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ORIGINAL ARTICLE

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Profound dysregulation of T cell homeostasis and function in patients with severe COVID-19

Sarah Adamo[1] | Stéphane Chevrier[2,3] | Carlo Cervia[1] | Yves Zurbuchen[1] | Miro E. Raeber[1] | Liliane Yang[1] | Sujana Sivapatham[2,3] | Andrea Jacobs[2,3] | Esther Baechli[4] | Alain Rudiger[5] | Melina Stüssi-Helbling[6] | Lars C. Huber[6] | Dominik J. Schaer[7] | Bernd Bodenmiller[2,3] | Onur Boyman[1,8] | Jakob Nilsson[1]

[1]Department of Immunology, University Hospital Zurich (USZ), Zurich, Switzerland

[2]Department of Quantitative Biomedicine, University of Zurich, Zurich, Switzerland

[3]Institute of Molecular Health Sciences, ETH Zurich, Zurich, Switzerland

[4]Clinic for Internal Medicine, Uster Hospital, Uster, Switzerland

[5]Department of Medicine, Limmattal Hospital, Schlieren, Switzerland

[6]Clinic for Internal Medicine, City Hospital Triemli Zurich, Zurich, Switzerland

[7]Department of Internal Medicine, USZ, Zurich, Switzerland

[8]Faculty of Medicine, University of Zurich, Zurich, Switzerland

Correspondence
Jakob Nilsson and Onur Boyman, Department of Immunology, University Hospital Zurich (USZ), Gloriastrasse 23, 8091 Zurich, Switzerland.
Emails: jakob.nilsson@usz.ch (JN); onur.boyman@uzh.ch (OB)

Bernd Bodenmiller, Department of Quantitative Biomedicine, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland.
Email: bernd.bodenmiller@imls.uzh.ch

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Abstract

Background: Coronavirus disease 2019 (COVID-19) is caused by infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and shows a broad clinical presentation ranging from asymptomatic infection to fatal disease. A very prominent feature associated with severe COVID-19 is T cell lymphopenia. However, homeostatic and functional properties of T cells are ill-defined in COVID-19.

Methods: We prospectively enrolled individuals with mild and severe COVID-19 into our multicenter cohort and performed a cross-sectional analysis of phenotypic and functional characteristics of T cells using 40-parameter mass cytometry, flow cytometry, targeted proteomics, and functional assays.

Results: Compared with mild disease, we observed strong perturbations of peripheral T cell homeostasis and function in severe COVID-19. Individuals with severe COVID-19 showed T cell lymphopenia and redistribution of T cell populations, including loss of naïve T cells, skewing toward $CD4^+$T follicular helper cells and cytotoxic $CD4^+$ T cells, and expansion of activated and exhausted T cells. Extensive T cell apoptosis was particularly evident with severe disease and T cell lymphopenia, which in turn was accompanied by impaired T cell responses to several common viral antigens.

Abbreviations: COVID-19, coronavirus disease 2019; CyTOF, cytometry by time-of-flight; T_{FH}, T follicular helper cell.

Sarah Adamo and Stéphane Chevrier contributed equally.

Jakob Nilsson lead contact

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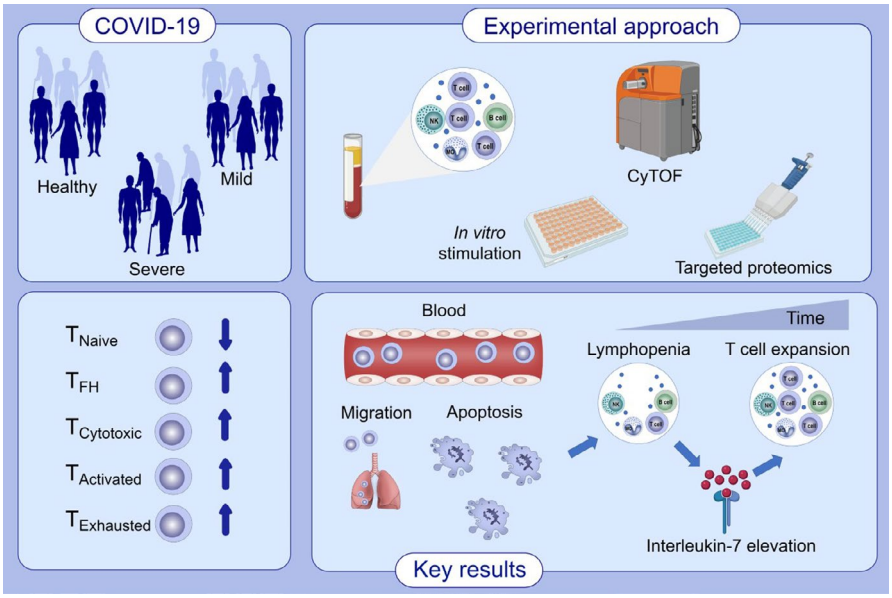
Patients with severe disease showed elevated interleukin-7 and increased T cell proliferation. Furthermore, patients sampled at late time points after symptom onset had higher T cell counts and improved antiviral T cell responses.

Conclusion: Our study suggests that severe COVID-19 is characterized by extensive T cell dysfunction and T cell apoptosis, which is associated with signs of homeostatic T cell proliferation and T cell recovery.

