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Prognostic immune markers identifying patients with severe COVID-19 who respond to tocilizumab

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Introduction: A growing number of evidences suggest that the combination of hyperinflammation, dysregulated T and B cell response and cytokine storm play a major role in the immunopathogenesis of severe COVID-19. IL-6 is one of the main pro-inflammatory cytokines and its levels are increased during SARS-CoV-2 infection. Several observational and randomized studies demonstrated that tocilizumab, an IL-6R blocker, improves survival in critically ill patients both in infectious disease and intensive care units. However, despite transforming the treatment options for COVID-19, IL-6R inhibition is still ineffective in a fraction of patients.

Methods: In the present study, we investigated the impact of two doses of tocilizumab in patients with severe COVID-19 who responded or not to the treatment by analyzing a panel of cytokines, chemokines and other soluble factors, along with the composition of peripheral immune cells, paying a particular attention to T and B lymphocytes.

Results: We observed that, in comparison with non-responders, those who responded to tocilizumab had different levels of several cytokines and different T and B cells proportions before starting therapy. Moreover, in these patients, tocilizumab was further able to modify the landscape of the aforementioned soluble molecules and cellular markers.

Conclusions: We found that tocilizumab has pleiotropic effects and that clinical response to this drug remain heterogenous. Our data suggest that it is possible to identify patients who will respond to treatment and that the administration of tocilizumab is able to restore the immune balance through the re-establishment of different cell populations affected by SARS-COV-2 infection, highlighting the importance of temporal examination of the pathological features from the diagnosis.

KEYWORDS

SARS-CoV-2, COVID-19, tocilizumab, cytokines, B cells, T cells

Introduction

Most SARS-CoV-2 infections are asymptomatic or paucisymptomatic. However, a proportion of individuals develop a severe disease, characterized by a progressive respiratory failure after the onset of dyspnea and hypoxemia (1). It is well known that the combination of hyperinflammation, dysregulation in T and B cell response and cytokine storm are responsible for the development of severe and eventually fatal COVID-19 (2–4). Plasma levels of several inflammatory cytokines, such as interleukin (IL)-6, tumor necrosis factor (TNF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and granulocyte colony-stimulating factor (G-CSF) are indeed increased after SARS-CoV-2 infection, in patients with the severe disease (3–6).

IL-6 is one of the main pro-inflammatory cytokines. It regulates several aspects of innate and adaptive immunity, including the differentiation of B lymphocytes, cytotoxic T cells, and macrophage/monocyte functions (7, 8). IL-6 exists both as a soluble and membrane-bound molecule, and is typically found in the blood of healthy individuals at very low concentrations (1–5 pg/mL) (9). Its levels increase during acute and/or chronic inflammation (10, 11). IL-6 binds to IL-6 receptor (IL-6R, or CD126) and glycoprotein 130 (gp130) to form a hexameric complex that transduces IL-6 signal through the Janus kinase (JAK) and signal transducer and activator of transcription (STAT) pathway (10). In the context of disease, IL-6 can have both local and systemic deleterious inflammatory effects. Indeed, the association between high IL-6 concentration (> 80 pg/mL) and respiratory failure and/or death has been confirmed in several studies and posed the rationale for the use of IL-6 blockers in the management of patients with severe COVID-19 (12, 13).

Tocilizumab is a humanized antibody that targets IL-6R and inhibits the binding of IL-6 to both soluble and membrane forms of the receptor. It interferes with IL-6 signaling and dampen inflammation. The single-cell RNA-seq analysis of peripheral blood mononuclear cells (PBMCs) isolated from two severe COVID-19 patients treated with tocilizumab, at the severe and remission disease stages, revealed that a monocyte-associated cytokine storm is present in patients at the severe stage and that tocilizumab can weaken this inflammatory response (14). Both

case-control studies and randomized trials of tocilizumab in COVID-19 have demonstrated that it improves survival and reduces the chances of progressing to invasive mechanical ventilation in hospitalized COVID-19 patients with hypoxia and systemic inflammation (15, 16). Data from the REMAP-CAP and RECOVERY trials revealed that combining corticosteroids with tocilizumab yields cumulative benefits for mortality and morbidity in severe COVID-19 (15, 17). However, as already observed for other immunotherapeutic drugs in other diseases, including chronic inflammatory diseases and cancer, a proportion of patients did not derive benefits from tocilizumab treatment (18, 19).

In the present study, we aimed at identifying possible prognostic biomarkers related to the response to this drug. For this reason, we profiled the plasma levels of several cytokines, chemokines and other soluble factors in patients with severe COVID-19 who responded or not to tocilizumab, and we examined how tocilizumab impacted the composition of peripheral immune cells.

Results

Modulation of plasma cytokines and chemokines during tocilizumab treatment

The plasma profiling of 62 cytokines, chemokines and other soluble factors involved in several immune responses was first performed in 23 patients with severe COVID-19, responding or not to tocilizumab. The drug was given twice, 12 hours apart, by intravenous or subcutaneous route (see Methods for details). Plasma molecules were quantified at the baseline (T0, before the beginning of treatment), two days (T2) and seven days (T7) after the first dose of the drug. Most patients were sampled during the acute phase of the infection: 13 patients responded to therapy, whereas 10 patients did not. Demographic information, clinical data, blood parameters and arterial haemogas analysis, collected at T0, are reported in [Supplementary Table 1](#).

[Figure 1](#) shows plasma levels of several cytokines/chemokines and other soluble factors in responder and non-responder patients. Concerning pro-inflammatory cytokines, we found that after 2 days

