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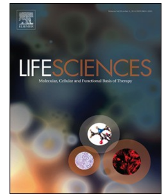

Immunocytometric analysis of COVID patients: A contribution to personalized therapy?

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ABSTRACT

Aims: This study aims to cast light on immunocytometric alterations in COVID-19, a potentially fatal viral infection with heterogeneous clinical expression and a not completely defined pathophysiology.
Methods: We studied 35 COVID patients at hospital admission testing by cytofluorimetry a large panel of lymphocyte subpopulations and serum tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-17A and the soluble receptor of IL-17A (IL-17RA).
Key findings: At hospital admission, total lymphocytes and most T and B subpopulations were reduced in 50–80% of patients, with close relationship to disease severity. While activated T helper 1 (TH1) and TH17 cells resulted normal or higher. Serum IL-6 was increased in all patients, while TNF-α and IL-17A were higher in advanced stages. A patient subset with low severity had very high IL-17RA levels. Tocilizumab treatment caused an increase of IL-17A in 3/6 patients and a reduction in 3 others, while the lymphocyte number increased in 3 patients and did not change in the others.
Significance: Cytofluorimetry revealed a functional exhaustion of most lymphocyte populations in COVID patients not involving activated TH1 and TH17. Consequently, there was a relevant cytokines production that contributes to impair the respiratory inflammation. The increase of TH17 and IL-17 in a subset of cases and the evidence of a significant increase of IL-17RA (that prevents the interaction of IL-17 with the cell receptor) in patients with low severity suggest that some patients could benefit from monoclonal antibodies treatment targeting IL-17 pathway. Immunocytofluorimetric markers may contribute to a personalized therapy in COVID patients.

1. Introduction

Coronavirus disease 2019 (COVID-19) caused by Severe Acute Respiratory Syndrome - Coronavirus-2 (SARS-CoV-2) has a widely variable clinical expression from asymptomatic or paucisymptomatic forms to severe clinical conditions with respiratory failure, sepsis and multiple organ dysfunction syndrome [1,2].

The pathophysiology of COVID-19 infection is not completely defined. Circulating lymphocytes are reduced for still unknown pathogenic mechanisms and there is a massive release of cytokines [3] that contribute to the progression of the disease promoting viral sepsis and leading to severe respiratory complications [4]. Among inflammatory cytokines, interleukin (IL) 6 is increased in serum from patients with severe COVID-19 infection [5] and its levels relate to serum viral load and to the outcome of the disease [6]. Due to the pivotal role of IL-6 as mediator of inflammatory toxicity [7], a monoclonal antibody (Tocilizumab) against IL-6 receptor (IL-6R) was proposed in a preliminary clinical trial of severe COVID patients in China

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and after encouraging results, larger trials were promoted [8–10]. However, the role of IL-6 in the pathogenesis of COVID-19 remains to be fully clarified since in experimental models IL-6 can either inhibit or promote viral replication [11,12]; furthermore, this cytokine is essential for viral clearance in early stages of COVID pneumonia and to drive the correct tissue remodeling after the infection [12].

Emerging evidences support the role of IL-17 in SARS-CoV-2 pathogenesis. A high number of T helper (TH) 17 lymphocytes was found in the alveolar space of a COVID-19 patient [13] and serum IL-17 was increased in COVID-19 patients, particularly in those that required intensive care [14]. In view of this, blocking IL-17 directly [15,16] or through the inhibition of JAK2 [17] could contribute to improve the aberrant immune response and acute respiratory distress of COVID patients.

To cast light on immunological and cytological response during SARS-Cov-2 infection, we studied a group of COVID-19 patients during their recovery in a specialized hospital, evaluating either at admission or after 7-day follow-up the levels of serum cytokines and the lymphocyte subpopulation profile.

2. Patients and methods

2.1. Patients

All consecutive adult patients with a diagnosis of COVID-19 (SARS-CoV-2 infection) admitted at one of the following hospitals were enrolled: Department of Clinical Medicine and Surgery - Section of Infectious Diseases, University Hospital Federico II, Naples; Department of Infectious Disease and Infectious Urgencies - Division of Respiratory Infectious Disease, Cotugno Hospital, AORN dei Colli, Naples. The study period started on 20 March 2020 with the enrollment of the first patient and was concluded on 5 May 2020 with the 7-days follow-up of the last patient. The study was approved by the Ethical Committee of the University Federico II of Naples; the lone exclusion criterion was the refusal or the impossibility to sign the informed consent. However, none of the patients admitted to our Institutions during the period of our study was excluded. All the patients were informed on the aims of the study and all of them were able to decide. The diagnosis of COVID infection was confirmed by molecular analysis (RT-PCR) on nasopharingeal swab using the kit by Ab-analitica (REALQUALITY RQ-2019-nCoV). All the enrolled patients were classified on the basis of the seven ordinal scale made by the World Health Organization (WHO)-Research and Development Blueprint expert group and used in previous influenza studies [18,19]. According to this classification patients can be identified as: 1, not hospitalized with resumption of normal activities; 2, not hospitalized, but unable to resume normal activities; 3, hospitalized, not requiring supplemental oxygen; 4, hospitalized, requiring supplemental oxygen; 5, hospitalized, requiring nasal high-flow oxygen therapy, non-invasive mechanical ventilation, or both; 6, hospitalized, requiring extra corporeal membrane oxygenation, invasive mechanical ventilation, or both; and 7, death. For each patient we considered the worst WHO stage during the infection.