KEYWORDS
COVID-19, lymphopenia, SARS-CoV-2, T cells



GRAPHICAL ABSTRACT
In severe COVID-19 T cell populations show perturbations, including loss of naïve T cells, $CD4^+$ T cell skewing toward T follicular helper and cytotoxic phenotypes and expansion of activated and exhausted T cells. Apoptosis and migration contribute to the lymphopenia of severe disease, which is accompanied by Interleukin-7 elevation. Functional responses to viral antigens are reduced in severe COVID-19.
Abbreviations: COVID-19, coronavirus disease 2019; CyTOF, cytometry by time-of-flight; TFH, T follicular helper cell

1 | INTRODUCTION

The global epidemic of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which is the causative agent of coronavirus disease 2019 (COVID-19), has resulted in almost 100 million confirmed cases and 2 million deaths worldwide as of January 2021. Patients with COVID-19 have a wide spectrum of symptoms ranging from asymptomatic infection to severe acute respiratory distress syndrome (ARDS).[1–5] Advanced age and comorbidities are risk factors for the development of severe disease.[6–8] Furthermore, individuals with severe disease have increased amounts and longer duration of SARS-CoV-2 viral shedding in the respiratory mucosa and of viral RNA in blood as compared to individuals with mild COVID-19.[9–12] Several reports have demonstrated associations between severe disease and elevation of systemic inflammatory markers such as C-reactive protein (CRP), procalcitonin, and Interleukin-6 (IL-6).[6,13–17] Taken together these data suggest that inefficient adaptive antiviral immunity and ensuing hyperinflammation might underlie the pathogenesis of severe COVID-19.[18]

T cells are central players in antiviral immunity. Effector T cells eliminate virus-infected cells, assist in innate antiviral response and support B cell responses, which culminate in the production of virus-specific antibodies.[12] It has been convincingly shown that severe COVID-19 is associated with reduced amounts of $CD3^+$ T cells in peripheral blood and that the extent of T cell decrease correlates with disease severity.[10,13,19,20] The reduction in peripheral T cells appears to be particularly prominent within the $CD8^+$ T cell compartment, but it remains unclear if this is due to trafficking of $CD8^+$ T cells into tissues with ongoing SARS-CoV-2 replication, increased elimination of $CD8^+$ T cells during COVID-19, or pre-existing low levels of $CD8^+$ T cells in individuals who experience severe disease.

Besides a quantitative reduction, qualitative perturbations of the T cell compartment have been observed. Several studies have reported an increased frequency of activated T cell phenotypes,[21] as well as increased expression of surface markers typical of T cell exhaustion, such as PD-1 and TIM-3.[19,22] While a number

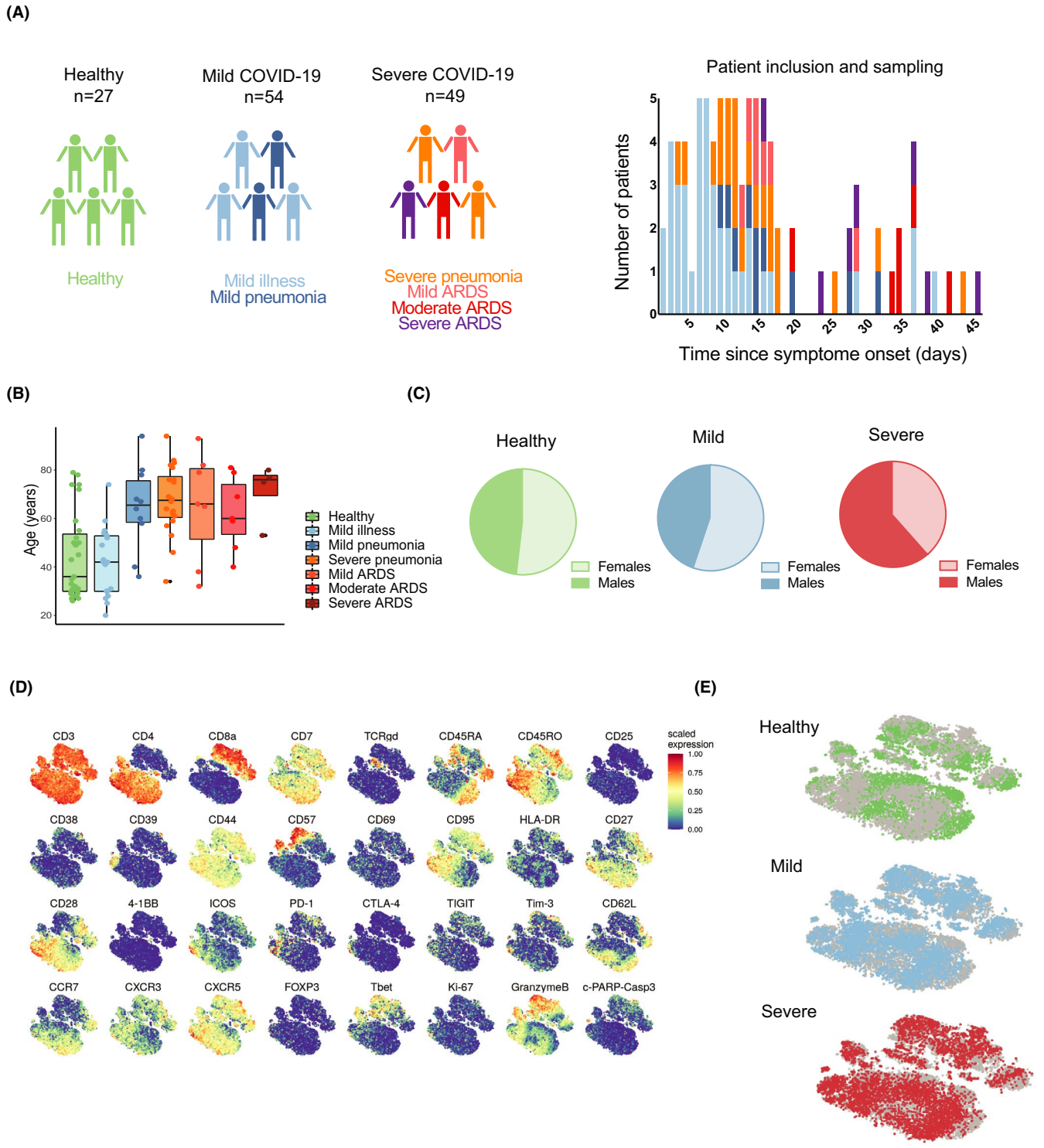


FIGURE 1 Characteristics of COVID-19 patients and healthy subjects included in the cross-sectional study. (A) Number of subjects recruited into the study (left) and time since onset of symptoms at sampling (right). (B) Age distribution of controls and of patients grouped by disease severity subcategories. (C) Gender distribution of healthy subjects, patients with mild disease, and patients with severe disease. (D) t-SNE plots of normalized marker expression for up to 1,000 T cells from each sample analyzed by mass cytometry. Regions with high expression of specific markers appear red. (E) t-SNE plot of the T cells of our study colored by disease severity. Areas occupied prevalently by events corresponding to patients can be visualized on the marker specific t-SNE plots to derive the phenotypes

of reports have dealt with T cell reactivity to SARS-CoV-2 proteins,[23–25] little is known about how functional responses of T cells are affected during COVID-19.

