Flow Cytometric Analysis of Lymphocyte Subsets of Covid-19 Patients from A Single Centre in Turkey

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ABSTRACT

Purpose: Lymphocyte counts have been shown to negatively correlate with the severity in Covid-19. The aim of this study is to analyse the distribution of lymphocyte subsets in response to SARS-CoV-2 infection and its relation to the severity of the disease.

Methods: Blood samples were obtained from 67 consecutive patients between April 2020 and July 2020. Data on other laboratory parameters, and clinical course were collected retrospectively from patient files and patients were defined to have as severe or non-severe (mild/moderate) disease. Leukocyte subsets to be studied were identified by using flow cytometric analysis (Beckman Coulter Navios Ex V2.0). Patients were allocated into 3 groups based on the day of blood sample collection: Days 0-7, 8-14 and >14 as Group I, Group II and Group III, respectively. In 10 of 67 patients an additional analysis was done 7-10 days after the initial sampling.

Results: A total of 67 patients (30 female, 37 male) with a median age of 57 were evaluated. Lower total lymphocyte, CD3 positive, CD4 positive and B-cell counts were identified in severe infection compared to non-severe infection group which were also correlated with high serum CRP, D-dimer and ferritin levels. NK and monocyte counts were not different between the two groups. Activation markers CD38 and HLA-DR on CD4 and CD8 positive lymphocytes also were not different in either group.

Conclusion: CD3 and CD4 lymphopenia were lower in accordance with previous studies and were associated with severe disease. The expectancy of high activation markers was not met. Future studies with detailed subgroup analyses at different time-points will shed more light on our general knowledge of the immune response to COVID-19.

Keywords: Flow Cytometry, COVID-19, Immune Profile, Lymphocyte Subsets, Monocytes

Türkiye'deki Tek Bir Merkezden Covid-19 Hastalarinin Lenfosit Alt Kümelerinin Akim Sitometrik Analizi

ÖZET

Amaç: Lenfosit sayılarının Covid-19'de hastalık şiddeti ve farklı gidişatla negative yönde ilişkisi olduğu gösterilmiştir. Bu tek merkezli çalışmanın amacı, SARS-CoV-2 enfeksiyonuna yanıt olarak lenfosit alt gruplarının dağılımını analiz etmek ve hastalığın şiddeti, seyri ve prognozuyla ilişkisini incelemektir.

Yöntemler: Nisan 2020 ile Temmuz 2020 arasında ardışık olarak 67 hastadan kan örnekleri alındı. Diğer laboratuvar parametreleri ve klinik seyirle ilgili veriler, hastaların dosyalarından geriye dönük olarak toplandı ve hastalık, ciddi veya ciddi olmayan (hafif / orta) hastalık olarak tanımlandı. İncelenecek lökosit alt grupları akım sitometri analizi (Beckman Coulter Navios Ex V2.0) kullanılarak belirlendi. Hastalar, akım sitometri analizi için kan örneği alınma gününe göre üç gruba ayrıldı: 0-7.gün, 8-14.gün ve >14.gün için sırasıyla Grup I, Grup II ve Grup III. 67 hastanın 10'unda, başlangıç örneğinden 7-10 gün sonra ek bir akım sitometri analizi yapıldı.

Bulgular: Ortanca yaşı 57 olan 67 hasta (30 kadın, 37 erkek) incelendi. Ciddi hastalık grubunda, ciddi olmayan hastalık grubuna göre daha düşük toplam lenfosit, CD3 pozitif, CD4 pozitif ve B-hücre sayıları belirlendi; aynı zamanda yüksek serum CRP, D-dimer ve ferritin seviyeleri ile korelasyon gösterdi. NK ve monosit sayıları ise iki grup arasında farklı değildi. CD4 ve CD8 pozitif lenfositlerdeki aktivasyon belirteçleri CD38 ve HLA-DR, her iki grupta da farklı değildi.

Sonuç: CD3 ve CD4 lenfopenisi, önceki çalışmalarla uyumluydu ve ciddi hastalıkla ilişkilendirildi. Yüksek aktivasyon belirteçlerinin beklentisi karşılanmadı. Gelecekte, farklı zaman noktalarında detaylı alt grup analizleri COVID-19'a bağışıklık tepkisi hakkındaki genel bilgimizi daha da aydınlatacaktır.