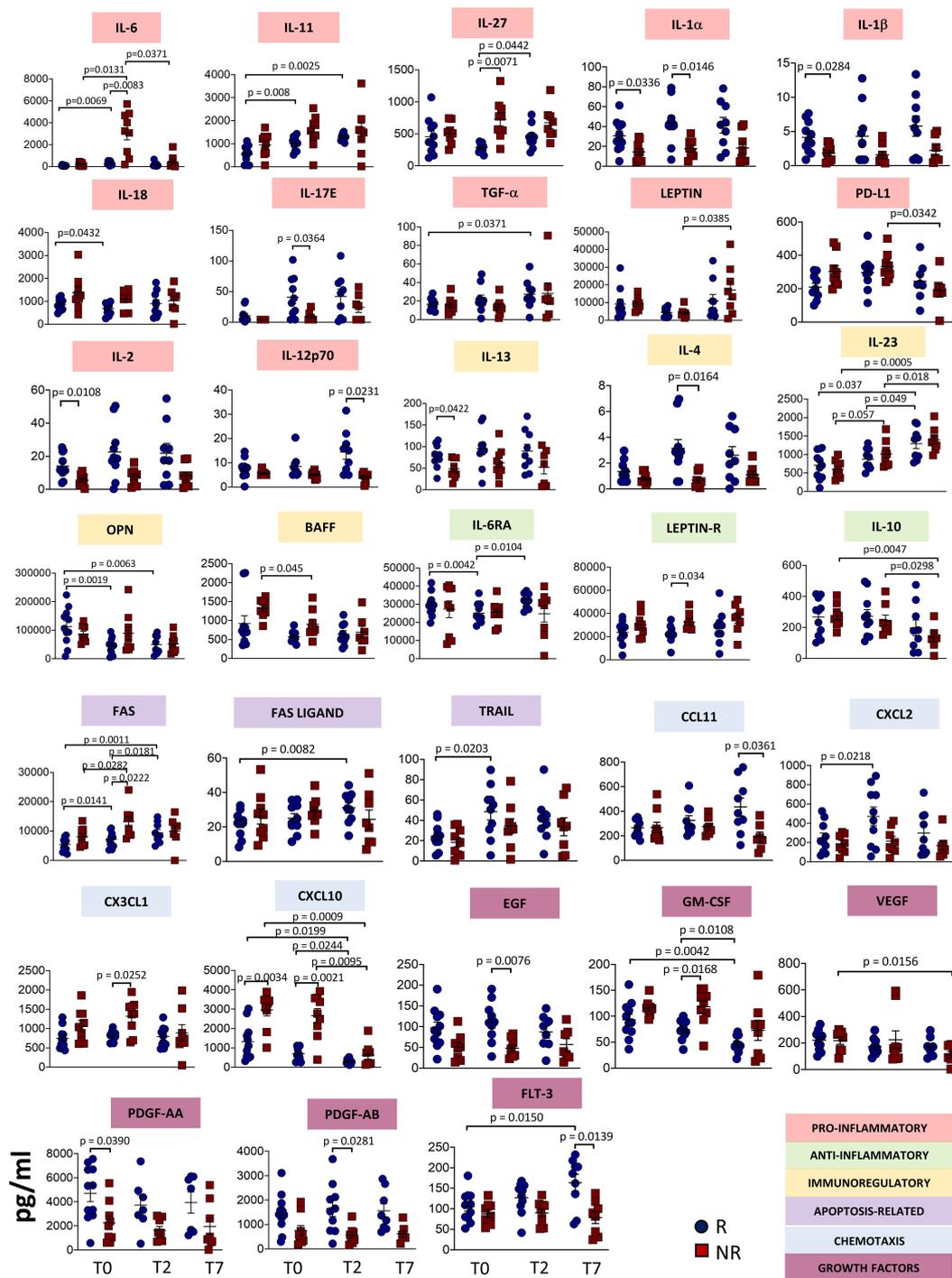


FIGURE 1
Plasmatic level of different soluble molecules in responder and non-responder patients. Quantification of cytokines and other mediators in plasma obtained from COVID-19 patients responding (R) or not (NR) to tocilizumab (n = 23). Data represent individual values, mean (centre bar) ± SEM (upper and lower bars). Statistical analysis by two-way ANOVA followed by Bonferroni correction; if not indicated, p-value is not significant.

of therapy IL-6 and IL-27 plasma concentrations strongly increased in non-responders. IL-11 levels did not differ between responders and non-responders, but increased in responders after therapy. Non-responders had lower basal levels of interleukin IL-1α, IL-1β, while in responders at T2 IL-18 decreased and IL-17E increased. Level of TGF-α increased over time in responders. Leptin level

increased from T2 to T7 in non-responders, while PD-L1 decreased. IL-2 level was lower in non-responders at T0 and significant differences were observed at T7 between responders and non-responders for IL-12p70.

The concentrations of IL-6RA reached the minimum value at T2 in responders. At T2, Leptin receptor (Leptin R) was different

between responders and non-responders, with higher level in the latter. IL-10 decreased during therapy in non-responders. IL-2 and IL-13 plasma levels were lower in non-responders at T0, while IL-4 level was lower at T2. Significant differences were observed at T7 between responders and non-responders for IL-12p70. We also noted that IL-23 increased after one week of treatment in both groups of patients. Levels of optineurin (OPN) decreased during therapy in patients responding to tocilizumab. The level of B-cell activating factor (BAFF) decreased in non-responders during therapy.

Apoptosis-related molecules have been investigated. FAS levels increased during therapy in responder and non-responder patients, but higher levels have been detected in non-responders at T2. FAS ligand and TRAIL levels increased in responders, at T7 and T2, respectively.

Plasma concentrations of C-C Motif Chemokine Ligand (CCL)-11 were lower in non-responders at T7, while C-X-C motif chemokine ligand (CXCL)-2 was higher at T2 in responders. CX3CL1 was higher at T2 in non-responders. CXCL10 plasma levels decreased over time in both groups of patients, but non-responders displayed higher levels at T0. This difference was maintained at T2 and at T7.

Regarding growth factors, in responders epidermal growth factor (EGF) was higher at T2 whereas GM-CSF decreased with time. Vascular endothelial growth factor (VEGF) decreased in non-responders over time. Levels of platelet-derived growth factor (PDGF)-AA were lower at the baseline in non-responders. (PDGF)-AB was lower in non-responders at T2. Fms related receptor tyrosine kinase 3 (FLT-3 ligand, also known as CD135) decreased after seven days in non-responders, but increased in responders. All the other soluble molecules remained unchanged in responders and non-responders (Supplementary Figure 1).

Circulating plasmablasts decreased in responders after therapy

To investigate how immune responses vary in the presence of tocilizumab, we profiled B cells and T cells by using polychromatic flow cytometry. After unsupervised analysis, nine distinct cell clusters have been identified (UMAP and heatmap are reported in Figures 2A, B) on the basis of the surface protein expression (Supplementary Figure 2). Besides a population of cells negative for all the activation/differentiation markers analyzed, naïve B cells are defined as $IgM^+IgD^+CD24^+CD21^+CD38^-CD27^-$ while transitional B cells are $IgM^+IgD^+CD24^+CD21^+CD38^+CD27^-$ memory unswitched B cells are defined as $IgM^+IgD^+CD24^+CD21^+CD38^-CD27^+$ while memory switched B cells are $IgM^-IgD^-CD24^+CD21^+CD38^-CD27^+$. Memory IgM only B cells are $IgM^+IgD^-CD24^+CD21^+CD38^-CD27^+$. Plasmablasts are $IgM^{-/+}IgD^-CD24^-CD21^-CD38^+CD27^+$. Finally, exhausted B cells are those $CD21^-$ and $CD24^-$ (2).

Before therapy (T0), responders showed a higher percentage and absolute number of total B cells and a higher absolute number of memory IgM-only subpopulation compared to non-responders. After two days of therapy, plasmablasts not expressing IgM increases in non-responders compared to responder patients.

Responders show an increase in both absolute number and percentage of memory switch B cell population compared to non-responders. After seven days of therapy, we observed an increase in absolute number of total B cells and naïve B cell subpopulation in both responder and non-responder patients, an increase in percentage of plasmablast IgM^+ in responder patients, a decrease in percentage of plasmablast IgM^- and an increase in absolute number of exhausted B cell and surface- subpopulation in both groups. Finally, non-responder patients display a decrease of absolute number of transitional B cells (Figure 2C).