Whole blood samples were collected at admission and after one week of hospitalization in tubes containing EDTA or free from anticoagulant and then immediately analyzed by flow cytometry. Serum samples were separated from blood cells after the collection in tubes without anticoagulant and stored at −80 °C until cytokine analysis.

2.2. Lymphocyte subset analysis

Immunophenotyping analysis was perfomed by multicolour flow cytometry (Facs Canto II; Becton Dickinson Italia, Milan, Italy). The cells were collected and analyzed with Facs Diva software [20]. For each sample, we prepared three mixes of monoclonal antibodies against specific cell populations. Specifically, CD45 was used to gate the viable lymphocyte cells. From this gate, CD3+ CD4+ cells were identified as T helper [21], while CD3+ CD8+ as cytotoxic T lymphocytes. Among T-helper cells, TH1 and TH17 were distinguished by specific surface markers, i.e., CXCR3 and CCR6 [22], respectively. Moreover, CD38 and HLA-DR were used as activation markers expressed on activated TH1 and TH17 cells. In the second mix, beside CD3 and CD45, CD56 and CD19 were used to identify Natural Killer (NK) and B cells, respectively. From this last mix, we obtained the whole picture of T (CD3+ CD45+), NK (CD45+ CD56+) and B (CD45+ CD19+) cell distribution for each patient. In the third mix, we analized the levels of regulatory (CD3+ CD4+ CD25+ CD127low), naïve (CD45RA+ CD3+ CD4+ CD25+ CD127low) and memory (CD45RO+ CD3+ CD4+ CD25+ CD127low) T cells [23].

2.3. Serum cytokines analyses

Serum IL-17A, IL-6 and TNF-α levels were analyzed using human-specific enzyme-linked immunosorbent assay (ELISA) Max™ Set Deluxe kits (BioLegend, Inc., San Diego, USA), in accordance with the manufacturer's instructions. In particular, one day prior to perform the assay, 96-well plates were coated with the specific capture antibody and incubated overnight at 4 °C. Serum IL-17A receptor (IL-17RA) was measured by ELISA kit from Thermo Scientific™ Pierce™ (Frederick, MD 21704, USA), in accordance with the manufacturer's instructions. The assay was performed using anti-human IL-17RA precoated 96-well strip plates. All serum samples and standard solutions for calibration curves were analyzed in duplicate using 100 μL of diluted or undiluted sample and mean concentrations were calculated. In particular, IL-17A and TNF-α were analyzed using undiluted and 2-fold diluted samples, while IL-6 was analyzed on 2-fold and 10-fold diluted samples. For IL-17RA measurement, the serum samples were diluted 2-fold and 5-fold. The concentration values (pg/mL) of each cytokine were obtained by interpolating the absorbance values on the respective calibration curve.

2.4. Statistical analysis

Continuous data were reported as mean (standard error, SE) for normal distributions or median (interquartile range, IQR) for non-parametric distributions. Categorical data were reported as frequency and percentage. Comparisons of paired data with non-parametric distributions were performed by Wilcoxon signed-rank test. Statistical differences between three groups with normal distributions were assessed by ANOVA test and Bonferroni test as post-hoc test. The correlations between variables were evaluated by Spearman's rank-order correlation and Spearman's rank correlation coefficient (r_s) was calculated. Graphics have been performed by KaleidaGraph software (version 4.1.1, Synergy, Reading, PA, USA). *P* values < 0.05 were considered as significant.

3. Results

Thirty-five patients with COVID-19 infectious were enrolled (27/35 males, 80.0%), with median age of 61 years (IQR: 50–73). All patients started different treatments in the day of hospitalization. Most patients received Lopinavir/Ritonavir (22/35, 62.8%) and/or Hydroxychloroquine (24/35, 68.5%) as treatment for COVID-19. Only 2/35 (5.7%) patients were treated with Darunavir/Cobicistat. Seven patients (20.0%) were also treated with Tocilizumab according to the following inclusion/exclusion criteria. Inclusion criteria: clinical/instrumental diagnosis of pneumonia and oxygen saturation at rest in ambient air ≤93% or requiring oxygen therapy or mechanical

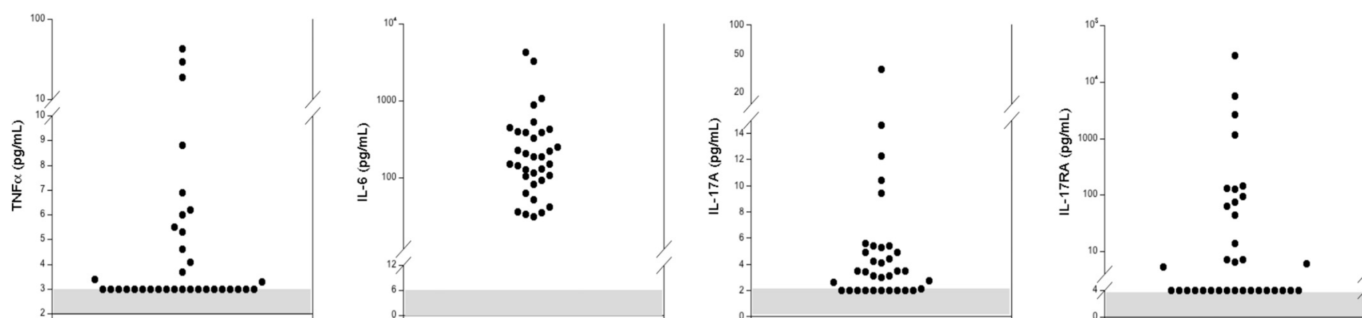

Fig. 1. Serum levels (pg/mL) of TNF-α, interleukin-6, interleukin-17A and interleukin-17 RA in 35 COVID patients at hospital admission. Gray areas indicate the reference ranges.