Anahtar Kelimeler: Akım Sitometri, COVID-19, İmmun Profil, Lenfosit Alt Grupları, Monositler

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Severe Acute Respiratory Syndrome Corona Virus 2 (SARS-CoV-2) has resulted in one of the greatest pandemics in the history changing life on earth in almost every aspect. Extensive and devoted research has shed some light into the biology and pathogenesis of the disease and potential immune response mechanisms against the virus, yet there is still much to learn. Hyper-inflammation complicated by endotheliitis and thrombosis seems to be the major pathology underlying the organ damage which mainly involves the lungs and leads to death of most of the infected patients with SARS-CoV-2. Both, the direct viral cytotoxicity and the immune response of the host against the replicating virus have been reported to contribute to the resulting organ damage.

Lymphocyte counts have been shown to negatively correlate with the severity and different outcomes of the disease in several studies and meta-analyses (1-4). The aim of this single centre study is to analyse the distribution of lymphocyte subsets in response to SARS-CoV-2 infection and its relation to the severity, course and prognosis of the disease.

MATERIALS and METHODS

Patients

Blood samples were obtained from 67 consecutive patients who were either admitted to the COVID-19 Outpatient Clinics or were followed in the COVID-19 wards at Cerrahpasa Medical Faculty, Istanbul University-Cerrahpasa between April 2020 and July 2020 and who accepted to enter the study by signing the written informed consent. The study received approval from the ethical committee of Cerrahpasa Medical Faculty (03.06.2020 - 67534) and Ministry of Health of Türkiye.

Definitions

Data on other laboratory parameters, and clinical course were collected retrospectively from patient files. Patients with the following criteria were defined to have severe/critical COVID-19: (1) breathing rate ≥30 times/min; (2) oxygen saturation (SpO_2) ≤93% at rest; and (3) ratio of partial pressure of arterial oxygen (PaO_2) to fraction of inspired oxygen (FiO_2) ≤300 mmHg according to Fifth Revised Trial Version of the Novel Coronavirus Pneumonia Diagnosis and Treatment Guidance criteria (4,10,11,23). Furthermore, patients who had progressive disease, were transferred to intensive care unit (ICU) and/or died from COVID-19 were also classified as severe/critical. All other patients were defined as non-severe (mild/moderate) (1). Computerised Tomography (CT) images were evaluated by the radiology department according to RSNA evaluation criteria as described elsewhere (16). All patients were treated and followed according to the National COVID-19 Guidelines released and regularly updated by the Turkish Ministry of Health (24).

Day 0 was accepted as the first day of symptoms in symptomatic patients and the day of first nasopharyngeal SARS-CoV-2 polymerase chain reaction (PCR) positivity in patients with no symptoms. Patients were allocated into 3 groups based on the day of blood sample collection for the flow cytometric analysis: Days 0-7, Days 8-14 and Days >14 as Group I, Group II and Group III, respectively. In 10 available of 67 patients an additional flow cytometric analysis was done 7-10 days after the initial sampling to study the evolution in the of lymphocyte subsets during the course of the disease.

Flow- cytometric Analysis

Leukocyte subsets to be studied were identified by using flow cytometric analysis. The evaluation panel consisted of CD19 (+) B-cells, CD3(-)16(+) (NK cells), CD3(-)19(-)14(+) (monocytes), CD3(+)4(+)8(-) T-Helper cells CD3(+)4(-)8(+) cytotoxic T-cells and CD38 and/or HLA-DR expression on T-helper and cytotoxic T cells as activation markers. Flow cytometric analysis was done with Beckman Coulter Navios Ex V2.0 10 Colours/3 lasers machine with the following monoclonal antibodies (Beckman Coulter): CD14 FITC (fluorescein isothiocyanate), CD4 PE (phycoerythrin), CD45 ECD (phycoerythrin-Texas Red conjugate), HLA-DR PC5 (phycoerythrin-cyanine5 conjugate), CD19 APC (allophycocyanin), CD38 AF700 (APC-Alexa Fluor 700), CD8 AF750 (APC-Alexa Fluor 750), CD16 KrO (Krome Orange). Ten thousand events were acquired per sample. Results were evaluated using Kaluza C analysis software (2017), calculated as percentages and given as absolute numbers based on total leukocyte counts.

