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T Cell Phenotyping in Individuals Hospitalized with COVID-19

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Coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has become pandemic. Cytokine release syndrome occurring in a minority of SARS-CoV-2 infections is associated with severe disease and high mortality. We profiled the composition, activation, and proliferation of T cells in 20 patients with severe or critical COVID-19 and 40 matched healthy controls by flow cytometry. Unsupervised hierarchical cluster analysis based on 18 T cell subsets resulted in separation of healthy controls and COVID-19 patients. Compared to healthy controls, patients suffering from severe and critical COVID-19 had increased frequencies of activated and proliferating $CD38^+Ki67^+$ $CD4^+$ and $CD8^+$ T cells, suggesting active antiviral T cell defense. Frequencies of $CD38^+Ki67^+$ Th1 and $CD4^+$ cells correlated negatively with plasma IL-6. Thus, our data suggest that patients suffering from COVID-19 have a distinct T cell composition that is potentially modulated by IL-6. ***The Journal of Immunology*****, 2021, 206: 1478–1482.**

Coronavirus disease 2019 (COVID-19) caused by infection with the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has become a pandemic within 3 months of its first description (1). A subset of these patients show signs of excessive release of proinflammatory cytokines, such as IL-6 and TNF-α, termed "cytokine release syndrome" (CRS) (2, 3). CRS is thought to be caused by betacoronavirus infection of macrophages, leading to their activation and secretion of proinflammatory cytokines (4). As an increase in IL-6 may predict respiratory failure and is associated with increased mortality (5–7), agents blocking IL-6 signaling, such as the anti–IL-6R Ab tocilizumab, have been administered early in the pandemic (8). Lymphopenia is another hallmark of severe COVID-19. It mainly results from a decrease in T lymphocytes (3, 9), and $CD8^+$ T cell lymphopenia was reported to predict COVID-19 severity (10). Recent studies characterizing the T cell composition in COVID-19 patients focused on developmental stages of T cells (3, 11, 12), T cell activation (3, 11–13), and exhaustion of T cells (11, 12, 14, 15). A large study analyzing the lymphocyte populations of 125 COVID-19 patients found an increase in activated and proliferating $CD8^+$ and $CD4^+$ T cells compared with healthy controls (HC) (12). The Th cell polarization of these activated and proliferating $CD4^+$ cells and the composition of T cells during CRS in COVID-19 are unknown.

Materials and Methods

Study design

Retrospective analysis was conducted of all patients hospitalized with confirmed COVID-19 at the Department of Internal Medicine, Medical University of Graz, from February to June 2020 for whom a highly sensitive analysis of T cells had been obtained. COVID-19 pneumonia and critical illness such as need for high-flow oxygen therapy, continuous positive airway pressure therapy, or mechanical ventilation were classified according to the criteria of the American Thoracic Society and Infectious Diseases Society of America (16). Demographic data, medical history, COVID-19–specific medication, and laboratory data were obtained through electronic medical records (Supplemental Tables I, II). Two HC were age- and sex-matched to each COVID-19 patient. Exclusion criteria for the HC group were neoplasia (present or past), infectious diseases, pregnancy, severe anemia (Hb <9 mg/dl), autoimmune diseases, acute or chronic diseases associated with organ damage, and increased C-reactive protein (CRP) levels.

Laboratory measurements

SARS-CoV-2 infection was diagnosed by RT-PCR using the cobas SARS-CoV-2 test (Roche Molecular Systems, Branchburg, NJ) for use on the cobas 6800/8800 system according to the manufacturer's instructions. Amplification was achieved by targeting ORF1a/b (target 1), a nonstructural region that is unique to SARS-CoV-2 and a conserved region in the E-gene (target 2) for pan-Sarbecovirus detection. Cycle threshold (Ct) values of target 1 were chosen for correlation with the frequency of T cell subsets. In addition, clinical laboratory measurements including lymphocytes and T cell counts, ferritin, CRP, and plasma IL-6 were performed at the Clinical Institute of Medical and Chemical Laboratory Diagnostics, Medical University of Graz.