To provide a detailed investigation of the peripheral T cell compartment, we performed mass cytometry, flow cytometry, targeted proteomics, and functional assays to phenotypically and functionally

characterize the changes associated with symptomatic COVID-19 and relate them to disease severity. We observed peripheral T cell loss in both naïve and memory populations, especially among $CD8^+$ T cells, which was most prominent in severe disease. This was accompanied by increased T cell apoptosis, perturbations of the T cell compartment, and impaired T cell responses toward other viral antigens. Furthermore, we observed stronger T cell responses in patients sampled at later time points after onset of symptoms, which, together with the increased Interleukin-7 (IL-7) serum concentrations and evidence of substantial T cell proliferation, suggests a role for lymphopenia-induced proliferation in severe COVID-19.

2 | RESULTS

2.1 | Characteristics of COVID-19 patients and healthy subjects included in the cross-sectional study

To characterize the immune response associated with SARS-CoV-2 infection we conducted a prospective, observational, and cross-sectional study on symptomatic COVID-19 patients recruited at hospitals in the Canton of Zurich, Switzerland. The included patients were stratified based on clinical disease severity at the time of sampling (mild, n = 54; severe, n = 49); a group of healthy controls was included for comparison (n = 27) (Figure 1A). Patients were sampled at a single time point during their symptomatic phase (Figure 1A). Standard laboratory parameters and clinical characteristics of the included patients are presented in Table 1. In agreement with previous studies,[21,22] we observed that patient age was positively correlated with disease severity (Figure 1B) and that males were overrepresented in the severe COVID-19 subgroup (Figure 1C).

We performed a comprehensive T cell characterization making use of a mass cytometry panel (Table S1 and Figure S1A–C) in a subset of patients (mild, n = 28; severe, n = 38, healthy, n = 22). These data are available at http://dx.doi.org/10.17632/s84vd72fsz.1. T cell-related markers were visualized on t-distributed Stochastic Neighbor Embedding (t-SNE) maps focusing on a subset of T cells from each sample, as identified based on a random forest classification (Figure 1D, Figure S1D).[26] By assessing the distribution of events on separate t-SNE maps for healthy controls, patients with mild disease and patients with severe disease, we observed differences between healthy donors and COVID-19 patients (Figure 1E). Notably, cells from healthy individuals showed accumulation in an area of the t-SNE map enriched for CD45RA, whereas events from patients were more represented in areas enriched for CD45RO, CD57, and Granzyme B (Figure 1D–E).

2.2 | Profound reduction in naïve and memory T cell populations in severe COVID-19

We determined the frequencies of naïve, central memory, effector memory, and terminal effector memory expressing CD45RA (TEMRA) $CD4^+$ and $CD8^+$ T cell subsets by manual gating among all T cells in COVID-19 patients and controls (Figure 2A,B, top panels). The frequency of the subsets within $CD4^+$ or $CD8^+$ cells is shown in stacked bar plots for individual patients (Figure 2A,B, bottom panels). As previously reported,[21] patients with severe disease had decreased frequencies of naïve cells within the $CD4^+$ and $CD8^+$ T cell compartments compared to controls (Figure 2A–D). At the same time, the percentages of central memory $CD4^+$and $CD8^+$ T cells were increased in severe COVID-19 (Figure 2C,D).

In order to investigate how the relative changes observed within the different T cell subsets related to absolute cell counts, we performed flow cytometry of whole blood. By measuring absolute cell counts for $CD4^+$ and $CD8^+$ T cells we could calculate absolute numbers for the different T cell subpopulations within our mass cytometry data set (Figure S2A). The marked reduction in naïve $CD4^+$ and $CD8^+$ T cells was confirmed by absolute counts and was especially pronounced for naïve $CD8^+$ T cells (Figure 2C,D). In contrast, central memory and effector memory populations within the $CD4^+$ T cell compartment also showed a reduction in COVID-19 patients compared to healthy controls (Figure 2C).

Absolute numbers of central memory $CD8^+$ T cells, effector memory $CD8^+$ T cells, and TEMRA $CD8^+$ T cells were also reduced in patients with severe disease compared to controls (Figure 2D). Thus, in severe COVID-19, naïve T cell reduction was not accompanied by memory T cell expansion but rather by a slight decrease of the memory compartment, especially among $CD8^+$T cells. The impression of a relative increase in memory (especially central memory) populations was mainly created by the predominant reduction in naïve cells in the peripheral blood. Of note, the T cell reduction in peripheral blood was accompanied by strong T cell activation across all the main subsets (Figure S2B).

Patients with severe COVID-19 in our cohort were older than patients with mild disease (Figure 1B), which could account for some differences in the distribution of T cell populations as previously proposed.[27] Generally, both lymphopenia and naïve T cell reduction correlated with age (Figure S3A). Since age and clinical severity are strongly linked, some of the alterations within the T cell compartment might be due to immunological aging. Upon stratification of patients according to age, we still detected differences between mild and severe COVID-19 patients in terms of total and naïve T cells, although the $CD8^+$ T cell reduction in patients above 60 years of age was not statistically significant (Figure S3B). The same was true when we stratified individuals according to gender (Figure S3C). In our healthy control group, we saw a correlation between age and $CD8^+$ T cell decline (Figure S4A) while this was not the case for $CD4^+$ cells. Conversely, $CD4^+$ T cells were reduced in male individuals compared to females (Figure S4B).