ventilation either noninvasive or invasive (intubated). Exclusion criteria: known hypersensitivity to tocilizumab or its excipients; known active infections or other clinical condition that contraindicate tocilizumab and cannot be treated or solved according to the judgement of the clinician; ALT/AST > 5 times the upper limit of the normality; neutrophils < 500/mmc; platelets < 50.000/mmc; bowel diverticulitis or perforation. All patients underwent arterial blood gas test (ABG) at admission to evaluate pO_2 and start an eventual oxygen supplement therapy; after one week ABG was repeated. At admission, most patients (28/35, 80.0%) needed oxygen therapy. Among these, the median value of the ratio of arterial oxygen partial pressure to fractional inspired oxygen (P/F ratio) was 243 (IQR: 172–338). Most patients had a WHO score of 4 (20/35, 57.1%); the remaining patients had a WHO score of 3 (7/35, 20.0%), 5 (3/35, 8.5%) and 7 (5/35, 20.0%).

Fig. 1 shows serum levels of TNF-α, IL-6, IL-17A and IL-17RA in the COVID patients at admission. For each of the four markers a percentage of patients showed values above the upper reference value: 14/35 (40.0%) for TNF-α; 35/35 (100%) for IL-6; 25/35 (71.4%) for IL-17A; and 19/35 (54.2%) for IL-17RA (see Additional file 1, Table S1).

Fig. 2 shows the number of total, T, B and NK lymphocytes in the 35 patients at admission. For each population of lymphocytes a percentage of patients showed values below the lower reference value: 28/34 (80.0%) for total lymphocytes; 14/34 (40.0%) for T lymphocytes; 18/35 (51.5%) for B lymphocytes, and 5/35 (14.3%) for NK lymphocytes (see Additional file 1, Table S2). Fig. 3A shows the number of naïve, activated and memory lymphocytes in the same patients. Three (8.6%) patients had a number of naïve lymphocytes above the upper reference value; 8/35 (22.8%) had a number of activated lymphocytes below the lower reference value; and finally, 1/35 (2.9%) had a higher and 13/35 (37.1%) had a reduced number of memory lymphocytes (see Additional file 1, Table S2).

Fig. 3B shows the number of helper and suppressor T lymphocytes in the 35 COVID patients at admission. Nineteen (54.2%) patients had helper lymphocytes below the lower reference value and 20/35 (57.1%) had a lower number of suppressor lymphocytes. Furthermore, 3/35 patients (8.5%) had a lower and 3/35 (8.5%) had a higher helper/suppressor ratio (see Additional file 1, Table S2). Fig. 4 shows the number of other T lymphocyte subpopulations in our patients at admission. Seven (20.0%) patients had a number of T regulatory lymphocytes below the lower reference value; 7/35 (20.0%) patients had a number of T activated lymphocytes below the lower reference value; 4/35 (11.4%) patients had lower, and 5/35 (14.3%) had higher TH1 lymphocytes; and 9/35 (25.7%) patients had a higher TH17 lymphocyte number, while only one patient had a number of activated TH17 lymphocytes below the reference values. Finally, 4 patients (11.4%) had a higher number of TH1 activated lymphocytes, and 5 (14.3%) a higher number of TH17 activated lymphocytes (see Additional file 1, Table S2).

Table 1 shows the correlations between serum cytokines and WBC subpopulations. The levels of TNF-α were significantly correlated to the number of circulating monocytes, T helper and TH1 activated lymphocytes, while serum IL-6 levels significantly correlated to the number of monocytes and granulocytes. Furthermore, IL-6 levels were inversely correlated to the number of T suppressor and TH1. Serum IL-17A was positively correlated with TNF-α and IL-17RA. On the other hand, we found that the highest levels of IL-17RA (Fig. 1) were negatively correlated with IL-17A levels ($n = 6$, $r_s = -0.899$, $p = 0.038$). Finally, IL-17RA levels were significantly correlated with the number of TH17 and TH17 activated lymphocytes.

Table 2 shows serum levels of cytokines and circulating lymphocyte subpopulations (expressed either as % and as absolute number) in the 35 COVID patients at admission, classified on the basis of the WHO score. TNF-α and IL-17A were higher in WHO 4 patients as compared to WHO 3 ones, although not significantly for TNF-α. While, IL-6 resulted higher in WHO 4 as compared to WHO 3 patients and significantly higher in WHO 5–7 as compared to WHO 4 ones. Finally, IL-17RA was lower in WHO 4 patients as compared to WHO 3 ones, and again lower in WHO 5–7 as compared to WHO 4, although not significantly.

As shown in Table 2, the percentage and the number of total lymphocytes gradually reduced with the severity score. All the lymphocyte subpopulations expressed as % of total lymphocytes did not significantly varied within the three severity levels; for some subpopulations (i.e., total activated lymphocytes, T activated lymphocytes, TH17, TH1) the percentage increased, although not significantly, in patients in the advanced stage. Only the percentage values of TH1 activated on the total of T Helper lymphocytes significantly increased in patients with advanced WHO stages of the disease. Going to the absolute number of lymphocyte subpopulations, all showed a trend of reduction in patients with advanced WHO stages of the disease, that resulted significant for several subpopulations, (i.e., T, memory, helper, regulatory and TH1). The number of other lymphocyte subpopulations (i.e., activated total lymphocytes and activated TH17) resulted stable within the stages, while the number of activated TH1 increased with the stage of the disease.