Flow cytometry and T cell phenotyping

Flow cytometry analyses were based on published guidelines (17) from whole blood. In brief, cells were stained for 15 min at room temperature using fluorochrome-conjugated anti-human Abs against CD3, CD4, CD8, CD197/CCR7, CD127, CD28, CD25, and CD45RA or CD3, CD4, CD8,

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Abbreviations used in this article: COVID-19, coronavirus disease 2019; CRP, C-reactive protein; CRS, cytokine release syndrome; Ct, cycle threshold; HC, healthy control; SARS-CoV-2, severe acute respiratory syndrome coronavirus-2.

CD189/CXCR3, CD196/CCR6, CD194/CCR4, and CD38. Erythrocytes were lysed using BD Lysing solution (Becton Dickinson, Heidelberg, Germany). For intracellular staining of Ki67, cells were incubated for 30 min on ice with fixation/permeabilization solution (Thermo Fisher Scientific, Waltham, MA) and incubated with an anti-Ki67 Ab for 30 min at room temperature. Cells were analyzed using a BD Canto II cytometer (Becton Dickinson) and FlowJo software (TreeStar, San Diego, CA). The gating strategy is depicted in Supplemental Fig. 1.

Statistics

Kolmogorov-Smirnov tests were used to define the distribution of the values (parametric or nonparametric). Mann–Whitney *U* tests were used to compare data of two independent groups after correction for outliers using the robust regression and outlier removal method ($Q = 1$). Statistics comparing multiple groups were done using the Kruskal–Wallis one-way ANOVA in conjunction with Dunn posttests. Spearman correlations were performed to study the relationship between IL-6 and the antiviral subsets. Wilcoxon signed-rank tests were used to compare paired samples (prior/posttherapy). Statistical analysis, scatter blots, and correlation analysis were performed using SPSS software version 26 (IBM, New York, NY) and GraphPad Prism software version 8 (TreeStar). Cluster analysis was performed using R (https://www.R-project.org/). Unsupervised hierarchical clustering was calculated based on Ward minimum variance method (18). Transformed frequencies of T cell subsets and clustering results were summarized in a heatmap (19).

Study approval

This study was performed after approval of the local ethics review board (institutional review board registry number IRB00002556, approval number 32-434 ex 19/20). Data of HC derive from an earlier study establishing reference values for flow cytometry (approval number 26-599 ex 13/14). All experiments were conducted according to Austrian laws, the declaration of Helsinki, and the principles of good scientific practice.

Results

T cell composition in COVID-19

Various studies reported lymphopenia in SARS-CoV-2–infected patients, which correlated with disease severity and mortality (3, 9, 20–23). As lymphopenia is mostly a result of reduced T lymphocyte counts (3, 9, 23), we profiled T cell subsets of 20 hospitalized COVID-19 patients and compared them to 40 age- and sex-matched HC. In our cohort, 11 patients (55%) required mechanical ventilation due to COVID-19 pneumonia, and total mortality was 30%. Patient characteristics are listed in Supplemental Table I. In line with previous reports (3, 9, 10), we confirmed the finding of lymphopenia, including decreased B and T cell counts (Supplemental Fig. 2A–C). Because absolute cell counts of all subsets were diminished, we further investigated frequencies of the $CD4^+$ and $CD8^+$ subpopulations. Unsupervised hierarchical cluster analysis based on 18 T cell subsets revealed separation of HC and COVID-19 patients (Fig. 1, Supplemental Fig. 3). Interestingly, four of the six patients that succumbed to COVID-19 were enriched in a subcluster characterized by lower frequencies of naive $CD4^+$ T cells compared with other COVID patients. Based on the results of the cluster analysis, we further examined the differences of the $CD4^+$ T effector cells between healthy and SARS-CoV-2–infected individuals. Intriguingly, no significant differences were identified between severe and critical COVID-19 patients (Supplemental Fig. 2). Compared to HC, we identified significant changes, including elevated $CXCR3^-CCR6^+$ Th17 cells and higher $CD8^+$ T cell frequencies in SARS-CoV-2–infected patients. Furthermore, we found a trend of reduced $CXCR3^+CCR4^-CCR6^-$ Th1 cell frequencies in COVID-19 (Fig. 1B–E). Moreover, frequencies of $CD4^+$ central memory (decreased in COVID-19) and $CD8^+CD28^-$ (increased in COVID-19) differed significantly between healthy and SARS-CoV-2–infected subjects. Proliferating ($Ki67^+$) and activated ($CD38^+$) T cells were increased in COVID-19.