2.3 | Expansion of $CD4^+$ T cells, with cytotoxic, activated and exhausted phenotypes in COVID-19

We next investigated how the distribution of $CD4^+$ T cell subpopulations (Figure S5A) was altered during COVID-19. We saw a significant

TABLE 1 Clinical and laboratory characteristics of healthy subjects and COVID-19 patients

Disease severity[a]		Mild cases (n = 54)		Severe cases (n = 49)			
Disease grade[b]	Healthy (n = 27)	Mild illness	Pneumonia	Severe pneumonia	Mild ARDS	Moderate ARDS	Severe ARDS
Grade at sampling – no.	-	42	12	24	9	9	7
Maximal grade – no.	-	38	10	23	10	11	11
Demographical characteristics							
Age (median (IQR) [yrs])	36.0 (30.0–53.5)	42.00 (30.25–57.75)		69.00 (58.0–79.0)**			
Gender (m/f)	13/14	28/26		31/18			
Time since symptom onset (days)	-	9.96 ± 8.73		18.55 ± 11.36*			
Level of care at blood sampling time point							
Outpatient – no. (%)	-	35 (64.8)		-			
Inpatient – no. (%)	-	19 (35.2)		49 (100)*			
Ward – no. (%)	-	19 (35.2)		34 (69.4)			
ICU – no. (%)	-	-		15 (30.6)			
Outcome							
Released/Recovered	-	53 (98.1)		48 (98)			
Deceased	-	1 (1.9)		1 (2)			
Laboratory characteristics							
C-reactive protein (mean ± SD, [mg/L])	1.20 ± 1.51	19.5 ± 40.09*		106.71 ± 99.04**			
LDH(% of patients above reference value)	5%	16.7%		79.1%*			
Hemoglobin (mean ± SD, [g/L])	141.5 ± 11.77	139.68 ± 17.43		131.10 ± 15.38**			
Absolute platelet count (mean ± SD, [G/L])	254.68 ± 56.45	211.21 ± 63.39*		209.96 ± 109.21*			
Total white blood cell count (mean ± SD, [G/L])	5.86 ± 1.52	5.62 ± 2.50		7.14 ± 4.55			
Monocytes (mean ± SD, [G/L])	0.45 ± 0.14	0.51 ± 0.32		0.45 ± 0.34			
Neutrophils (mean ± SD, [G/L])	3.35 ± 1.15	3.42 ± 2.25		5.25 ± 3.34**			
Eosinophils (mean ± SD, [G/L])	0.15 ± 0.08	0.07 ± 0.08*		0.03 ± 0.07**			
Basophils (mean ± SD, [G/L])	0.04 ± 0.02	0.02 ± 0.02*		0.01 ± 0.02**			
Lymphocytes (mean ± SD, [G/L])	1.85 ± 0.65	1.56 ± 0.72		0.82 ± 0.45**			
CD3- $CD56^{bright}$ $CD16^{dim}$ NK cells (mean ± SD, [cells/μL])	10.5 ± 5.39	9.17 ± 5.63		6.14 ± 5.09**			
CD3- $CD56^{dim}$ $CD16^{bright}$ NK cells (mean ± SD, [cells/μL])	204.69 ± 110.88	191.98 ± 139.98		152.14 ± 99.75			
Comorbidities							
Hypertension – no. (%)	4 (14.8)	12 (22.2)		29 (59.2)*			
Diabetes – no. (%)	2 (7.4)	7 (13)		15 (30.6)*			
Heart disease – no. (%)	1 (3.7)	6 (11.1)		22 (44.9)*			
Cerebrovascular disease – no. (%)	-	2 (3.7)		5 (10.2)			
Lung disease – no. (%)	1 (3.7)	6 (11.1)		7 (14.3)			
Kidney disease – no. (%)	-	9 (16.7)		14 (28.6)*			

(Continues)

TABLE 1 (Continued)

Disease severity[a]		Mild cases (n = 54)		Severe cases (n = 49)			
Disease grade[b]	Healthy (n = 27)	Mild illness	Pneumonia	Severe pneumonia	Mild ARDS	Moderate ARDS	Severe ARDS
Malignancy – no. (%)	1 (3.7)	2 (3.7)		5 (10.2)			
Systemic Immunosuppression – no. (%)	-	3 (5.6)		5 (10.2)			
Treatment at sampling							
Hydroxychloroquine – no. (%)	-	2 (3.7)		19 (38.8)*			
Remdesivir– no. (%)	-	3 (5.6)		10 (20.4)*			
Glucocorticoids – no. (%)[c]	-	-		5 (10.2)*			
Lopinavir-Ritonavir– no. (%)	-	-		1 (2)			
Tocilizumab – no. (%)	-	-		3 (6.1)			
Glucocorticoids pre-COVID – no. (%)		2 (3.7)		5 (10.2)			
Mycophenolate Mofetil– no. (%)	-	1 (1.9)		-			
Calcineurin Inhibitors – no. (%)	-	1 (1.9)		1 (2)			
Azathioprine – no. (%)	-	-		2 (4.1)			
Leflunomide – no. (%)	-	1 (1.9)		-			
Mesalazine– no. (%)	-	1 (1.9)		-			
Methotrexate – no. (%)	-	1 (1.9)		-			
Rituximab – no. (%)	-	1 (1.9)		-			

Note: Mann-Whitney-Wilcoxon test was used to test for differences between continuous variables adjusted for multiple testing using the Holm method. * Indicates significance (p-value threshold <.05) compared to the healthy, ** in the severe indicates significance in comparison to the healthy and the mild. Categorical variables were compared using Chi-square test, * indicates significance (p-value threshold <.05) overall.

Abbreviations: ARDS, acute respiratory distress syndrome; ICU, intensive care unit; IQR, interquartile range; LDH, Lactate Dehydrogenase.

[a]COVID-19 disease severity at the time of blood sample collection. Mild illness and pneumonia are considered mild COVID-19 disease and severe pneumonia as well as any grade of ARDS are considered severe COVID-19 disease.

[b]COVID-19 grade according to WHO guidelines, recorded at sampling and prospectively followed until recovery.