Leucocyte subset patterns of patients with COVİD-19 and their relevance to clinical severity (severe vs. non-severe), survival and the need for intensive care was evaluated.

Statistical Analysis

Categorical data were described as percentages and continuous data as median with interquartile range (IQR). Parametric and nonparametric comparative tests for continuous data was used to compare variables between groups, where appropriate. All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences) version 23.0 software (SPSS, Inc.). Two-sided

P values of less than .05 were considered statistically significant.

RESULTS

Characteristics of the 67 COVID-19 patients according to their clinical severity were given in Table 1. Age distribution was significantly different between severe and non-severe groups, severe patients being significantly older than the non-severe ones. Furthermore, a significant male predominance was observed in the severe group. By definition, ICU stay and deaths were only present in the severe group. PCR positivity rates and CT findings were similar in both groups. Although the percentage of hypertension and diabetes mellitus patients were higher in the severe group, this was statistically insignificant. Tocilizumab use was solely confined to severe patients. Similarly, favipiravir use was higher in this group; whereas there were no differences regarding the use of hydroxychloroquine, azithromycin and oseltamivir.

Table 1: Characteristics of the Study Group

	Total (n, %)	Severe (n, %)	Non-severe (n, %)	p-value
	67 (100)	**36 (54)**	**31(46)**	
Age [years, median (range)]	57 (24–93)	64(35-93)	53 (24-81)	**0.0200***
Gender				
· Male [n, (%)]	37 (55)	25 (69)	12 (39)	**0.012****
· Female [n, (%)]	30 (45)	11 (31)	19 (61)	**0.012****
Co-morbidities				
· Hypertension [n, (%)]	23 (34)	13 (36)	10(32)	0.740**
· Diabetes mellitus [n, (%)]	21 (31)	14 (39)	7 (22)	0.151**
· Coronary artery disease [n, (%)]	10 (15)	8 (22)	2 (6)	0.070**
· Congestive heart failure [n, (%)]	1 (1)	1(3)	0(0)	0.537**
· Dementia [n, (%)]	1 (1)	1 (3)	0(0)	0.537**
· Cerebrovascular event [n, (%)]	1 (1)	1(3)	0(0)	0.537**
· COPD/Asthma (n, %)	11 (16)	8 (22)	3 (10)	0.147**
· Chronic renal failure [n, (%)]	8 (12)	6 (16)	2 (6)	0.183**
· Rheumatoid disease [n, (%)]	2 (3)	2 (6)	0(0)	0.285**
· Cancer [n, (%)]	4 (6)	3 (8)	1(3)	0.366**
CT positive [n, (%)]	53 (79)	29 (80)	24 (78)	0.771**
CT non-diagnostic [n, (%)]	10 (15)	5 (14)	5 (16)	0.771**
CT negative [n, (%)]	4 (6)	2 (6)	2 (6)	0.771**
PCR positive [n, (%)]	62 (92)	33 (92)	29 (93)	0.572**
PCR negative [n, (%)]	5 (8)	3 (8)	2 (7)	0.572**
Therapy				
· Hydroxychloroquine [n, (%)]	63 (94)	35 (97)	28 (90)	0.252**
· Favipiravir [n, (%)]	46 (68)	36(100)	10 (32)	**0.000****
· Azithromycin [n, (%)]	50 (74)	27 (75)	23 (74)	0.940**
· Oseltamivir [n, (%)]	31 (46)	18 (50)	13 (42)	0.509**
· Tocilizumab [n, (%)]	16 (23)	16 (44)	0 (0)	**0.000****
· Other antibiotics [n, (%)]	29 (43)	26 (72)	3 (10)	**0.000****
Patients requiring ICU [n, (%)]	5 (7)	5 (14)	0 (0)	**0.039****
Deaths [n, (%)]	4 (6)	4 (11)	0 (0)	0.077**

**Mann-Whitney; **Chi-square / Fisher; COPD, Chronic obstructive pulmonary disease; CT, Computerised tomography; ICU, Intensive care unit; PCR, Polymerase chain reaction*

Flow cytometric results and other relevant laboratory tests taken on the day of flow-cytometric analysis are listed in Tables 2 and 3. Table 4 depicts the flow cytometry results of leucocyte subsets obtained at 2 different time points 7-10 days apart from 10 patients during the course of the disease.