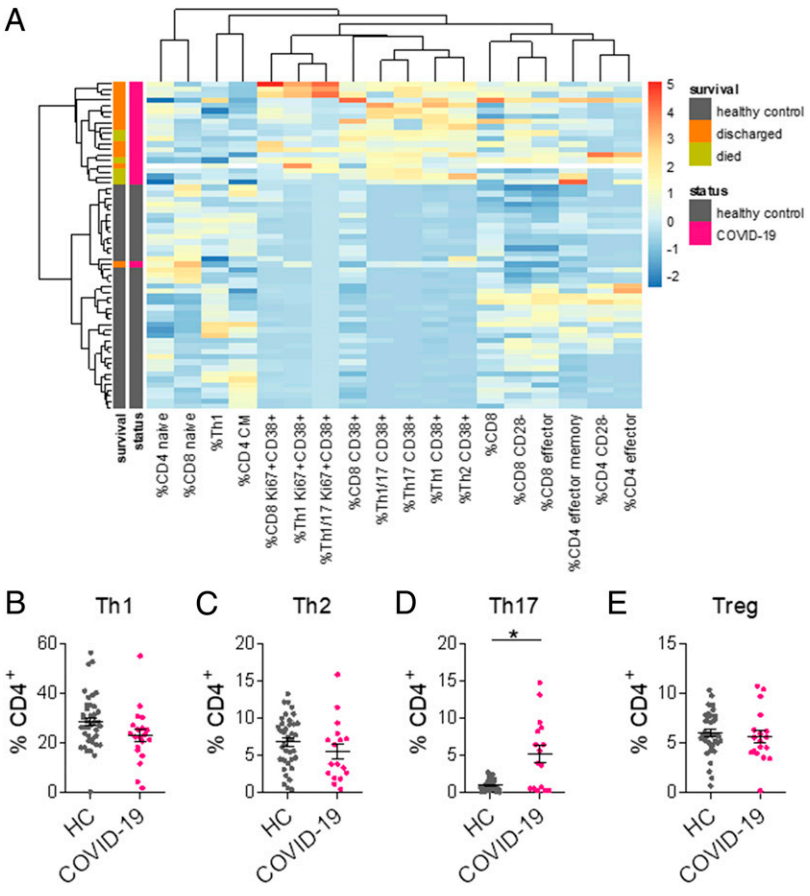

FIGURE 1. T cell composition segregate between healthy and COVID-19. (**A**) Unsupervised hierarchical clustering based on transformed T cell subset frequencies present in HC and COVID-19 patients. Individuals are color coded based on disease and survival. T cell subset frequencies are shown in a heatmap. (**B**–**E**) Effector Th subsets Th1, Th2, and Th17 and regulatory T cells (Tregs) shown as percentage of $CD4^+$. Kruskal–Wallis ANOVA (Dunn posttest) was performed for multiple comparisons (B–E). Data are presented as mean ± SEM. $*p \leq 0.05$.

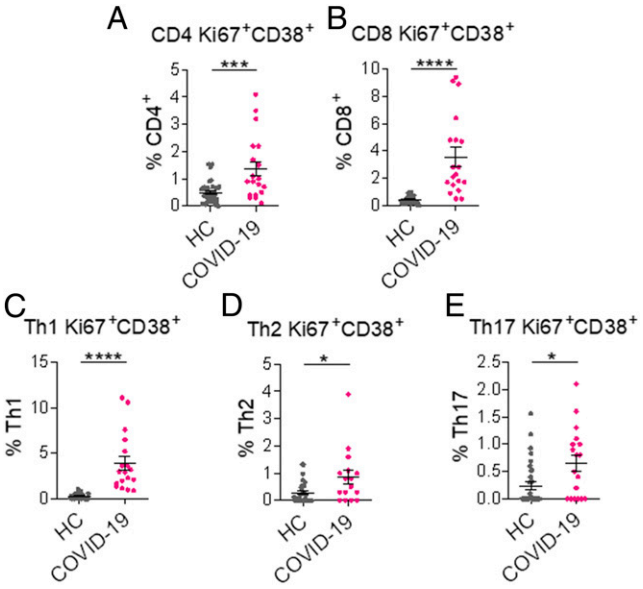

FIGURE 2. Altered T cell function within COVID-19. (**A** and **B**) Activated and proliferative $CD4^+$ and $CD8^+$ ($CD4^+Ki67^+CD38^+$, $CD8^+Ki67^+CD38^+$) displayed as percentage of $CD4^+$ and $CD8^+$, respectively. (**C**–**E**) $Ki67^+CD38^+$ of Th subsets shown as percentage of Th1, Th2, and Th17. Forty HC and 20 severe to critical cases were included. Kruskal–Wallis ANOVA (Dunn posttest) was performed for multiple comparisons. Data are presented as mean ± SEM. $^{****}p \leq 0.0001$, $^{***}p \leq 0.001$, $^{*}p \leq 0.05$.