Tocilizumab induces a redistribution in memory T cell pool

T cell compartment of patient with COVID-19 pneumonia displays marked T cell activation, senescence, exhaustion and skewing towards Th17 (3). Given that also T cells expressed CD126 and could be tuned by Tocilizumab administration, we investigated the T cells landscape of patients undergoing anti-IL6R-therapy.

Eighteen clusters of $CD4^+$ T cells were recognized after unsupervised analysis on the basis of different expression of differentiation ($CD45RA$, $CCR7$, $CD25$, $CD95$, $CD27$, $CD28$, $CD57$) and activation markers ($HLA-DR$, $CD28$, $PD1$) (Figures 3A, B; Supplementary Figure 3, 4). Naïve T cells (N) were defined as expressing $CD45RA$, $CCR7$, $CD27$, $CD28$, $CD127$, $CD25$ and lack of expression of $CD95$, $CD38$ and $HLA-DR$. Moreover, a population of recently activated naïve T cells has been recognized as those expressing $CD38$. $CD38$ is a multifunctional molecule, belonging to the family of ectoenzymes and it induces intracellular calcium release. The expression of $CD38$ identifies a hypo-proliferative $CD4^+$ T-cell subset that, following TCR stimulation, retains expression of naïve cell surface markers including $CD45RA$ and $CCR7$ (20). We were able also to identify T cells stem memory (T_{SCM}) as naïve cells expressing $CD95$ and $CD38$. Central memory T cells (CM) were those expressing $CCR7$, $CD95$ and not $CD45RA$, then four subsets of central memory were found: one expressing $CD38$, one expressing $PD-1$, one co-expressing $CD38$ and $PD-1$ and one activated ($CD38^+HLA-DR^+$) expressing also $PD-1$. Three populations of transitional memory (TM) were found as they do not express $CD45RA$ and $CCR7$, but they express $CD28$. Moreover, one population of TM is phenotypically resting, one population is expressing $CD38$ and $PD1$ and another subset is highly activated as it expresses high level of $HLA-DR$, $CD38$ and $PD-1$. In particular, $PD-1$ is expressed during the early phase of T cell activation when naïve T cells differentiate into effector cells: it is rapidly expressed after antigen stimulation of naïve T cells with a kinetics of expression similar to the expression of early activation markers ($CD69$ and $CD25$) (21, 22). Effector memory T cells (EM) are characterized by the lack of the expression of $CD28$, $CD45RA$ and $CCR7$. Two different populations of EM have been identified: one has been identified as a population of phenotypically resting cells and the other as exhausted cells as it expresses $CD57$ and $PD-1$. A population of terminally differentiated T cells (TE) were found as effector memory

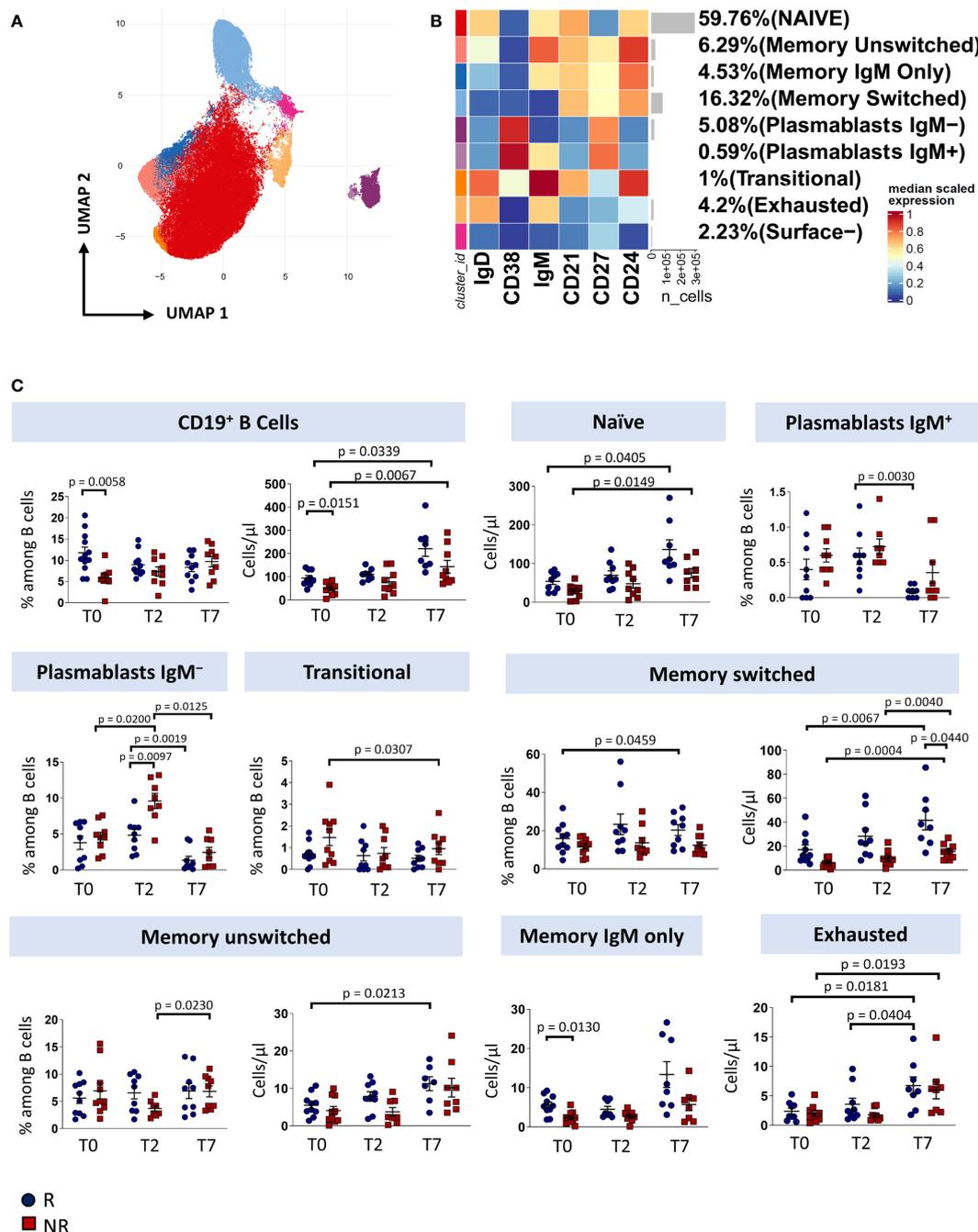


FIGURE 2

B cells landscape of COVID-19 patients treated with tocilizumab. (A) Uniform Manifold Approximation and Projection (UMAP) representation of the B-cell landscape. Each color is assigned according to the cluster identification palette. (B) Heatmap representing different B-cell clusters identified by FlowSOM, with relative identity and percentages. The colors in the heatmap represent the median of the arcsinh, 0-1 transformed marker expression calculated over cells from all the samples, varying from blue for lower expression to red for higher expression. Each cluster has a unique color assigned (bar on the left). (C) Statistical analysis of the different cell populations identified as in (B) Comparison between responders (R, blue circles) and non-responders (NR, red squares) at different treatment timepoints. The dot plots show the relative abundancies (left plot) and the absolute numbers (right plot) of populations found within B cells. Data represent individual percentage values (dots), median (center bar) and SEM (upper and lower bars). Statistical analysis by two-way ANOVA test with Bonferroni correction has been used. Exact p-value is indicated in the figure.