[c]Glucocorticoids initiated as part of the COVID-19 treatment.

increase in T follicular helper cells and T regulatory cells, as well as terminally differentiated, activated, and exhausted T cells (Figure 3A). Interestingly, a population of Granzyme B$^+$ terminally differentiated (CD28$^-$) cells, compatible with cytotoxic CD4$^+$ T cells, that was absent in healthy controls was strongly increased in COVID-19. This population likely corresponds to the one identified at the transcriptional level by others.[28] Given the profound lymphopenia of severe COVID-19 patients (Figures S2A,S6A), we sought to relate our findings to absolute counts. This was especially important because a disproportionate reduction in specific populations could create the illusion of an expansion of other populations as observed for naïve and memory cells. By investigating absolute counts, we saw that the increased percentage of T follicular helper cells reflected a real expansion of this population in peripheral blood, likely contributing to the robust antibody responses observed in COVID-19,[12] while Th1 cell counts were reduced. T regulatory cells were strongly altered in terms of frequency but not in absolute counts (Figure 3B), perhaps reflecting a shift in the global distribution of CD4$^+$ T cell subsets rather than T regulatory cell proliferation. Expansion of CD4$^+$ T cells with a cytotoxic phenotype, terminally differentiated and exhausted cells was also confirmed by absolute counts (Figure 3B).

In order to visualize the most prominent changes within the CD4$^+$ compartment we calculated the fold change of absolute counts for the described T cell populations between mild and severe COVID-19 as well as healthy controls (Figure 3C). Indeed, we observed that the most prominent changes in patients with severe COVID-19 compared to healthy controls was the increase in cytotoxic CD4$^+$ T cells, as well as the increase in terminally differentiated and exhausted cells. We next analyzed populations within the CD8$^+$ compartment (Figure S5B) and observed an increase of activated and exhausted CD8$^+$ T cells in terms of frequency (Figure 3D) and absolute counts (Figure 3E). CD8$^+$ T cells with a cytotoxic phenotype (Granzyme B$^+$, CD28$^-$) were increased in percentage but not in absolute number (Figure 3D–F). Among CD8$^+$ cells, the most prominent change observed was the increase in exhausted T cells, which also showed a strong difference between mild and severe patients (Figure 3F). PhenoGraph unsupervised clustering of T cell populations revealed a similar picture (Figure S7A–B).

2.4 | T cell apoptosis and indirect signs of T cell migration in severe COVID-19

We next investigated apoptosis and cell migration as possible mechanisms contributing to peripheral T cell loss in patients with severe