CRP, ferritin and D-dimer levels were all higher and total lymphocyte, as well as CD3+CD4+ lymphocyte counts were lower in the severe group; no significant differences were observed for monocytes, NK cells (represented as CD16+ positive cells), B lymphocytes, CD3+CD8+ cells and activated lymphocytes (described as HLA-DR+38+CD4+ and HLA-DR+38+CD8+ cells). CD4/8 ratio was found to be decreased in the severe group in accordance with lowered CD4+ and unchanged CD8+ cell counts. Neutrophil / lymphocyte ratio (NLR) was elevated and lymphocyte / CRP ratio (L/CRP) was lowered in the severe group, accordingly.

According to their initial blood sampling time points for flow cytometric analysis patients had been assigned into 3 groups: Groups I, II, III (see Methods Section for details). Intergroup comparison revealed a slight decrease in the total lymphocyte counts of patients who were in the first few days of the disease. However, none of the parameters tested (including CRP, D-dimer, ferritin and total neutrophil and lymphocyte counts) showed a significant difference between groups.

10 patients with results at two time intervals were also compared, only CRP levels showed a tendency to fall while median ferritin, D-dimer and complete blood count parameters remained unchanged.

Table 2: Laboratory Tests and Leucocyte subset distribution at the Day of Flow Cytometric Analysis

	Total (n=67)*	Severe (n=36)*	Non-severe (n=31)*	P-value**
CRP (mg/L)	18.4(4.6-68.7)	44(15-108)	7(3-18)	**0.000**
Ferritin (ng/mL)	313(152-590)	469(167-670)	190(128-331)	**0.025**
D-Dimer (mg/L)	0.68(0.37-1.43)	0.86(0.48-1.75)	0,55(0.3-0.9)	**0.013**
Haemoglobin (g/dL)	12.1(10.6-12.9)	11.7 (9.7-12.8)	12.1 (11.2-13)	0.220
Platelets (x10^3/μL)	243(185-296)	246 (160-282)	237 (185-326)	0.950
Leucocytes (/μL)	6000(4800-7500)	5900(5100-8400)	6100(4300-7200)	0.225
Neutrophils (/μL)	3700(2400-5000)	4000(2900-6300)	3200(1900-4400)	**0.013**
Monocytes (/μL)	500(400-700)	545(300-775)	500(400-800)	0.695
Lymphocytes (/μL)	1600(1000-2100)	1100(750-1700)	1900(1350-2100)	**0.002**
Neutrophil/ Lymphocyte	2.4(1.4-3.9)	3.8(2.3-5)	1.7(1-2.3)	**0.000**
CD3+ (/μL)	1350(690-1800)	832(476-1445)	1486(1218-1908)	**0.004**
CD16+ (/μL)	187(94-270)	176(67-277)	171(94-268)	0.782
CD19+ (/μL)	135(80-260)	96(36-218)	214(124-312)	**0.001**
CD14+ (/μL)	550(275-775)	596((269-840)	459(309-712)	0.308
CD3+ 4+ 8- (/μL)	770(360-1080)	440(254-786)	917(774-1190)	**0.000**
CD3+ 4- 8+ (/μL)	420(230-590)	360(179-584)	457(312-608)	0.094
CD3+ 4+ 8+(/μL)	21(8-36)	19(6-28)	20(10-37)	0.227
CD3+ 4- 8-(/μL)	56(25-99)	50(23-97)	60(33-109)	0.300
CD4+ / CD 8+	1.8(1.2-2.3)	1,68(1,19-2,1)	1,95(1,5-2,9)	**0.018**
CD3+ 16+ (/μL)	10.5(5-34.5)	11(7-34)	7(3-43)	0.314
CD14+ 16+ (/μL)	50(23-85)	54(20-186)	58(29-85)	0.758
CD3+ 4+ 38+(/μL)	84(40-174)	51(33-116)	127(58-220)	**0.016**
CD3+ 4+ DR+(/μL)	101(40-206)	95(28-192)	102(36-273)	0.414
CD3+ 4+ 38+ DR+ (/μL)	21(11-36)	19(11-34)	21(12-43)	0.611
CD3+ 8+ 38+ (/μL)	30(12-68)	29(12-65)	31(17-85)	0.252
CD3+ 8+ DR+ (/μL)	91(37-231)	88(42-201)	133(35-319)	0.606
CD3+ 8+ 38+ DR+ (/μL)	19(9-34)	20(10-42)	17(7-29)	0.489
Lymphocyte/CRP	87.8(19-489)	24(6-134)	249(73-767)	**0.000**