re-expressing CD45RA. Finally, putative T regulatory cells (T_{reg}) were defined as those expressing CD25 and not expressing CD127. Four populations were found: naïve T_{reg} expressing CD45RA and CCR7, central memory T_{reg} as those not expressing CD45RA and expressing CCR7, central memory T_{reg} expressing also CD38 and

effector memory T_{reg} as those characterized by the lack of expression of both previous markers.

The percentage of CD4⁺ T cells was influenced by therapy, as in responders it increased at T2 and decreased at T7. The absolute number increased at T2 and T7 in responders, and only from T0

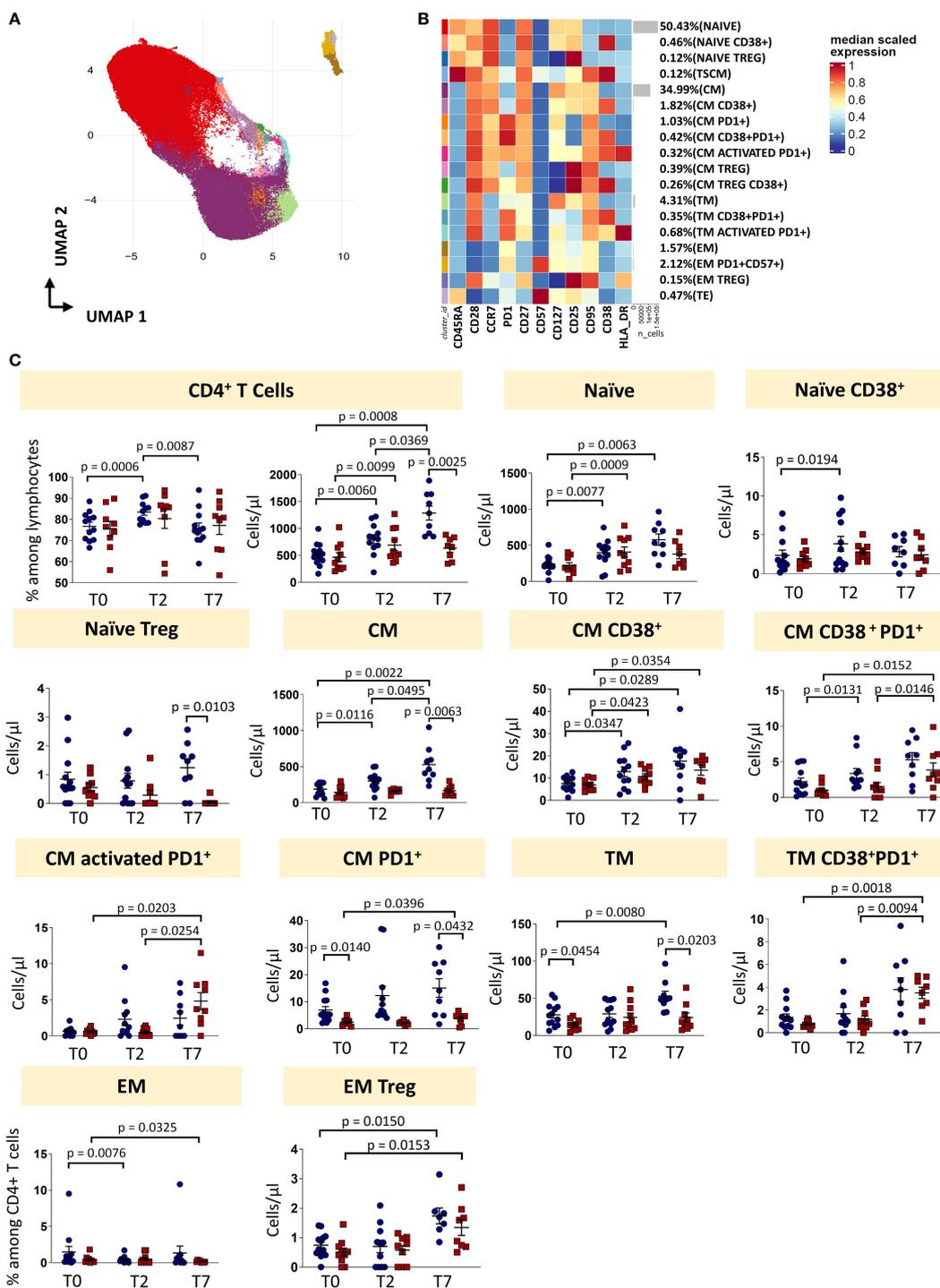


FIGURE 3

CD4 T cells landscape of COVID-19 patients treated with tocilizumab. (A) Uniform Manifold Approximation and Projection (UMAP) representation of the CD4 T cell landscape. Each color is assigned according to the cluster identification palette. (B) Heatmap representing different cell clusters identified by FlowSOM, with relative identity and percentages. The colors in the heatmap represent the median of the arcsinh, 0-1 transformed marker expression calculated over cells from all the samples, varying from blue for lower expression to red for higher expression. Each cluster has a unique color assigned (bar on the left). (C) Statistical analysis of the different CD4 T cells populations identified as in (B) Comparison between responders (R, blue circles) and non-responders (NR, red squares) at different treatment timepoints. The dot plots show the relative abundances and/or absolute numbers of subpopulations found within CD4 T cells. Data represent individual percentage values (dots), median (center bar) and SEM (upper and lower bars). Statistical analysis by two-way ANOVA test with Bonferroni correction has been used. Exact p-value is indicated in the figure. T_{SCM}, T cells stem memory; CM, central memory; TM, transitional memory; EM, effector memory; TREG, T regulatory cells; TE, terminally differentiated T cells re-expressing CD45RA.

and T2 in non-responders. At T7, the absolute number of CD4⁺ T cells was lower in non-responders if compared to responders. The absolute number of naïve T cells increased in responders (until T7) and non-responders (until T2). Naïve CD38⁺ T cells increased in responders from T0 to T2, while the absolute number of naïve Treg at T7 was higher in responders vs non-responders. Absolute number of CM T cells increased after treatment in responders, but not in non-responders. The same trend was observed for the absolute number of CM CD38⁺ T cells and CM CD38⁺ T cells expressing PD-1 for responders and non-responders. The absolute number of activated CM expressing PD-1 increased in non-responders, but did not change in responders. Absolute numbers of CM T cells expressing PD-1 were higher in responders at each time points. Absolute number of TM cells was higher at T0 and T7 in responders and increased during therapy for responder patients. The absolute number of TM expressing CD38 and PD-1 increased in non-responders after therapy, but did not change in responders. The percentage of EM T cells decreased in non-responders from T0 to T7 and in responders from T0 to T2. The absolute number of EM Treg increased in both responders and non-responders after therapy (from T0 to T7).