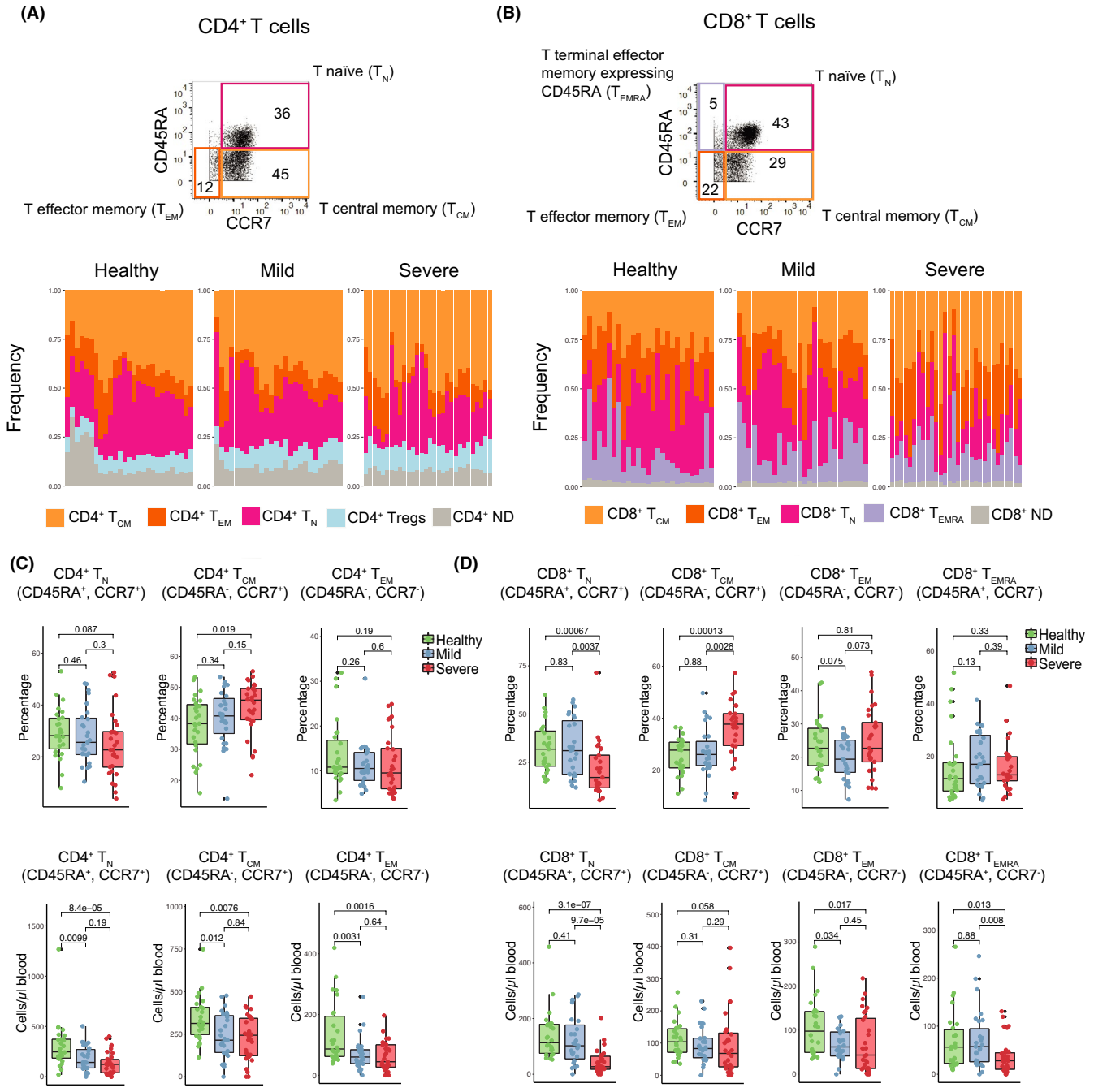


FIGURE 2 Naïve and memory T cells are profoundly reduced in severe COVID-19. (A) Gating strategy for naive and memory populations as shown on representative mass cytometry plots for $CD4^+$ (top) and stacked histograms with frequencies of regulatory T cells and naive and memory (central memory, effector memory) $CD4^+$ T cells for healthy controls and mild and severe disease categories (bottom). (B) Gating strategy for naive and memory populations as shown on representative mass cytometry plots for $CD8^+$ (top) and stacked histograms with frequencies of naive and memory (central memory, effector memory, TEMRA) $CD8^+$ T cells for healthy controls and mild and severe disease categories (bottom). (C) Percentages (top) and absolute counts (bottom) of $CD4^+$ T cell subsets in healthy subjects and patients with mild and severe COVID-19 shown as individual dots. Box plots display median and interquartile ranges. Indicated *p* values were calculated with a Mann-Whitney-Wilcoxon test and adjusted for multiple comparisons with the Holm method. (D) Percentages (top) and absolute counts (bottom) of $CD8^+$ T cell subsets in healthy subjects and patients with mild and severe COVID-19 shown as individual dots. Boxplot display median and interquartile ranges. Statistical testing was performed as in (C)

COVID-19. Several factors can contribute to T cell apoptosis in severe COVID-19 including cytokine signaling, direct interaction of the virus with T cells via CD26 or CD147 and elevated plasma Fas ligand levels as described for SARS-CoV.[29,30]

Excessive pro-inflammatory cytokine signaling, especially mediated by TNF-α, can directly lead to T cell apoptosis.[31] As TNF-α levels were considerably elevated in our cohort (Figure S8A) in agreement with previous reports,[17,32] we hypothesized that

apoptosis could drive T cell loss during severe COVID-19 disease. We thus determined the percentage of apoptotic cells among the main CD4$^+$ and CD8$^+$ T cell subsets. We defined apoptotic cells as cells positive for cleaved caspase-3 and/or cleaved PARP, as cleavage of both of these substrates occurs during apoptosis and positivity for the cleaved form of these proteins can be used as evidence of an apoptotic state.

Indeed, we observed significant increases in the percentages of apoptotic cells among central and effector memory CD4$^+$ T cells, as well as naïve, central memory, and effector memory CD8$^+$ T cells and