In 31/35 COVID patients (88.5%) we performed a second sampling after 7 days from the admission, testing again serum levels of the 3 cytokines (i.e., TNF-α, IL-6, IL-17A) and IL-17RA, and lymphocyte subpopulations. Among the 31 patients who repeated blood collection, most were in oxygen therapy (25, 80.6%). Among these, median P/F value was 245 (IQR: 184–342). We compared the data of the first and the second determination in each patient using the Wilkoxon test. All the variants were not significantly different except TH1 lymphocytes that resulted significantly higher ($p = 0.012$) in the second

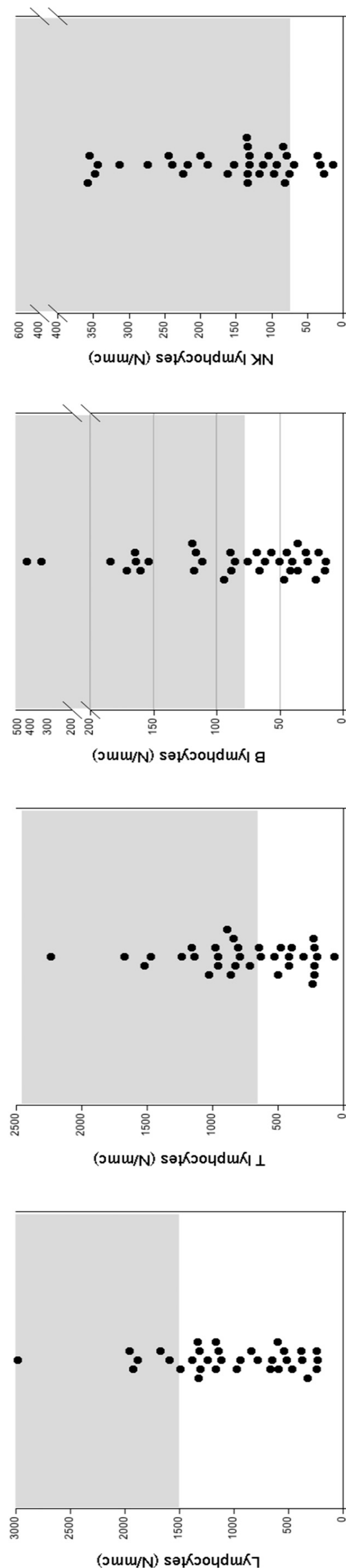

Fig. 2. Total, T, B and NK lymphocytes (N/mmc) in 35 COVID patients at hospital admission. Gray areas indicate the reference ranges.

determination (data not shown).

Finally, 7/35 (20.0%) patients were treated with a monoclonal anti IL-6 (Tocilizumab). One of these patients was transferred in another hospital two days later. While, for 6 patients we performed a second sampling after 7 days from the admission, testing again either serum levels of the 3 cytokines (TNF-α, IL-6, IL-17A) and IL-17RA, and all lymphocyte subpopulations (Table 3). In all six patients the WHO score did not change after the treatment. The values of serum cytokines and of IL-17RA had a variable trend: in three patients (i.e., cases n. 1, 2 and 3) serum levels of IL-6 were reduced after the treatment, as it occurred also for TNF-α in cases n. 2 and 3. While, in three other patients (i.e., cases 4, 5 and 6) the values of IL-6 increased, while the other parameters, already normal or slightly increased before the treatment did not change.

4. Discussion

We observed a reduced number of lymphocytes in 80% of the 35 COVID patients at admission, in agreement with previous studies [24,25]. Such reduction involves B and T lymphocytes, both reduced in about a half of COVID patients, differently from previous studies that reported a main reduction of T lymphocytes [3,26] and an increase of the percentage and of the absolute number of B lymphocytes, particularly in severe COVID patients [6]. While, NK lymphocytes resulted within the reference range in more than 80% of patients, differently from a previous study that reported a remarkable reduction of such cells, particularly in severe COVID patients [26]. Among the common subpopulations (B + T lymphocytes), the reduction involves mainly memory lymphocytes (in more than 35% of patients), while naïve and activated lymphocytes are normal or slightly reduced, in agreement with studies that report the accumulation of B memory and naïve cells in lung of COVID patients [4]. Among T lymphocytes, we observed a reduction of helper, suppressor and regulatory subpopulations, while most patients showed a normal or increased number of TH activated lymphocytes, differently from previous studies that reported a global T cell lymphopenia [3,6,24–26]. However, these studies tested only T helper and suppressor lymphocytes not extending the analysis to the activated T cells. Thus, the cytofluorimetric analysis reveals a picture of lymphocyte depression, a known effect induced by COVID that may be due to a functional exhaustion [26], recently demonstrated for T lymphocytes in COVID patients [24]. In fact, the percentage of most lymphocyte subpopulations was stable even in patients with a more severe disease, while the absolute number of most subpopulations was reduced in a high percentage of COVID patients and declined with the severity of the disease, according to previous studies [6]. However, some specific subpopulations (i.e., TH1 and activated TH1 and TH17 lymphocytes), seem to be less involved by the exhaustion, because they were not reduced in COVID patients (in some patients they are above the upper reference range) and they showed a trend to increase with the WHO disease stage, partially in agreement with a previous study [6], confirming that the hyperactivation of CD8 T cells contributes to enhance the severity of the disease [17]. In fact, activated TH lymphocytes contribute to produce cytokines that represent the second-line defense mechanism against the virus [3]. A consequence of the cytokine release is the recruitment of macrophages and granulocytes, particularly stimulated by IL-17 [4] that in turn amplifies the production of cytokines [3], as it is confirmed by the higher number of circulating granulocytes in most COVID patients (data not shown) and by the correlations between serum TNF-α and IL-6 and the number of circulating monocytes, and of serum IL-6 with the number of circulating granulocytes. A recent study found an inverse correlation between serum IL-6 levels and circulating helper and suppressor lymphocytes, suggesting that the increase of IL-6 may further contribute to lymphopenia [24]. We confirm such hypothesis, since we found an inverse correlation between serum IL-6 and the number of TH1 and suppressor lymphocytes.