To sum up, even if the percentage of total CD4⁺ T is modulated by the therapy, the proportion of different CD4⁺ T cell subpopulations remained unchanged. However, the increased of the absolute number of CD4⁺ T cells in responders was likely responsible for all the differences found in the main subpopulations (Figure 3C, exact p-values are reported in the figures).

Regarding CD8⁺ T cell subsets, twenty-two clusters of CD8 T cells have been identified by unsupervised analysis on the basis of different expression of differentiation (CD45RA, CCR7, CD25, CD95, CD27, CD28, CD57) and activation markers (HLA-DR, CD28, PD-1) (Figures 4A, B; Supplementary Figures 5, 6). These populations span the entire spectrum of differentiation and activation status. As for CD8⁺ T cell subsets, naïve cells, naïve cells expressing CD38, T_{SCM}, T_{SCM} expressing CD38, CM, CM co-expressing CD38 and PD-1, TM, EM and TE were identified. Of the TM population, we found four different subsets: a subset of phenotypically resting cells, a subset of TM expressing HLA-DR, a subset of TM expressing CD38 and PD-1, a subset of activated TM also expressing PD1 and a subset of exhausted TM (CD57⁺PD1⁺). Moreover, we found four populations of EM: EM phenotypically resting, EM expressing only CD57, EM expressing CD38 and CD57, EM co-expressing CD57 and HLA-DR. Finally, seven populations of TE cells have been identified: TE phenotypically resting, TE expressing HLA-DR, TE expressing CD38⁺, TE expressing CD57, TE activated (CD38⁺HLA-DR⁺), TE co-expressing CD57 and HLA-DR, and TE co-expressing CD38 and CD57.

The percentage of CD8⁺ T cells decreased from T0 at T2 in both groups of patients, even if in responders the percentage increased at T7, reaching a similar level of T0. As far as the absolute number is concerned, responders were characterized by an increase of CD8⁺ T cells at T7. Similar absolute numbers of CD8⁺ T cells were observed in non-responders. At T7, responders were characterized by increased absolute number of naïve, naïve CD38⁺, CM, TM and EM T cells. The absolute number of T pedix SCM CD38⁺ was different at T0 and T2 between responders and non-responders,

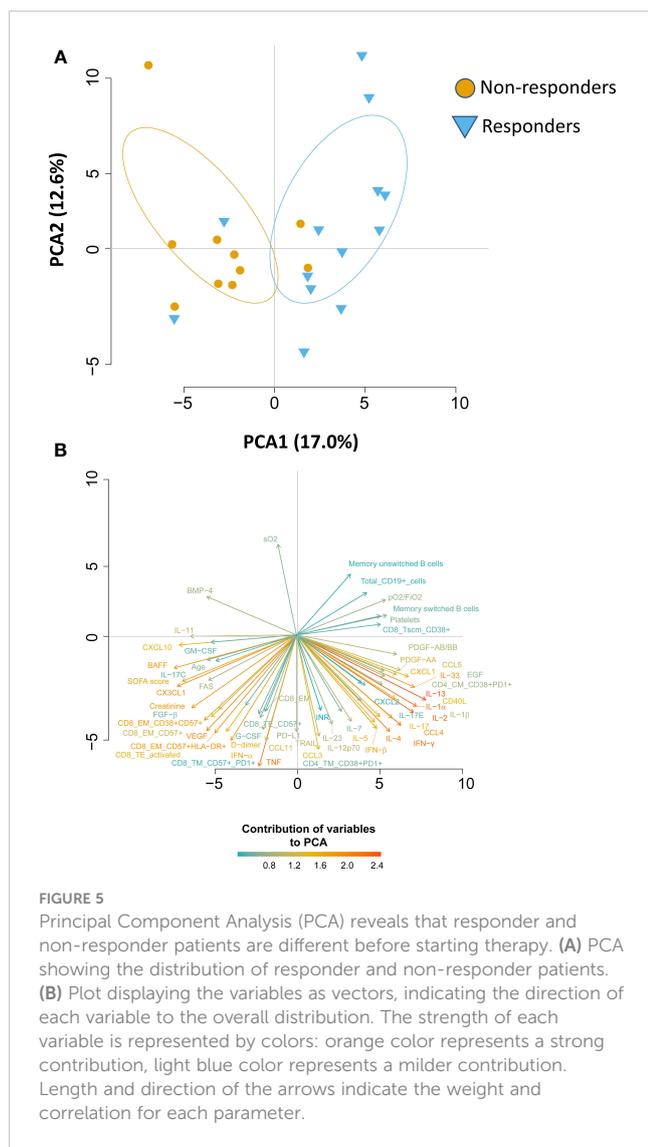
with higher level in responders. Non-responders had higher absolute number of CM CD38⁺PD-1⁺ and TM CD38⁺PD-1⁺, at T7. Moreover, at T7, responders showed higher levels of TM and EM if compared to non-responders. The percentages of TE decreased at T7 in non-responders and at T2 in responders. The percentage of TE CD38⁺CD57⁺ decreased at T2 in non-responders (Figure 4C, exact p values are reported in the figures).

We wondered whether the two groups of patients could be identified based on immunological and clinical parameters before therapy initiation (T0), predicting who will benefit from therapy and who will not. By using the principal component analysis (PCA), we saw that non-responders clusterize on the left side of the first dimension (PC1, representing 17% of variance), while responder patients clusterize on the right side (Figure 5A), meaning that before starting therapy they were extremely different from both immunological and clinical point of view. Besides the level of pO2/FiO2, the immunological parameters that were the main responsible for the clusterization in responders were plasma levels of IL-2, IL-13, IL-1 α , the absolute number of B cells as well as the memory unswitched B subset and among CD4⁺ T cells those TM expressing CD38 and PD1. Plasma levels of CXCL10, CXC3CL1, BAFF and the absolute number of different subpopulations of CD8⁺ T cells such as those EM expressing CD38, CD57 and HLA-DR were the major contributors for the clusterization of non-responders along PC1 (Figure 5B). Moreover, as previously reported, age, levels of D-dimer, SOFA score and levels of creatine are the major demographic/clinical features that are mostly associated to worse disease outcome and lack of response to therapy (2–6).