**Values are given in median, (IQR25-75); **Mann-Whitney; CRP, C-reactive protein*

Table 3: Laboratory Results and Leucocyte Subsets Grouped According to the Day of Initial Sampling

	DAY 0 -7*	DAY 7-14*	DAY > 14*	P-value**
Number of Patients (n)	15	16	12	-
CRP (mg/L)	27(6-95)	19.3(5-65)	11.8(2.5-19)	0.061
Ferritin (ng/mL)	253(129-664)	313(118-516)	368(154-585)	0.789
D-Dimer (mg/L)	0.65(0.4-1.5)	0.72(0.36-1.3)	0.9(0.36-1.68)	0.837
Haemoglobin (g/dL)	11.7(10.1-12.7)	12.2(10.8-13.1)	12.4(11.2-13.1)	0.595
Platelets (x10^3/μL)	220 (167-258)	256(187-326)	296(206-427)	0.064
Leucocytes (/μL)	6000(4100-7800)	6000(5200-7100)	6700(4500-8000)	0.723
Neutrophils (/μL)	3800(2000-5200)	3550(3050-4400)	4000(1800-5100)	0.991
Monocytes (/μL)	500(300-600)	600(400-830)	600(500-900)	0.052
Lymphocytes (/μL)	1210(780-1900)	1900(1000-2300)	1700(1300-2200)	0.110
Neutrophil/ Lymphocyte	2.6(1.1-5.2)	2.3(1.5-3.4)	2.2(1.4-3.9)	0.609
CD3+ (/μL)	1200(500-1600)	1420(820-1860)	1400(1120-1900)	0.369
CD16+ (/μL)	188(48-258)	212(72-291)	153(99-272)	0.819
CD19+ (/μL)	106(64-210)	113(79-281)	251(102-300)	0.142
CD14+ (/μL)	522(312-716)	459(243-673)	774(329-1088)	0.448
CD3+ 4+ 8- (/μL)	772(324-950)	768(357-1190)	902(587-1210)	0.254
CD3+ 4- 8+ (/μL)	386(205-585)	467(306-702)	492(312-608)	0.752
CD3+ 4+ 8+(/μL)	23(7-40)	13(8-26)	23(10-47)	0.247
CD3+ 4- 8-(/μL)	53(25-97)	53(21-174)	78(32-107)	0.898
CD4+ / CD 8+	1.86(1.19-2.4)	1.76(1.19-2.2)	2.25(1.28-2.61)	0.428
CD3+ 16+ (/μL)	8.6(0-60)	12(6-33)	9(5-33)	0.634
CD14+ 16+ (/μL)	48(19-73)	68(31-85)	48(23-151)	0.710
CD3+ 4+ 38+ (/μL)	47(35-154)	99(33-156)	122(62-315)	0.154
CD3+ 4+ DR+ (/μL)	126(46-286)	68(33-192)	104(29-142)	0.331
CD3+ 4+ 38+ DR+ (/μL)	20(12-32)	15(10-28)	31(11-74)	0.416
CD3+ 8+ 38+ (/μL)	29(10-69)	28(16-64)	31(12-73)	0.970
CD3+ 8+ DR+ (/μL)	133(30-342)	91(47-231)	60(37-169)	0.588
CD3+ 8+ 38+ DR+ (/μL)	17(7-34)	18(10-29)	22(11-44)	0.967
Lymphocyte/CRP	34(7-298)	95(24-456)	152(47-850)	**0.045**

**Values are given in median, (IQR25-75); **Kruskal-Wallis; CRP, C-reactive protein*

Table 4: Comparison of the leukocyte subset samples taken at 2 different time points (10 patients)