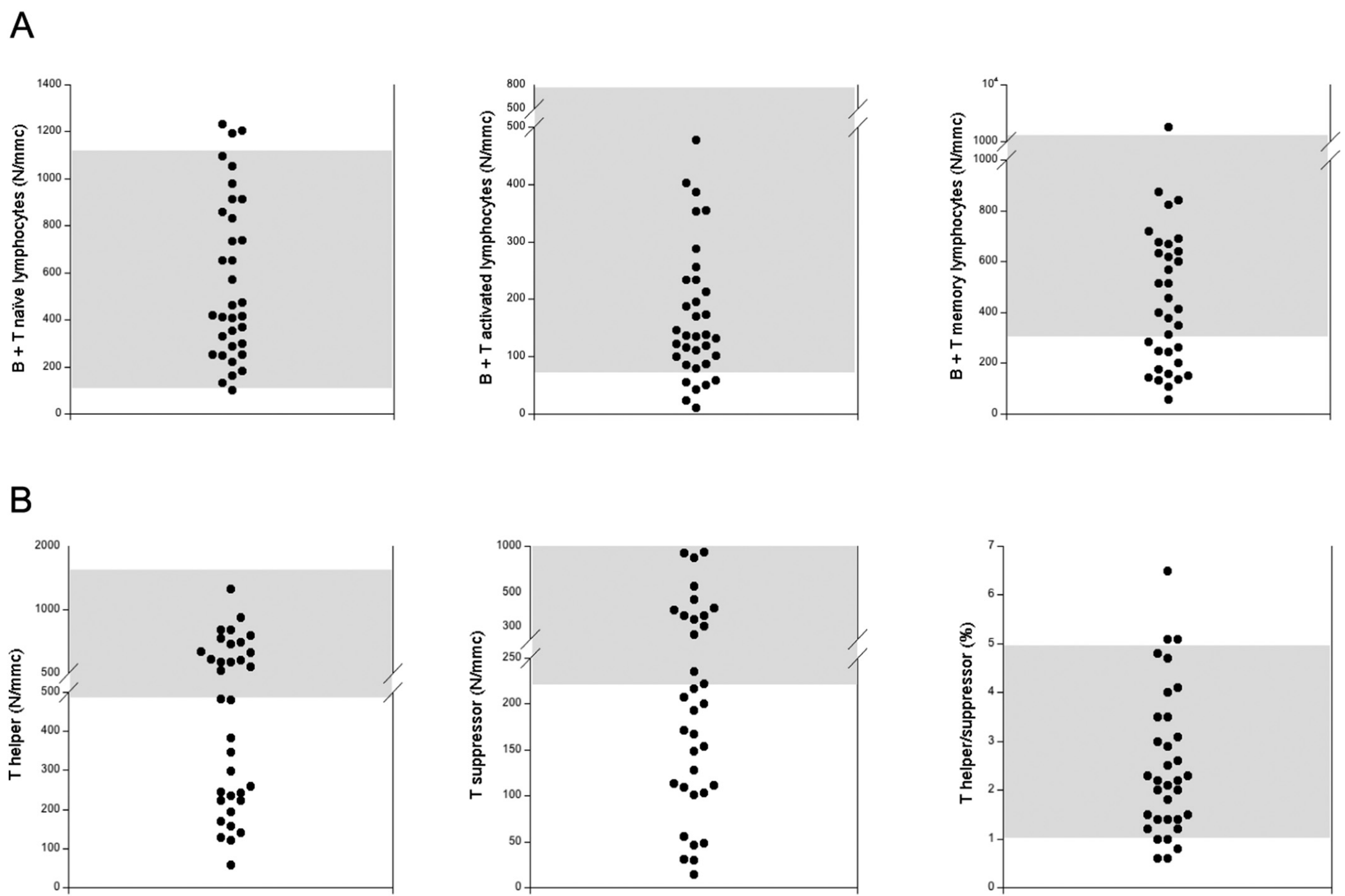

Fig. 3. A) Naïve, activated and memory lymphocytes (N/mmc) in 35 COVID patients at hospital admission. B) Helper, suppressor and helper/suppressor ratio in 35 COVID patients at hospital admission. Gray areas indicate the reference ranges.

Thus, the lymphocyte depression in COVID patients helps the spreading of the virus [4], and was not modified in our patients after 1 week of hospitalization and treatment, differently from a previous study that reported an increase of lymphocyte subpopulations in 66% of COVID patients after 1 week of treatment [27]. A consequence of the lymphocyte depression is the cytokine storm, more pronounced in severe patients as demonstrated by the higher serum levels of TNF-α, IL-6 and IL-17A in advanced stages of the disease, in agreement with previous data [6]. It strongly contributes to acute respiratory distress [28] and to multiple organ dysfunction [29] syndromes typically observed in COVID patients.