T cell proliferation and activation in COVID-19

Activated and proliferating $CD8^+$ and Th1 cells expressing CD38 and Ki67 are considered the antiviral response of virus-specific and activated bystander T cells (12, 24, 25). Cells coexpressing both markers were significantly enriched in the $CD4^+$, $CD8^+$, Th1, Th2, and Th17 cell compartments of SARS-CoV-2–infected patients compared with HC. The largest fold-change was found in $CD8^+$ and Th1 cells, which are implicated in the antiviral defense (Fig. 2).

Correlations of T cell populations with parameters of inflammation and disease severity

We correlated clinically relevant parameters of COVID-19, such as oxygen supplementation, duration of symptoms, viral load, CRP, ferritin, and IL-6, with frequencies of T cell populations. There was no difference in T cell populations between patients receiving 2–10 l of oxygen supplementation per minute compared with those receiving high-flow oxygen or mechanical ventilation. The Ct of the SARS-CoV-2 PCR can be used as a proxy for viral load (26). In our cohort, the Ct correlated inversely with the frequency of $CD8^+$ central memory T cells, implying a positive correlation with viral load (Fig. 3A). The inflammatory markers CRP, ferritin, and IL-6 have been used to describe the inflammatory burden of COVID-19. We did not find significant correlations of T cell populations with serum ferritin or CRP. In contrast, plasma IL-6 correlated inversely with the frequency of T cell counts, $Ki67^+CD38^+CD4^+$ cells, and $Ki67^+CD38^+$Th1 cells (Fig. 3B–D). Therefore, we hypothesized that IL-6 may directly impair T cell proliferation. To test this hypothesis in vitro, we performed cell culture experiments investigating whether increasing IL-6 concentrations could inhibit anti-CD3/CD28–induced T cell proliferation. However, IL6 treatment did not alter proliferation, activation, apoptosis, or expression of exhaustion markers PD-1 and CTLA-4 in Th1 or $CD8^+$ cells in vitro (data not shown).

Clinical and immunologic effects of IL-6 blockade

In vitro blockade of IL-6 during chronic virus infection has been reported to result in enhanced virus-specific $CD8^+$ T cell response, increased IFN-γ production, and decreased virus loads (27). Therefore, we assessed the cellular and clinical effects of IL-6 blockade by the anti–IL-6R Ab tocilizumab in SARS-CoV-2–infected patients. As expected, CRP levels of all four patients declined (Fig. 4A) after tocilizumab treatment. Lymphocyte counts increased in three of four patients 5 d post therapy, except in one patient who died (Fig. 4B). Interestingly, the frequency of Th1 cells and their activated and proliferative forms increased in