Discussion

Several observational and randomized studies, including TESEO, RECOVERY and REMAP-CAP, demonstrated that tocilizumab is effective in COVID-19 patients with hypoxemia and in need of oxygen therapy and that it improves survival in critically ill patients in intensive care units (ICUs) (15–17, 23). Despite transforming the treatment options for COVID-19, IL-6R inhibition is still ineffective in a fraction of patients. Thus, to identify possible differences between patients responding or not, we deeply investigated the humoral and adaptive immune compartments of COVID-19 patients undergoing tocilizumab treatment. We found that patients who respond to therapy, are characterized by high basal level of plasmatic IL-1 β , IL-1 α , IL-2, IL-13 and PDGF and lower level of CXCL10, higher number of B, CD4⁺ and CD8⁺ T cells which increase after therapy (Figure 6).

Tocilizumab is a monoclonal antibody that blocks interleukin-6 signaling, reducing downstream effects on inflammation and the immune response, which is largely used for years by rheumatologists. Tocilizumab binds to both soluble (s)IL-6R and membrane (m)IL-6R, thus inhibiting IL-6 binding to its receptors and leading to the blockade of the IL-6 signaling without interfering with other cytokines of the IL-6 family (24). IL-6 is essential for the maturation of B cells and it acts as a T cell survival factor driving T cell expansion under inflammatory conditions, but not during normal homeostasis (25, 26). IL-6



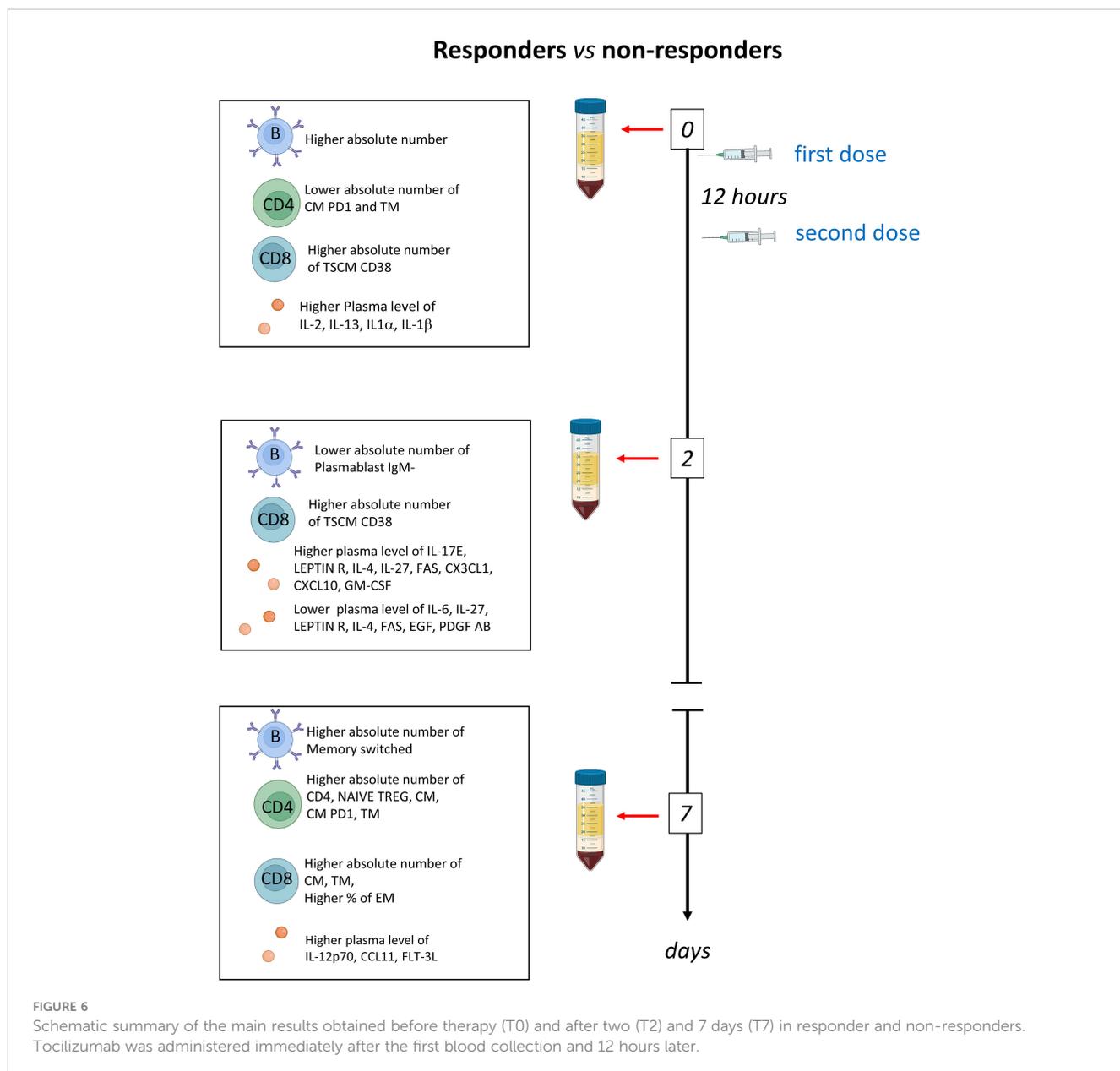
also inhibits TGF- β -induced Treg differentiation (27), and this reinforces its inflammatory aspect.

We found that, at the time of treatment initiation, COVID-19 patients who responded to tocilizumab had higher levels of several pro-inflammatory cytokines, including IL-1 β , IL-1 α and IFN- β . This is in agreement with a previous observation showing that the efficacy of tocilizumab is higher in COVID-19 patients with a hyperinflammatory state (28). Similarly, in patients with high IL-6, early administration of tocilizumab was associated with improvement in oxygenation, indicated as the ratio between arterial oxygen tension and fraction of inspired oxygen (29). In our cohort, IL-6 plasma levels were not different between patients responding or not to therapy at the time of treatment initiation, but in non-responders such levels increased after two days of therapy. In other conditions, including rheumatoid arthritis (RA) and Castleman disease, both serum IL-6 and sIL-6R increase in patients after administration of tocilizumab while the disease symptoms ameliorates (30). In this setting, it is likely that free serum IL-6 increases because IL-6R-mediated consumption of IL-6 is inhibited by the unavailability of tocilizumab-free IL-6R, and thus

that serum IL-6 during the inhibition of its receptor by tocilizumab represents the actual endogenous production of IL-6 (30). In our cohort, IL-6 levels increased after two days of therapy in non-responders. We could hypothesize that, also in this case, plasma IL-6 levels represent the actual endogenous production of IL-6 and the true disease activity of patients when the causal factors of IL-6 overproduction are not adequately counterbalanced. Conversely, in responders, causal factors are neutralized and IL-6 level decreases by natural protein degradation.