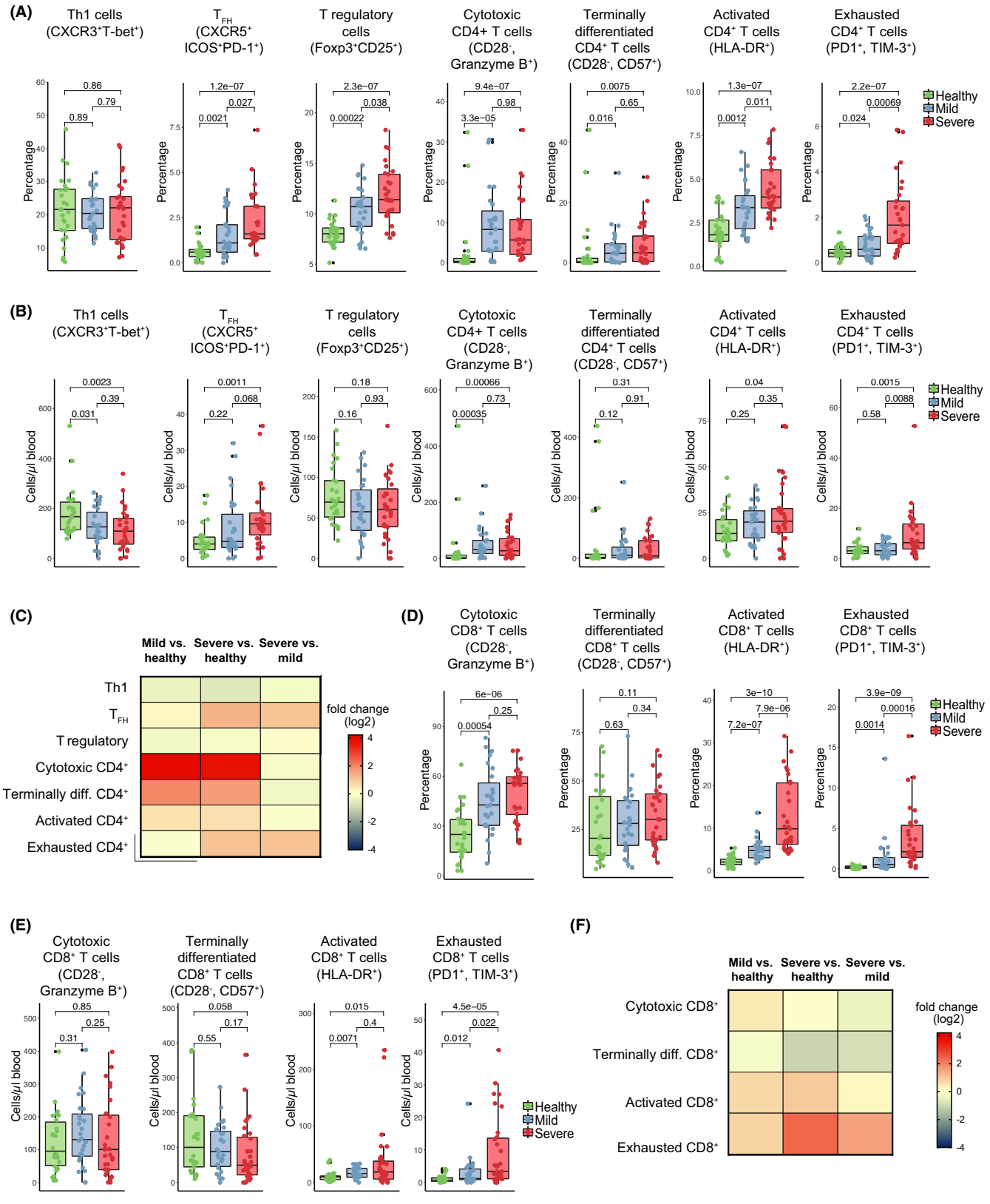


FIGURE 3 Several T cell subsets are increased in mild and severe COVID-19. (A) Percentages of $CD4^+$ T cell subsets identified with mass cytometry in healthy subjects and patients with mild and severe COVID-19 shown as individual dots. Box plots display median and interquartile ranges (shown are percentages of total $CD4^+$). Indicated *p* values were calculated with a Mann-Whitney-Wilcoxon test and adjusted for multiple comparisons with the Holm method. (B) Absolute counts of $CD4^+$ T cell subsets identified with mass cytometry in healthy subjects and patients with mild and severe COVID-19 shown as individual dots. Box plots display median and interquartile ranges. Statistical testing was performed as in (A). (C) Log2 fold change of absolute counts of $CD4^+$ T cell subsets for mild COVID-19 compared to healthy (mild vs. healthy), severe COVID-19 compared to healthy (severe vs. healthy) and severe COVID-19 compared to mild COVID-19 (severe vs. mild) is shown as a heatmap. (D) Percentages of $CD8^+$ T cell subsets identified with mass cytometry in healthy subjects and patients with mild and severe COVID-19 shown as individual dots. Box plots display median and interquartile ranges (shown are percentages of total $CD8^+$). Statistical testing was performed as in (A). (E) Absolute counts of $CD8^+$ T cell subsets identified with mass cytometry in healthy subjects and patients with mild and severe COVID-19 shown as individual dots. Box plots display median and interquartile ranges. Statistical testing was performed as in (A). (F) Log2 fold change of absolute counts of $CD8^+$ T cell subsets for mild COVID-19 compared to healthy (mild vs. healthy), severe COVID-19 compared to healthy (severe vs. healthy) and severe COVID-19 compared to mild COVID-19 (severe vs. mild) is shown as a heatmap

TEMRA $CD8^+$ T cells (Figure 4A). Among $CD8^+$ T cells, the extent of apoptosis was greater in samples from patients with severe as compared to mild COVID-19 (Figure 4A). Even within the group with mild disease, the extent of T cell apoptosis was strongly associated with symptom severity in the CD8 subset as shown by increased apoptosis in patients with mild pneumonia (Figure 4B). Increased T cell apoptosis could thus contribute to the lymphopenia that is a hallmark of severe COVID-19.

T cell reduction in peripheral blood can also be a consequence of T cell migration to tissues and lymphocyte accumulation in the lungs during COVID-19 has been reported by some [33,34] but not by others.[35,36] We therefore looked for indirect signs of T cell migration in the peripheral blood of COVID-19 patients. T cell migration into inflamed lung parenchyma is primarily mediated by CXCR3 signaling,[37,38] so we investigated the level of CXCR3 ligands in the serum of COVID-19 patients. CXCR3 ligands CXCL9, CXCL10, and CXCL11 were significantly increased in sera from COVID-19 patients, especially in sera from patients with severe disease (Figure 4C). Furthermore, IFN-γ, a potent inducer of CXCR3 ligands,[39] was markedly elevated (Figure 4C and Figure S8B). We also observed a very strong reduction in CXCR3 expression in all the main T cell subsets with the exception of naïve $CD4^+$ T cells in COVID-19 patients (Figure 4D and Figure S8C). Reduced CXCR3 expression on MAIT cells and $CD8^+$ T cells has previously been reported,[40] whereas reduced CXCR3 levels on memory $CD4^+$ populations have been associated with a negative prognosis.[41] CXCR3 abundance inversely correlated with levels of CXCL9, CXCL10, CXCL11, and IFN-γ, and was positively correlated with $CD3^+$, $CD4^+$, and $CD8^+$ T cell frequencies (Figure 4E). The frequency of apoptosis in $CD4^+$ and $CD8^+$ T cell populations also seemed to inversely correlate with absolute T cell numbers (Figure 4E). Taken together, our data suggest that both migration and apoptosis might contribute to T cell lymphopenia.

2.5 | Reduced in vitro T cell function in patients with COVID-19

We next investigated whether the observed changes within the T cell compartment associated with COVID-19 had an impact on T cell function. We took advantage of our clinically validated flow cytometric assay for specific cell-mediated immune responses in activated whole blood (FASCIA).[42,43] This routine assay measures the ability of peripheral T cells in whole blood to form blasts in response to mitogens, super-antigens, and a selection of common viral antigens. In patients with severe COVID-19, we observed reduced blast formation upon stimulation with adenovirus, Cytomegalovirus (CMV), Herpes simplex virus 1 (HSV1), Herpes simplex virus 2 (HSV2) and Varicella Zoster Virus (VZV) in COVID-19 as compared to healthy controls or patients with mild disease (Figure 5A). The same tendency was not observed when blood cells were incubated with pokeweed mitogen or *Staphylococcal* super-antigens (SEA/SEB), but we did observe a reduction blast formation upon stimulation with concavalin A (Figure 5A). The discrepancy between different mitogens is difficult to interpret, as pokeweed mitogen and concavalin A are both plant lectins, known to induce mitosis in T cells, but their exact mechanism of action is not known. We next investigated whether the reduction in antiviral T cell responses was associated with the time between sampling and symptom onset in our cross-sectional study. Interestingly, we observed a positive correlation between blast formation and time after symptom onset upon stimulation with HSV1, HSV2, and adenovirus (Figure 5B and Figure S9A). A trend toward a positive correlation was also observed for CMV and VZV stimulation of samples from patients with severe COVID-19, but not for stimulation with mitogens or super-antigens (Figure 5B and Figure S9A). Taken together, these data suggest that antiviral T cell responses are impaired in patients with severe COVID-19 and that these responses tend to improve in patients sampled at later time points from symptom onset.