	1 st*	2 nd*	p-value**
CRP (mg/L)	153(86-188)	14(8.5-27)	**0.005**
Ferritin (ng/mL)	589(477-814)	446(409-753)	0.074
D-Dimer (mg/L)	0.65(046-1.43)	0.78(0.46-1.16)	0.374
Haemoglobin (g/dL)	11.7(10.6-13.1)	11.6(10.2-15.1)	0.646
Platelets (x10^3/µL)	220(160-266)	378(262-471)	0.139
Leucocytes (/µL)	8400(4200-11500)	6300(5000-9000)	0.646
Neutrophils (/µL)	6600(2900-9000)	4300(2900-8100)	0.878
Monocytes (/µL)	540(200-560)	700(500-800)	0.284
Lymphocytes (/µL)	1000(490-1700)	1200(1100-1700)	0.766
CD3+ (/µL)	697(487-1216)	1070(705-1480)	0.878
CD16+ (/µL)	142(40-199)	88(27-270)	0.241
CD19+ (/µL)	76(11-99)	75(29-117)	0.241
CD14+ (/µL)	402(267-747)	705(580-1020)	0.285
CD3+ 4+ 8- (/µL)	264(81-855)	575(295-615)	0.721
CD3+ 4- 8+ (/µL)	340(210-511)	496(296-730)	0.878
CD3+ 4+ 8+(/µL)	23(8-59)	19(18-62)	0.678
CD3+ 4- 8-(/µL)	42(23-99)	54(27-94)	0.959
CD4+ / CD 8+	1.2(0.64-2.56)	0.84(0.41-2.5)	0.444
CD3+ 16+ (/µL)	34(4-201)	9(4.5-75)	0.740
CD14+ 16+ (/µL)	23(8-47)	126(58-315)	0.139
CD3+ 4+ 38+(/µL)	42(12-47)	55(31-87)	0.203
CD3+ 4+ DR+(/µL)	92(47-370)	135(87-265)	0.575
CD3+ 4+ 38+ DR+ (/µL)	17(8-28)	25(24-49)	0.139
CD3+ 8+ 38+ (/µL)	33(9-81)	10(9-302)	0.721
CD3+ 8+ DR+ (/µL)	231(133-391)	254(85-521)	0.508
CD3+ 8+ 38+ DR+ (/µL)	33(4-81)	10(9-260)	0.646

**Values are given in median, (IQR25-75); ** Wilcoxon; CRP, C-reactive protein*

10 patients with results at two time intervals were also compared, only CRP levels showed a tendency to fall while median ferritin, D-dimer and complete blood count parameters remained unchanged.

Five patients were admitted to ICU and four of them died, all were in the severe group, ICU group shared same flow characteristics as in the severe group.

Sixteen patients received tocilizumab, all in the severe group. None of the parameters differed from the rest of the severe patients group(n=20), a temporal change was available only in 3 patients (before and after tocilizumab) but no conclusions could be drawn.

DISCUSSION

Immune response, innate and adaptive immunity play great role in the pathogenesis of SARS-CoV-2 infection. (2) It has been postulated that the aberrant immune response against the virus and defective repair mechanisms of the body are responsible for the organ damage, morbidity and mortality rather than the cytotoxicity caused by the virus. (2) It is, therefore, vital to gain insight into the exact mechanisms of disease and immunity against the virus in order to better understand how and when to best intervene. Flow cytometry is a fast and easy tool for pursuing immune cells and their maturation, differentiation during viral infections, yet the enormous spectrum of different cell lines makes the interpretation rather difficult. Phenotypical identification of different cell lines may not directly reflect their functional status. Also their circulating numbers in the peripheral blood may not always represent their true quantity in the body. Since a scantiness of T cells in the peripheral blood, for example, might be due to the relocation of these cells at injury sites (residing T cells at tissues) (17).

Our data were in line with the previous publications indicating that patients with severe infection were significantly older compared to patients with non-severe infection (2,7-9). The proportion of men in the severe group (69%) were significantly different from the non-severe group. Although hypertension, diabetes mellitus and chronic renal disease are insistently pronounced in other studies, no significance between different types of co-morbidities and clinical severity was observed in our study with the only exception of cardiovascular disease. Other than possible alterations in the immune system for the worse, age and comorbidities might also contribute to the shortcomings of the body by diminished residual organ capacities (17).