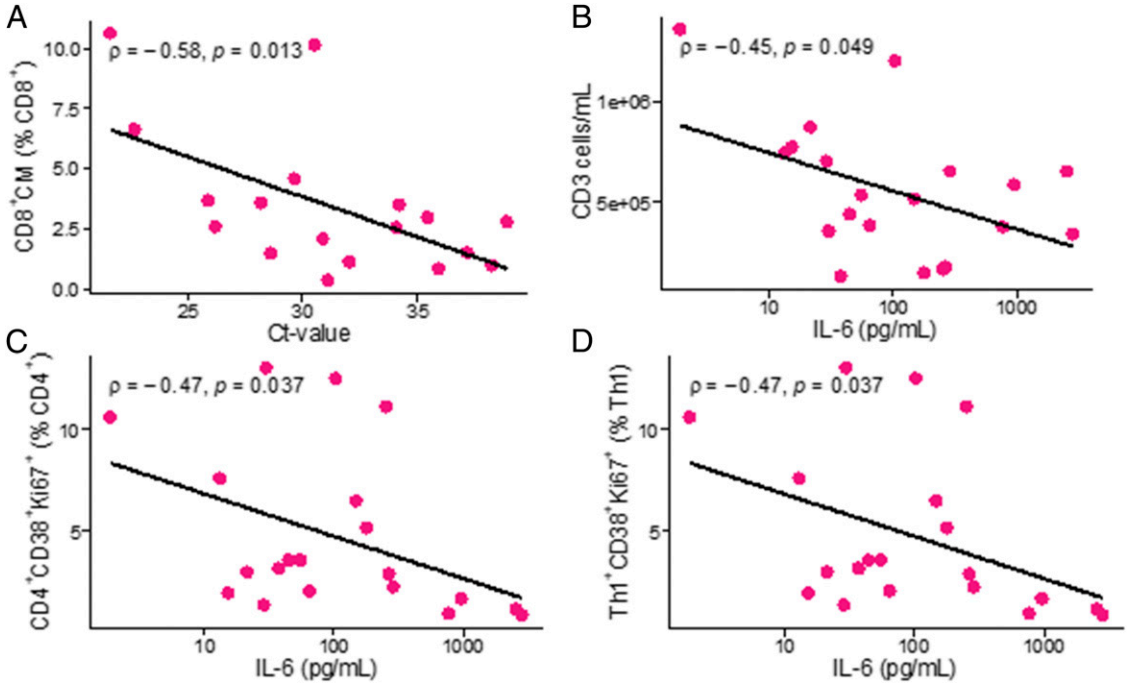

FIGURE 3. Elevated IL-6 levels negatively predict antiviral defense. Correlations of (**A**) the Ct value and $CD8^+$ central memory T cells. (**B**–**D**) Plasma IL-6 and (B) $CD3^+$ T cell counts, (C) $Ki67^+CD38^+$ $CD4^+$, and $Ki67^+CD38^+$ Th1 T lymphocytes. Correlation analysis was performed using Spearman correlation coefficient (ρ). The ρ and p values are presented in the figure.

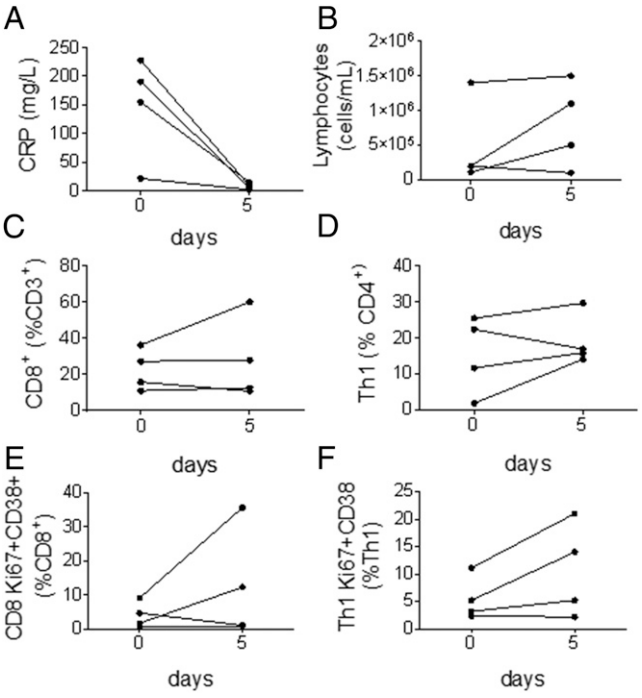

FIGURE 4. Clinical and immunologic effects of IL-6 blockade. Measurements of (**A**) CRP levels in milligrams per liter, (**B**) lymphocytes counts, and frequencies of antiviral T cell subsets (**C**) $CD8^+$, (**D**) Th1, (**E**) $CD8^+Ki67^+CD38^+$, and (**F**) $Th1Ki67^+CD38^+$ prior and after anti–IL-6 therapy. $n = 4$. Wilcoxon signed-rank test was performed to determine differences between the two different time points.

three out of four patients after administration of tocilizumab, whereas the effects were less pronounced for $CD8^+$ T cells (Fig. 4C–F).

Discussion

In this study, we profiled the T cell composition, activation, and proliferation in patients with severe to critical COVID-19 and matched HC by flow cytometry.