Tocilizumab acts by inhibiting IL-6 and its autocrine activity on B cell growth, and is expected to inhibit the differentiation of B cells and the survival of plasma cells (31, 32). We found that tocilizumab affects B cell homeostasis in all COVID-19 patients, responding or not to therapy. The number of B cells, and in particular the number of naïve B cells and of memory switched B cells, increases after tocilizumab administration irrespective of response to treatment. In neuromyelitis optica spectrum disorder (NMOSD), tocilizumab treatment led to an increase in the number of naïve B cells and decreases in the number of memory B cells and antibody-secreting B cells (31). B cell phenotype and IgD⁺CD27⁺ memory B cells are also affected by tocilizumab in patients with RA (33). Concerning the response to tocilizumab, we found that responders have a higher basal number of B cells if compared to non-responders. In particular, patients with high number of memory switched and memory IgM only cells will more likely benefit from the treatment if compared to patients with lower numbers. In other settings, i.e. patients with RA who had an inadequate response to disease-modifying antirheumatic drugs and a TNF inhibitor, tocilizumab proved more effective than rituximab in B cell-poor but not in B cell-rich patients (34).

IL-6 also acts on T cells, and its inhibition has several implications both on CD4⁺ or CD8⁺ T cells. Responder patients have higher number of CD4⁺ and CD8⁺ T cells, with a particular increase of naïve and recently activated memory T cell population after therapy. In inflammation due to RA, tocilizumab contributes to induce the increase of protective Treg and to inhibit the Th17 phenotype (35–37). Six hallmarks describe the main alterations occurring in the early infection phase with SARS-CoV-2 and in the course of the disease, which predispose to severe illness. These six hallmarks, that we have observed in our previous studies, are: i) dysregulated type I IFN activity; ii) hyperinflammation; iii) lymphopenia; iv) lymphocyte impairment; v) dysregulated myeloid response; and vi) heterogeneous adaptive immunity to the virus (38). Concerning tocilizumab treatment, we found that patients responding to therapy were characterized by higher level of inflammation and higher levels of B cells and T cells if compared to those not responding, whereas non-responders were characterized by leukopenia. Moreover, patients who respond to therapy were characterized by a higher activation of the immune system (identified as the expression of recent markers of activation such as CD38 and PD1) and the increase of naïve T cells, together with the decrease of plasmablasts. Accumulating evidence is implicating immunosuppression in the development of severe COVID-19. Therefore, COVID-19 management should aim to reverse immunosuppression and prevent resultant opportunistic infections. In line with these observations, timing of tocilizumab



administration plays a crucial role, as if patient is experiencing immunoparalysis, or, on the other hand, has poor or negligible symptoms, tocilizumab is likely unable to induce a clinically useful response.

We acknowledge that our study has some limitations. The observational nature and the limited number of patients represent a first weakness of this study. This is a single centre study, and the results may not be generalizable to the wider population. However, the longitudinal design with a close follow-up represents a remarkable strength together with the accurate assessment of cellular immune response and the comprehensive analysis of soluble factors, at different time points, before treatment and after two and seven days of treatment. To our knowledge this is the first study to date in which the immunological differences between COVID-19 patients responding or not to tocilizumab have been evaluated.

In conclusion, we found that tocilizumab has pleiotropic effects and that clinical response to this drug remain heterogenous. However, our data suggest that it is possible to identify patients who will respond to treatment and that the administration of tocilizumab is able to restore the immune balance through the re-establishment of different cell populations affected by SARS-COV-2 infection, highlighting the importance of temporal examination of the pathological features from the diagnosis.

Methods

Patients and study design

This is a case-control, longitudinal, single-center study, approved by the local Ethical Committee (Comitato Etico

dell'Area Vasta Emilia Nord, protocol number 177/2020, March 11th, 2020) and by the University Hospital Committee (Direzione Sanitaria dell'Azienda Ospedaliero-Universitaria di Modena, protocol number 7531, March 11th, 2020). Each participant provided informed consent according to Helsinki Declaration, and all uses of human material have been approved by the same Committees. A total of 23 patients with severe COVID-19 was included in the study; they were all treated with tocilizumab. Thirteen patients were responders to therapy, whereas ten patients were non-responders. All patients had severe pneumonia, defined as at least one of the following: (i) presence of a respiratory rate of 30 or more breaths per minute, (ii) peripheral blood oxygen saturation (SaO₂) of less than 93% in room air, (iii) a ratio of arterial oxygen partial pressure (PaO₂) to fractional inspired oxygen (FiO₂) of less than 300 mm Hg in room air, and (iv) lung infiltrates of more than 50% within 24–48 h, according to Chinese management guidelines for COVID-19 (version 6.0) (39). All patients received standard of care treatment at the time of hospital admission according to the regional COVID-19 guidelines of Emilia Romagna. In addition to the standard of care treatment, patients also received tocilizumab treatment. Depending on the availability of specific formulation at time of treatment, twelve patients received tocilizumab by intravenous administration at 8 mg/kg bodyweight (up to a maximum of 800 mg) given twice, 12 hours apart, while eleven patients received it by subcutaneous route at a dose of 162 mg administered in two simultaneous doses, one in each thigh (i.e., 324 mg in total). This particular subcutaneous approach was used to mimic, as much as possible, the pharmacokinetic activity of the intravenous formulation in order to achieve similar levels of drug exposure. After the administration of tocilizumab, patients were divided into 2 groups based on its clinical effects: 13 did not require invasive mechanical ventilation and were classified as responder, meanwhile 10 required invasive mechanical ventilation and were classified as non-responder. Three patients of the non-responder group died between 13 and 29 days after admission. We recorded demographic data, medical history, and main laboratory findings from each patient. For details, see [Supplementary Table 1](#). These data are referred to hospital admission, before starting therapy (T0).

Blood collection and isolation of mononuclear cells

Up to 20 mL of blood were collected from each patient in vacuettes containing ethylenediamine-tetraacetic acid (EDTA). Blood was immediately processed. Isolation of peripheral blood mononuclear cells (PBMC) was performed using ficoll-hypaque according to standard procedures. PBMC were stored in liquid nitrogen in fetal bovine serum (FBS) supplemented with 10% dimethyl sulfoxide (DMSO). Plasma was stored at -80°C until use. Measurements were taken from individual patients; in the case of plasma, each measurement was performed in duplicate and only the mean was considered and shown.

Quantification of cytokine plasma levels

The plasma levels of 62 molecular species were quantified using a Luminex platform (Human Cytokine Discovery, R&D System, Minneapolis, MN) for the simultaneous detection of the following molecules: G-CSF, PDGF-AA, EGF, PDGF-AB/BB, VEGF, GM-CSF, FGF, GRZB, IL-1A, IL-1RA, IL-2, IL-27, IL-4, IL-6, IL-10, IL-13, TNF, IL-17C, IL-11, IL-18, IL-23, IL-6RA, IL-19, IFN- α , IFN- β , IL-3, IL-5, IL-7, IL-12p70, IL-15, IL-33, TGF- β , IFN- γ , IL-1B, IL-17, IL-17E, CCL3, CCL11, CCL20, CXCL1, CXCL2, CCL5, CCL2, CCL4, CCL19, CXCL1, CXCL10, PD-L1, FLT-3, TACI, FAS, LEPTIN R, APRIL, OPN, BAFF, LEPTIN, BMP4, CD40 LIGAND, FAS LIGAND, BMP7, BMP2, TRAIL, according to the manufacturer's instruction. Data represent the mean of two technical replicates.