$CD4^+$ or $CD8^+$ T cell counts were independently linked to key patient outcomes including mortality, ICU admission, viral clearance, and recovery across many studies (4,6-11,). Lymphopenia and low CD3+ counts were signs of severe presentation in several datasets (1,2,4,10) as in this study.

Information regarding CD4$^+$ and CD8$^+$ T cells on the other hand is contradictory as either one or both were found decreased or unchanged in the literature (2,3,7-9, ,13). In many studies the CD4$^+$/CD8$^+$ ratio was reported to be unchanged since both the CD4$^+$ and CD8$^+$ cells were decreased (3). Lymphopenia obviously results from a decrease in both CD4$^+$ and CD8$^+$ T cells, although some studies suggested that the decline was more pronounced for CD8$^+$ T cells. It remains to be determined how lymphopenia might relate to CD4$^+$ T cell activation and/or dysfunction (2,11,17).

Not many reports can be found on activation markers, but deep immune-profiling and studies on immune signature in COVID-19 cases with different scenarios are expected to provide new insight into the immunity against the virus (8,15). In some of these studies, there were changes in CD38$^+$DR$^+$CD4$^+$ and CD38$^+$DR$^+$CD8$^+$ T cells (8,15). Other studies demonstrated that CD8$^+$ T cell activation was more salient than CD4$^+$ T cell activation but our study in line with other studies could not demonstrate an increase in the activated T cell fractions (2,17). In different studies other markers of activation including CD25 and Ki-67 have been studied at different time intervals with similar outcomes. These data should cautiously be interpreted since not all patients with COVID-19 might have this T cell activation phenotype; in fact, current data point out to potentially diverse patterns of CD8$^+$ T cell responses in patients with COVID-19 (11,17). Clonal expansion of CD8$^+$ T cells in peripheral blood has been associated with milder disease or better recovery rates; however, it is not clear whether this CD8$^+$ T cell clonal expansion was the cause or the consequence of the disease recovery (17).

In contrast to our results Chen et al. (7) could demonstrate the restoration of the distribution of CD3$^+$ T cells, CD4$^+$ T cells, CD8$^+$ T cells, and B cells following the recovery of patients. Our cohort, however, included a small group of patients and therefore was devoid of the power of showing significant difference in lymphocyte subset patterns over the course of the disease. This holds true for the 10 patients who gave 2 blood samples 7-10 days apart. Seven of those 10 were in the severe group and had delayed viral clearance and immune reconstitution. Studies on homogenous patient populations with multiple flow-cytometric analyses at previously specified time-points would help clarifying this issue.

We did not find a difference in the NK cell fractions over the course of the disease. Patients at different intervals of disease had similar NK cell populations. Some studies reported lower NK cell numbers with delayed NK cell improvement whereas others found normal levels (2, 7, 13). Interesting data on NK immune-phenotypes in COVID-19 have been retrieved from studies which also take KIR expression profiles into account striving more for functional abilities rather than sole phenotypical classification (14).

B lymphocyte profiling in comprehensive studies revealed elevated plasmablast levels and their relationship with antibody production after COVID-19 infection as well as other viral infections and vaccinations (8,20). CD19 counts alone were less remarkable, as they were unchanged in most (2,13) and but decreased in some studies (7,8). In our study, we found a significant decrease in CD19$^+$ cells in the severe group. We also noticed that CD19$^+$ cells were lower in patients within the first 14 days of infection when compared to levels after 14 days. However, this difference was not statistically significant.

In critical patients with COVID-19, decline in the number and HLA-DR expression of monocytes might potentially lead to decreased antigen presentation and thus immunosuppression, while increased number of CD16$^+$ pro-inflammatory monocytes might mediate hyper-inflammation. Studies reveal that the extent of HLA-DR$^+$ monocytes might help identifying the risk for developing critical/severe COVID-19 (22). CD16$^+$ monocytes were within normal limits in our study, mostly in line with the literature. There are, however, other studies, which have reported lower levels (2). Not only quantitative alterations in immune profile but also atypical monocytes with bizarre side scatter characteristics and mean fluorescent intensity patterns for common antigens like CD14 have been observed (19). Clinical significance of these findings and their relevance to COVID-19 has not yet been clarified.