Based on the T cell profile, healthy and COVID-19 patients could be assigned to two distinct clusters. Interestingly, four of the six patients that succumbed to COVID-19 were enriched in a subcluster characterized by low frequencies of naive $CD4^+$ T cells. Reduction of naive $CD4^+$ T cells was reported to be associated with poor response to vaccination (28). Similarly, viral infection in individuals with a limited number of naive $CD4^+$ T cells (and thus a limited TCR repertoire) may result in an insufficient T cell response and potentially a lethal course of the disease. Moreover, we identified significant changes in Th17 ($CXCR3^-CCR6^+$) and $CD8^+$ T cells. In line with our findings, De Biasi et al. (11) reported a shift toward Th17 cells and no changes in the expression of the master regulator of Th2 cells GATA3 in $CD4^+$ T cells in severe COVID-19 infection. In addition, they found a small but significant increase in regulatory T cells. Using the same gating strategy, however, we could not confirm this observation in our cohort.

Beside the changes in the effector memory subsets, we further identified decreased expression of $CD4^+$ central memory and increased $CD8^+CD28^-$ T cells. Loss of CD28 expression on cytotoxic T cells is a well-known immune response during viral infections (29–31). In HIV-infected individuals, disease progression is associated with $CD8^+CD28^-$ cell expansion (32). In addition to Th cell frequencies, proliferating $Ki67^+$ and activated $CD38^+$ T cells were increased in COVID-19. CD38 expression is associated with T cell activation (33) and decreased viral loads and protects from cell death in response to HIV infection (34). The nuclear protein Ki67 plays a role in the regulation of cell division and is expressed in all active phases of cell division but is absent in resting cells in G_0 (35). Thus, intracellular Ki67 staining can be used as a marker of T cell proliferation. Our data are in agreement with the findings recently described by Mathew et al. (12). Their work reported increased expression of CD38 and Ki67 in $CD4^+$ T cells but did not identify the $CD4^+$ subset(s) involved in this increase (12). We identified enrichment of $Ki67^+$ $CD38^+$ cells within the Th1, Th2, Th17, and $CD4^+$ and $CD8^+$ T cell population. Intriguingly, the effects were most pronounced in $CD8^+$ and Th1 cells; the T cell subsets implicated in the antiviral defense. In line with our findings, another study had previously shown increased activation of $CD8^+$ T cells in severe COVID-19 compared with patients with mild symptoms (13). Together these data suggest that SARS-CoV-2 triggers a typical antiviral immune program in infected patients.

CRS is associated with a massive release of proinflammatory cytokines, such as IL-6 (2, 3, 36). Briefly, IL-6 may induce the differentiation into Th17 cells and inhibit the induction of regulatory T cells (37) and can shift the Th1/Th2 balance toward Th2 (38). In line with previous reports (3), increased plasma IL-6 concentrations were associated with more-severe COVID-19 courses. In our patient cohort suffering from COVID-19, we found a negative correlation of activated and proliferating Th1 T cells with plasma IL-6. This might be the result of the cellular exhaustion reported in COVID-19 (11, 39). T cell exhaustion was previously suggested to predict a severe course of disease in COVID-19 (11, 13). Alternatively, cytokines produced during CRS could directly impact T cell proliferation or viability. However, IL-6 did not inhibit T cell proliferation in vitro. Therefore, IL-6 may not be directly responsible for reduced T cell activation and proliferation in CRS, thus suggesting a potential of immune regulation via other cytokines. Of note, Harker et al. (40) suggest that IL-6 positively influences T cell function, in the context of murine chronic viral infection, leading to decreased viral loads.

IL-6 blockade using tocilizumab has been widely used early in the pandemic to quench inflammation in COVID-19 CRS. However, recent studies did not find a better outcome for tocilizumab-treated patients when compared with placebo (41). In line with our observations, reduction of CRP and increase of lymphocyte counts in association with IL-6 blockade have been reported recently (28, 29). Although IL-6 did not directly decrease T cell viability or proliferation, blockade of IL-6 signaling by tocilizumab may indirectly affect other cytokines and chemokines implicated in lymphopenia. We are well aware of the preliminary nature of these findings limited by a small sample size. Thus, a larger sample size and longer time courses are necessary to evaluate significant changes within these cell subsets.

In conclusion, the peripheral T cell compartment in COVID-19 is characterized by a relative increase in activated and proliferating $CD4^+$ and $CD8^+$ T cells, a feature that is potentially affected by plasma IL-6 levels and IL-6 blockade therapy.

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Disclosures

The authors have no financial conflicts of interest.

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