-A – side scatter area.

pandemic and provide useful insight into the progression and prognosis of COVID-19 cases.

For a better understanding of the immune mechanisms occurring in the patients examined in this study, we analyzed the subpopulation of cells responsible for both the early and late immune response. Different types of pathogens require diverse types of immune effector cells for control. Viral infections require control of $CD4^+$ T cells, which induce B cells to produce high-affinity antibodies that can neutralize the pathogen, and cytotoxic $CD8^+$ T cells, which kill cells infected with the pathogen. The factor initiating the immune response is the recognition of antigens by lymphocytes, which, when stimulated, proliferate and mature into effector cells and memory cells. These cells are characterized by heterogeneity in terms of surface receptor expression, function and

location.[35] It appears that memory T cells can reduce the severity of COVID-19 infection by triggering a protective immune response.

Differentiation of T cell populations into effector and memory subsets is one of the most fundamental aspects of T cell-dependent immunity. Thus, the balance between naïve and memory T cells is crucial to maintain an effective immune response.[36] Very few reports were found comparing the composition of leukocyte and lymphocyte subsets from patients of different waves of the COVID-19 pandemic.

We showed the highest percentage of $CD8^+$ RTE cells and naïve $CD8^+$ cells in the HC group, indicating a muted immune system compared to the COVID-19 groups. In comparison, the proportion of memory effector cells was the highest in the 5th wave group of patients both in the case of $CD4^+$ and $CD8^+$ lymphocytes. The presence of effector memory cells could indicate re-contact with the antigen and residual immunological memory. Despite the lack of vaccination and confirmed infections with the SARS-Cov-2 virus, most patients from the 5th wave seem to have had contact with the virus during the first 4 waves of the pandemic.

The state of infection can also lead to the emergence of an adaptive immune response and the formation of memory cells responsible for protective immunity. Over time, the likelihood of developing immunological memory increases with subsequent exposures to the virus, either through vaccination or direct contact.

Our previous research showed an increase of T cells with immunological memory in response to COVID-19 infection. Among $CD8^+$ cells, effector cells were most abundant in COVID-19 patients. In contrast, we noted a significant growth in the proportion of $CD4^+$ central memory cells relative to the HC group. Our results indicated the development of immunological memory in patients with COVID-19 infection, without any correlation to changes in the lungs.[37,38] Netea and Li also showed more abundant effector and memory $CD8^+$ cells in COVID-19 survivors compared to healthy volunteers, highlighting their role in antiviral immunity.[39]

There was no consensus on what mechanisms might cause disease progression or inhibition. A significant body of literature has been published on the role of antibodies in COVID-19 disease, and it has been shown that $CD4^+$ T cell activity is necessary to produce antibodies against SARS-CoV-2 infection.[40] While controversy remains, it appears that the relief of COVID-19 symptoms is related to adaptive immunity and the production of memory cells. Peng et al. confirmed an association between the SARS-CoV-2-specific T cell response and recovery. The memory T cell response was shown to be greater in patients with severe disease than in those with mild COVID-19.[41] Liao et al. suggested that adaptive T cell responses are likely protective during SARS-CoV-2 infection.[42] Scalia et al. observed a decrease in most lymphocyte subsets in mild and moderate stages, a decrease in NK cells and regulatory T cells in 2nd-wave patients, and a more significant number of activated Th17 lymphocytes in all stages compared to the 1st wave. Less severe symptoms of SARS-CoV-2 infection were observed in 2nd-wave patients in advanced stages, while patients in the mild and moderate stages had a worse course compared to patients in the 1st wave. The authors suggested that in patients with mild COVID-19 at diagnosis, treatment with steroids and azithromycin appeared to blunt the immune reaction against the virus.[43] Asghar et al. found that most levels of inflammatory markers were lower in the 2nd wave, while the percentages of neutrophils and lymphocytes were higher compared to the 1st wave. Disease severity was also more predictable in the 2nd wave, which may be due to attenuation of the inflammatory response by the immediate use of immunosuppressants, antibiotics, antiviral drugs, or anticoagulants, according to treatment recommendations that were not available during the 1st wave.[44] Moreover, the course of the disease may depend on the adaptive immune response of patients. T-cell immunity plays a crucial role in controlling SARS-CoV-2, and its importance may have been relatively underestimated until now.[45] However, new data are emerging indicating that SARS-CoV-2-specific memory T cells are being produced. Long-term studies of patients who recovered from the closely related SARS virus (SARS-CoV-1) between 2002 and 2004 found that anti-SARS T cells were long-lived and remained nearly 2 decades later.[46] Therefore, the characteristics of the immune response among population groups may help develop personalized therapies for patients with severe disease.[47] Knowledge of the immune profile is also important for creating new vaccines against SARS-CoV-2, which should trigger the production of memory T cells.[46]

We proposed that memory effector $CD4^+$ and $CD8^+$ cells represent a reliable measure of immune status that may be useful for assessing recent major waves of COVID-19. Additionally, the reduced proportion of central memory $CD4^+$ cells, naive $CD8^+$ cells and RTE $CD8^+$ cells allowed for the distinguishing of patients in the last significant COVID-19 wave, which may indicate the direction of further research and comprise the next stage of diagnostics. Regular monitoring of lymphocyte subsets during SARS-Cov-2 infection will assess the patient's immune status and lymphocyte readiness for an immune response and may be essential to improve clinical outcomes.

Limitations

Our study has limitations that may introduce some potential bias. It was a study on a small group of patients, and data from a larger cohort of patients would be useful to evaluate subsequent changes in immune responses following SARS-CoV-2 infection. However, our study

provided much new information about the host immune response in COVID-19 patients that SARS-CoV-2 may act on lymphocytes, especially T cells. There has been a lack of studies assessing the virus variant in individual waves of the pandemic. Patients from only 2 waves of the pandemic were compared, although 2 extreme waves were selected, the 1st and, so far, the last (the 5th).

Conclusions

In this work, we analyzed basal peripheral leukocytes and T cell subpopulations of the maturation process and differences between COVID-19 waves compared to healthy controls. The number of characteristic changes in the maturation profile of T lymphocytes in the 5th wave group compared to the 1st wave group and the HC group indicated the switching of cell functions to effectors, ready for the immune response, and indicated the differentiation of the course of the disease depending on the wave of COVID-19.

Monitoring the memory cell population in healthy people and people at risk is very important for proper prevention or treatment. The characterization of T lymphocyte subpopulations allowed us to illustrate the phenomenon of immunological memory and readiness to effectively eliminate the virus in patients with COVID-19. The presented results allowed us to emphasize to some extent the importance of immunological memory in these patients, but further detailed studies are necessary.

Supplementary data

The Supplementary materials are available at https://doi.org/10.5281/zenodo.10803904. The package includes the following files:

Supplementary Table 1. Assessment of assumptions regarding normal distribution performed using the Shapiro–Wilk test and homogeneity of variances using the Brown–Forsythe test for the studied leukocyte subpopulations in 3 groups.

Supplementary Table 2. Assessment of assumptions regarding normal distribution performed using the Shapiro–Wilk test and homogeneity of variances using the Brown–Forsythe test for the studied lymphocytes T subpopulations in 3 groups.

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication

Not applicable.

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