The main significant change that could be observed between groups and time intervals was with CRP and ferritin to some extent, which are well established surrogate markers of inflammation. Just as in other clinical studies on COVID-19 they were correlated with severity of cases (1,2,4,10,13). Some previously defined ratios such as neutrophil-lymphocyte ratio (NLR) and lymphocyte-CRP ratio (LCR) were also lower and higher in our study in severe cases, respectively (2), but their clinical significance and true contribution needs further exploration and validation in clinical decision making algorithms.

Big part of the studies looking at immune profiling come from China and this study is one of the few reports from Türkiye and middle-eastern territory. There is a great polymorphism in inflammatory responses between different populations and it is important to reveal differences between them. Another study from Türkiye found reduced naive T cell/$CD4^+$ effector-memory T cell ratio, an indicator of the differentiation from naive T cells to memory cells and lower peripheral $CD4^+CD8^+$ double-positive T cells in severe disease (18). Conversely, double positive and double negative $CD3^+$ cells were similar between groups and no temporal changes were observed in our study; but we could not determine a baseline reference value for these subpopulations, therefore, we could not comment on a possible reduction.

Other than the universal finding of lymphopenia and low counts of $CD3^+$ cells and their correlation (causality yet to be shown!) to disease severity, the remainder of lymphocyte subsets have varying results among the vast majority of the studies. With the lack of proper and validated cut-off values, well-established subgroups of different cell lines and their interactions and their ever-changing numbers/proportions during reactive conditions, it is hard to draw exact conclusions just based on lymphocyte subsets. Small numbers and heterogeneous patient populations with highly variable study designs make this area more prone to speculation.

A recent study also reporting the subset of helper and cytotoxic T cells found, that not only $CD3^+$ and $CD4^+$ helper cells but also B cells, NK cells and also all subsets except EMRA $CD4^+$ and $CD8^+$ plus terminal effector $CD8^+$ cells, were all lower in COVID patients compared to healthy subjects and lower in the severe group to non-severe cases (25).

There were several limitations to this study that might cause some potential bias. First, it was a single-centre, small-sample study of patients admitted to the hospital. Second, patients with ICU admission and patients with residual lung damage were underrepresented. Second blood samples for flow-cytometric analysis were not necessarily obtained after recovery but after Day 14 according to the previously specified plan. Thus, second samples possibly did not always reflect a recovery phase. Another hurdle to overcome was the difficulty in determining the D0 of the disease since not all patients were symptomatic. Moreover, the use of chloroquine and azithromycin at the onset of symptoms may have affected the counts of some patients. Antigen presenting cells like dendritic cells and other representatives of innate immune system were not included in the panel, NK cells and monocytes were underrepresented as they were solely identified based on CD14 and CD16 positivity, respectively. Of note, HLA-DR and CD38 expression on lymphocytes might show inter-observer variability.

Deeper and more comprehensive immune profiling in larger cohort of patients reflecting different clinical scenarios and at different and well-planned time points will shed more light in the understanding of COVID-19 and the hyper-inflammatory states we see with viral infections. Also, the changes in the immune profile after immunomodulatory drugs and vaccination will inform us more about the complex structure of the immune response.

CONCLUSION

Total lymphocyte counts, CD4 counts and to some extent CD8 and CD19 counts correlate with COVID-19 infection as shown by previous studies. Associations between severity and lymphocyte activation markers, NK cells and monocytes could not be shown. A more comprehensive immune profiling with more frequent measurements will aid more in the understanding of the immunological process, the prognosis and outcomes of COVID-19.

Declarations

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Conflicts of interest/Competing interests

The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript

Ethics approval

Animal subjects were not part of this study. This was an all human subject study, they all have been asked for and given a written and signed informed consent (which was approved by the local ethical comitee. The study received approval from the ethical committee of Cerrahpasa Medical Faculty (03.06.2020 - 67534) and Ministry of Health of Türkiye

Availability of data and material

All additional data can be demanded from corresponding author.

Authors' contributions

All authors have participated in additional conception and design, or analysis and interpretation of the data, AKE contributed to data collection, TE and MCA planned the design, purpose of the study and wrote the article.

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