For this reason, various trials started with the use of Tocilizumab in COVID patients aiming to reduce the levels (and the effects) of IL-6. Such trials provided encouraging results in terms of the morbidity and mortality in severe COVID patients [30], but with a large variability and with a contrary voice [31], stimulating larger studies. An Italian study on 100 patients [10] concluded that 58% and 77% of 100 COVID patients treated with Tocilizumab had a clinical improvement at 72 h and 10 days, respectively, and that such patients had an increase of lymphocytes and of serum IL-6 after the treatment independently by the clinical effect. Another study on 20 COVID patients reported a clinical improvement after 5 days of treatment in about 70% of cases, an increase of the lymphocyte count in a half of cases and an early reduction of serum IL-6 mean values followed by an increase of the levels [9]. Finally, a study on 15 COVID patients treated with Tocilizumab reported a clinical improvement in two third of patients with a spike of serum IL-6 in the first day and then a rapid reduction of the cytokine in the following days and a dramatic increase of IL-6 in the other patients in which the treatment failed [8]. None of these studies evaluated lymphocyte subpopulations. In the present study, only 7 patients were treated with Tocilizumab; one of them was lost to follow-up, while the treatment caused a reduction of serum IL-6 in three cases and an increase in the others (particularly relevant in two of the three). This is in agreement with the mechanism of action of Tocilizumab, which is an antagonist of IL-6R. Finally, the treatment restored the number of total lymphocytes in three out of six patients and the number of T lymphocytes in two of the six patients. Thus, other studies are necessary to define the efficacy of the treatment and to select markers useful to predict and to assess the response of COVID patients to Tocilizumab.

Our data suggest that other cytokines, like IL-17A, may have a role in the pathogenesis of COVID and could became the target of therapies with monoclonal antibodies already available for the treatment of other diseases [32]. In fact, among our COVID patients, more than 70% had a serum increase of such cytokine (that in 5 cases was more than 5× the URL). In addition, TH17 and activated TH17 that produce most IL-17A [33] are among the few lymphocyte populations increased in our COVID patients (particularly in advances stages). Finally, a half of COVID patients had an increase of IL-17RA (among which 11 patients with an increase > 10× URL). It is known that high levels of receptor in serum (that we found mainly in COVID patients with a less severe WHO stage) may represent a system aimed to internalize some amount of IL-17A limiting its effect [34]. Such effect might be further reduced