Polychromatic flow cytometry

All data obtained by flow cytometric assays have been produced according to the state-of-the-art technologies, as described (40).

B cell characterization

Thawed PBMC were washed twice with RPMI 1640 supplemented with 10% fetal bovine serum and 1% each of l-glutamine, sodium pyruvate, nonessential amino acids, antibiotics, 0.1 M HEPES, 55 μ M β -mercaptoethanol and 0.02 mg/ml DNase. Thawed PBMC were stained with viability marker Promokine IR-840 (PromoCell GmbH, Heidelberg, Germany) for 20 min at room temperature in PBS. One million PBMC were washed with FACS buffer and stained with DuraClone IM B cells containing the following lyophilized directly conjugated mAbs: IgD-FITC, CD21-PE, CD19-ECD, CD27-PC7, CD24-APC, CD38-AF750, IgM-PB, CD45-KrO. Cells were washed with FACS buffer and acquired at Cytoflex LX flow cytometer (Beckman Coulter, Hialeah, FL). A minimum of 500,000 cells was acquired on a CytoFLEX LX flow cytometer (Beckman Coulter).

T cell characterization

Thawed PBMC were washed twice with RPMI 1640 supplemented with 10% fetal bovine serum and 1% each of l-glutamine, sodium pyruvate, nonessential amino acids, anti-biotics, 0.1 M HEPES, 55 μ M β -mercaptoethanol and 0.02 mg/ml DNase. Up to 1 million PBMC were stained with the Duraclone IM T cell panel (Beckman Coulter) added with another five fluorescent mAbs and a marker of cell viability. Along with side and forward scatter signals, signals were obtained from different fluorochrome-labeled mAbs, i.e., CD45 conjugated with Krome Orange, CD3 APC-A750, CD4-APC, CD8-AF700, CD27-PC7, CD57-Pacific Blue, CD279 (PD1)-PC5.5, CD28-ECD, CCR7-PE, CD45RA-FITC, HLA-DR-

BUV661, CD127-BV650, CD25-BV785, CD95-BUV395, CD38-BUV496, and PromoFluor-840 (Promokine, PromoCell, Heidelberg, Germany). A minimum of 500,000 cells per sample were acquired on a CytoFLEX LX flow cytometer (Beckman Coulter).

Representation of high parameter flow cytometry

Flow Cytometry Standard (FCS) 3.0 files were imported into FlowJo software version X (Becton Dickinson, San Jose, CA), and analyzed by standard gating to eliminate aggregates and dead cells, and to identify CD3⁺CD4⁺ T cells, CD3⁺CD8⁺ T cells (Supplementary Figures 7) and CD19⁺ B cells (Supplementary Figures 8). Data were exported for further analysis in R, by following a script that makes use of Bioconductor libraries and R statistical packages (CATALYST 1.10.1). The script is available at: <https://github.com/HelenaLC/CATALYST>. The selection of cofactor for data transformation was checked on Cytobank premium version (see: cytobank.org). Metaclustering (K=25) was performed by using FlowSOM algorithm. Dimensional reduction was performed using UMAP algorithm.

B cell UMAP graphs stratified by patient at time T0, T2 and T7 are reported in Supplementary Figures 9A–C, respectively. B cell projection of UMAP graphs stratified by patient showing the FlowSOM clusters at time T0, T2, T7 are reported in Supplementary Figures 10A–C, respectively. CD4⁺ T cell UMAP graphs stratified by patient at time T0, T2 and T7 are reported in Supplementary Figures 11A–C, respectively. CD4⁺ T cell projection of UMAP graphs stratified by patient showing the FlowSOM clusters at time T0, T2, T7 are reported in Supplementary Figures 12A–C, respectively. CD8⁺ T cell UMAP graphs stratified by patient at time T0, T2 and T7 are reported in Supplementary Figures 13A–C, respectively. CD8⁺ T cell projection of UMAP graphs stratified by patient showing the FlowSOM clusters at time T0, T2, T7 are reported in Supplementary Figures 14A–C, respectively.

Principal component analysis

We have then investigated the role of B and T cells along with several clinical and biochemical parameters. For this purpose, we used the principal component analysis (PCA), a dimension reduction method that retains the characteristics of a data set that contribute most to its variance, by keeping lower order principal components (PCs) and ignoring the others (41). PCA uses an orthogonal transformation to collapse the dataset containing correlated parameters to a smaller set of linearly uncorrelated variables known as PCs, such that each PC is a weighted combination of all the markers. We performed this analysis to test whether subject classification was possible based on the B and T cell profile, cytokines and/or clinical data. Thus, PCA was carried out on the clinical events (responder or non-responder), and a dataset comprising 137 parameters that included age, blood pH, pCO₂ (mmHg), pO₂ (mmHg), sO₂ (%), pO₂/FiO₂ ratio, ALT (U/L), total bilirubin (mg/dL), CK (U/L), creatinine (mg/dL), D-

Dimer (ng/mL), Hb (g/dL), white blood cells, WBC (N/ μ L), red blood cells, RBC (10⁶/ μ L), INR (ratio), LDH (U/L), CRP (mg/dL), platelets (10⁹/L), respiratory rate (breaths/minute), systolic blood pressure (mmHg), heart rate (beats/minute), SOFA score, different absolute numbers of B-cell populations (naïve, memory switched, memory IgM-only, transitional, memory unswitched, plasmablasts, exhausted, surface-) and T-cell subpopulations.

Statistical analysis

High-dimensional cytometric analysis was performed by using differential discovery in high-dimensional cytometry *via* high-resolution clustering. Quantitative variables were compared using Two-way ANOVA with Bonferroni correction. Data are represented as individual values, means, and standard errors of the mean. Statistical analyses were performed using Prism 8.4.3 (GraphPad Software Inc., La Jolla, USA).

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Comitato Etico dell'Area Vasta Emilia Nord, protocol number 177/2020 Direzione Sanitaria dell'Azienda Ospedaliero-Universitaria di Modena, protocol number 7531. The patients/participants provided their written informed consent to participate in this study.

Author contributions

LG, SDB, MMA and DT carried out experiments and drafted the figures. AP, RB, AN, LF, MMA, DT collected and stored PBMC samples. LG, SDB drafted and revised the figures and the tables. MME, GG, CM, SB, MG, FC, AM followed patients. SDB, LG, DT, MMA performed bioinformatic and statistical analyses. LG, SDB, CM and AC conceived the study and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2023.1123807/full#supplementary-material>

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