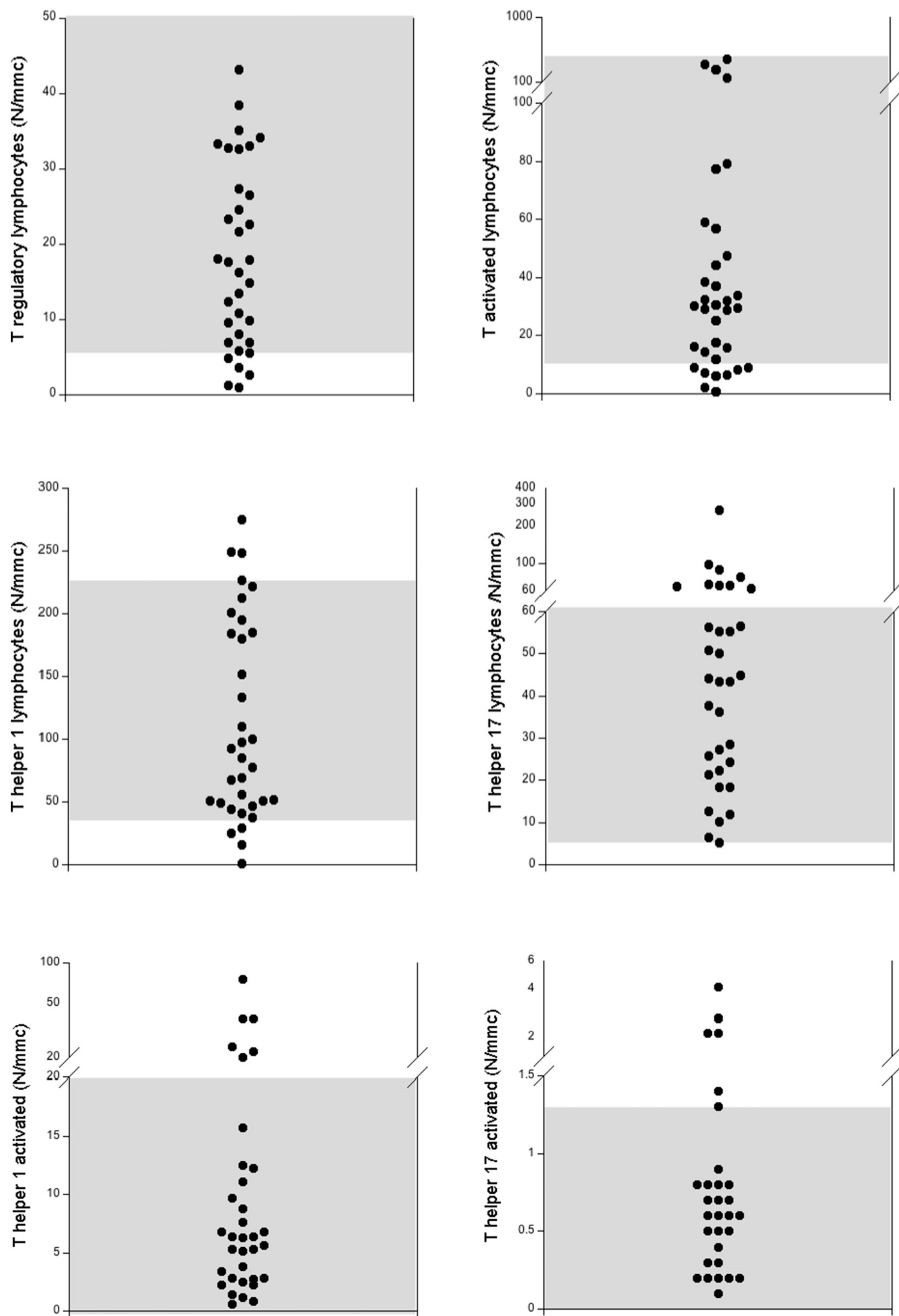

Fig. 4. Regulatory, activated, TH17, TH1, activated TH17 and activated TH1 lymphocytes (N/mmc) in 35 COVID patients at hospital admission. Gray areas indicate the reference ranges.

Table 1
Correlation between serum cytokines (pg/mL) and WBC subpopulations (N/mmc) in 35 patients with COVID at admission. *P* value and (r_s).

	TNF-α	IL-6	IL-17A	IL-17RA
IL-6	n.s.	–	n.s.	n.s.
IL-17A	0.038 (0.267)	n.s.	–	0.028 (0.273)
IL-17RA	n.s.	n.s.	0.028 (0.273)	–
Monocytes	0.024 (0.290)	0.043 (0.256)	n.s.	n.s.
Granulocytes	n.s.	0.031 (0.272)	n.s.	n.s.
Lymphocytes	n.s.	n.s.	n.s.	n.s.
B lymphocytes	n.s.	n.s.	n.s.	n.s.
T lymphocytes	n.s.	n.s.	n.s.	n.s.
NK lymphocytes	n.s.	n.s.	n.s.	n.s.
T helper	0.020 (0.381)	n.s.	n.s.	n.s.
T suppressor	n.s.	0.050 (−0.295)	n.s.	n.s.
TH1	n.s.	0.020 (−0.293)	n.s.	n.s.
TH17	n.s.	n.s.	n.s.	0.002 (0.371)
TH1 activated	0.021 (0.296)	n.s.	n.s.	n.s.
TH17 activated	n.s.	n.s.	n.s.	0.023 (0.281)

Table 2
Serum cytokine levels (pg/mL) and circulating lymphocytes in 35 COVID patients at admission with different severity according to worst WHO stage for each patient. Mean and (SE).

	WHO 3 (n = 7)	WHO 4 (n = 20)	WHO 5–7 (n = 8)	ANOVA (p value)
TNF-α	3.1 (0.2)	7.3 (2.3)	5.5 (1.9)	n.s.
IL-6	167.6 (59.8)	394.7 (160.6)	789.1 (503.2)*	0.021
IL-17A	2.5 (0.4)	6.6 (1.7)*	3.7 (1.0)	n.s.
IL-17RA	4867.2 (4862.5)	448.2 (301.9)	152.3 (142.2)	n.s.
Total (% of WBC)	34.1 (9.1)	16.3 (2.3)	12.5 (3.8)	n.s.
Total (N/mmc)	1600.2 (464.3)	1078.0 (100.8)	829.9 (324.9)	n.s.
T (% of lymphocytes)	74.7 (5.2)	72.1 (1.9)	66.8 (6.5)	n.s.
T (N/mmc)	1232.8 (394.4)	786.7 (81.0)*	594.4 (250.0)*	0.041
B (% of lymphocytes)	11.0 (3.0)	8.1 (1.1)	13.5 (3.7)	n.s.
B (N/mmc)	165.5 (53.3)	91.4 (17.0)	74.7 (17.1)	n.s.
NK (% of lymphocytes)	12.0 (2.6)	17.6 (2.1)	18.2 (2.8)	n.s.
NK (N/mmc)	165.2 (33.8)	180.5 (23.3)	119.9 (35.6)	n.s.
Naïve (% of lymphocytes)	58.3 (3.3)	52.9 (2.6)	59.8 (5.0)	n.s.
Naïve (N/mmc)	942.9 (272.0)	577.9 (67.6)	444.3 (138.4)	n.s.
Activated (% of lymphocytes)	14.7 (2.9)	16.5 (1.5)	19.1 (3.2)	n.s.
Activated (N/mmc)	244.6 (69.8)	172.1 (22.2)	139.9 (43.5)	n.s.
Memory (% of lymphocytes)	40.9 (3.6)	46.6 (2.6)	39.9 (4.9)	n.s.
Memory (N/mmc)	639.6 (191.3)	494.6 (49.9)	384.3 (203.8)*	0.05
T helper (% of lymphocytes)	49.7 (4.1)	44.5 (2.3)	43.6 (4.9)	n.s.
T helper (N/mmc)	834.1 (312.1)	482.4 (50.4)	349.1 (135.9)*	0.05
T suppressor (% of lymphocytes)	21.6 (4.6)	23.9 (2.2)	22.6 (3.8)	n.s.
T suppressor (N/mmc)	334.9 (105.3)	264.8 (44.1)	218.3 (108.7)	n.s.
Helper/suppressor	3.0 (0.6)	2.3 (0.3)	3.1 (0.7)	n.s.
T regulatory (% of lymphocytes)	2.1 (0.3)	1.8 (0.2)	1.4 (0.3)	n.s.
T regulatory (N/mmc)	25.6 (4.1)	19.2 (2.7)	9.7 (2.4)*	0.03
T activated (% of lymphocytes)	3.3 (0.7)	5.9 (0.9)	4.6 (1.1)	n.s.
T activated (N/mmc)	46.9 (16.8)	46.6 (10.3)	44.2 (27.0)	n.s.
TH1 (% of lymphocytes)	27.3 (1.3)	25.6 (2.6)	18.8 (4.1)	n.s.
TH1 (N/mmc)	230 (88.3)	121.4 (17.8)	69.3 (24.5)*	0.05
TH17 (% of lymphocytes)	9.8 (1.1)	14.2 (2.6)	16.2 (8.6)	n.s.
TH17 (N/mmc)	93.5 (50.2)	58.9 (12.3)	27.9 (7.3)	n.s.
TH1 activated (% of T helper)	1.1 (0.3)	2.5 (0.7)*	2.4 (0.5)	0.05
TH1 activated (N/mmc)	8.6 (2.6)	12.4 (3.9)	10.0 (4.4)	n.s.
TH17 activated (% of T helper)	0.1 (0.02)	0.17 (0.04)	0.26 (0.11)	n.s.
TH17 activated (N/mmc)	0.8 (0.3)	0.8 (0.2)	0.5 (0.1)	n.s.

* $p < 0.05$ versus WHO 3.

targeting IL-17A with a specific monoclonal antibody in order to limit its effect, particularly in COVID patients with high levels of serum IL-17A and with increased number of TH17 activated lymphocytes, as suggested also by a recent position paper [35].

The main limitation of the present study is the low number of enrolled patients that limits the statistical power of our analysis, suggesting to confirm such results on larger populations. However, IL serum levels and cytofluorimetric analysis could help to define and to monitor a personalized therapy (also by the use of monoclonal antibodies already available) by identifying the altered IL pathways and the TH populations in each COVID patient.

5. Conclusions

In most COVID patients there is a lymphocyte exhaustion that involves most subpopulations, and it is related to the severity of the disease. Activated TH1 and TH17 cells are not involved in lymphocyte exhaustion, but on the contrary they increase contributing to the

Table 3
Serum cytokines levels and lymphocyte subpopulations in 6 COVID patients before and 1 week after the treatment with Tocilizumab.

Patient (#)	Cytokines (pg/mL)				Lymphocytes subpopulations (N/mmc)						
	TNF-α	IL-6	IL-17A	IL-17RA	Total	B	T	NK	Naïve	Memory	Activated
1	3.0	254.7	3.4	143.3	1962.0	117.7	1471.5	313.9	1098.7	824.0	196.2
	3.0	173.6	2.6	143.0	1438.4	100.7	1064.4	172.6	661.7	776.7	158.2
2	19.0	4241.9	2.7	4.0	373.2	44.8	1064.4	93.3	220.2	145.5	59.7
	14.0	2315.4	2.0	4.0	1382.4	400.9	843.3	179.7	815.6	566.8	442.4
3	6.0	3275.1	2.0	4.0	841.7	75.8	631.3	75.8	420.9	412.4	134.7
	3.2	273.7	2.9	4.0	547.5	104.0	301.1	125.9	273.8	273.8	186.2
4	3.0	228.3	4.9	132.1	600.0	36.0	420.0	84.0	354.0	246.0	138.0
	3.0	2569.7	3.4	56.1	1470.0	73.5	1146	220.5	970.2	441.0	161.7
5	3.0	35.9	3.5	4.0	784.8	164.8	525.8	78.5	408.1	367.6	188.4
	3.0	106.0	6.0	4.0	390.0	97.5	253.5	35.1	179.4	210.6	117.0
6	3.0	426.7	2.0	4.0	387.6	50.4	220.9	112.4	251.9	135.7	85.3
	5.3	3717.9	2.2	6.7	925.8	157.4	574.0	157.4	666.6	268.5	194.4

Patient (#)	Helper	Suppressor	H/S	Activated	Regulatory	TH1	TH17	TH1 activated	TH17 activated
1	922.1	451.3	2.0	58.9	43.2	184.4	55.3	11.1	0.9
	690.4	330.8	2.1	42.6	34.5	234.7	48.3	8.3	0.7
2	194.1	29.9	6.5	9.0	4.9	50.5	21.3	6.4	0.8
	705.0	138.2	5.1	42.2	27.6	183.3	56.4	12.7	0.7
3	513.4	109.4	4.7	44.2	13.5	51.3	25.7	5.1	0.5
	202.6	93.1	2.2	27.1	5.5	54.6	22.3	11.7	0.4
4	246.0	114.0	2.2	25.2	9.6	44.3	66.4	2.7	0.7
	764.4	235.2	3.3	68.8	10.3	53.5	45.9	3.1	1.5
5	243.3	235.4	1.0	15.8	18.1	97.3	24.3	9.7	0.2
	144.3	105.3	1.4	12.7	10.9	53.4	11.5	3.5	0.6
6	158.9	31.0	5.1	6.6	5.8	16.2	12.7	0.8	0.3
	462.9	74.1	6.3	23.0	18.5	27.7	18.5	3.2	1.4

enhanced production of cytokines in serum of COVID patients. The treatment with Tocilizumab provided variable results in terms of cytokine reduction and lymphocyte restoration, while the relevant increase of IL-17A/IL-17RA levels and TH17 cells observed in a subset of COVID patients suggest that targeting IL-17A pathway with a specific monoclonal antibody could be contributory in a subset of COVID patients under the guidance of ILs levels and cytofluorimetric analysis.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

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Ethics approval and consent to participate

This prospective multicenter study was approved by the Ethical Committee of the University Federico II, Naples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2020.118355.

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