Reduced blast formation upon stimulation with specific viral antigens is likely mediated by a combination of factors, but reduced frequency of virus-specific memory T cells is likely to play a central role. In support of this, we found that blast formation positively correlated with absolute peripheral $CD4^+$ and $CD8^+$ T cell counts (Figure S9B,C). We therefore hypothesized that the improvement of T cell function at later stages of the infection might be partially due to reconstitution of the peripheral T cell compartment. Indeed, we observed higher absolute peripheral T cell counts in samples from patients with severe COVID-19 obtained at later time points after symptom onset as compared to samples obtained earlier in the disease course, especially

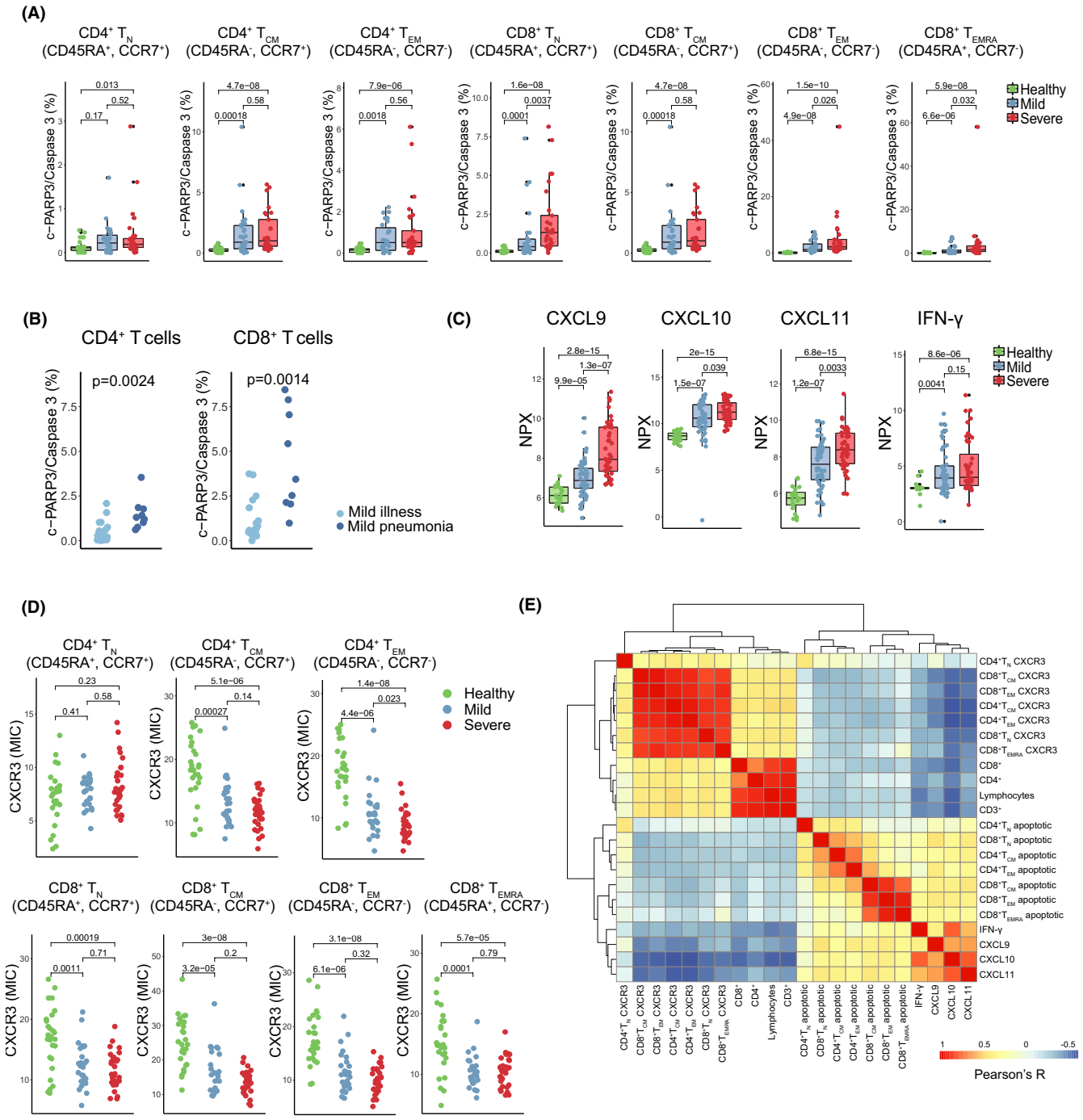


FIGURE 4 Severe COVID-19 is associated with lymphopenia, apoptosis and phenotypic changes of T cells. (A) Percentages of apoptotic (cleaved-PARP/cleaved Caspase 3$^+$) cells among CD4$^+$ T cell subsets and CD8$^+$ T cell identified with mass cytometry in healthy subjects and patients with mild and severe COVID-19 patients shown as individual dots. Box plots display median and interquartile ranges. Indicated *p* values were calculated with a Mann–Whitney–Wilcoxon test and adjusted for multiple comparisons with the Holm method. (B) Percentages of apoptotic (cleaved-PARP/cleaved Caspase 3$^+$) cells among CD4$^+$ T cells and CD8$^+$ T cell identified with mass cytometry in patients with mild illness vs. mild pneumonia. Indicated *p* values were calculated with a Mann–Whitney–Wilcoxon test. (C) CXCL9, CXCL10, CXCL11, and IFN-γ serum levels in healthy subjects and patients with mild and severe COVID-19 measured with an Olink proximity extension assay shown as individual dots. Box plots display median and interquartile ranges. Statistical testing was performed as in (A). (D) CXCR3 mean ion count (MIC) of CD4$^+$ T cell subsets (top) and CD8$^+$ T cell subsets (bottom) in healthy subjects and patients with mild and severe COVID-19. Statistical testing was performed as in (A). (E) Pearson correlation analyses among CXCR3, CXCL9, CXCL10, CXCL11, and IFN-γ levels, lymphocyte and T cell counts, and percentage of apoptotic cells in samples from all subjects

for CD8$^+$T cells (Figure 5C). To explore whether T cell reconstitution was a potential mechanism, we investigated T cell proliferation in the different T cell populations. We could indeed observe increased frequencies of proliferating (Ki-67$^+$) in multiple subsets of CD4$^+$ and CD8$^+$ T cells in COVID-19 patients, especially in patients with severe disease (Figure 5D and Figure S10A–B).

Given the extent of the lymphopenia in severe COVID-19 we hypothesized that lymphopenia-induced proliferation might play a role. IL-7 is known to be a critical homeostatic factor for T cells, and since IL-7 production by stromal cells is relatively constant,[44,45] IL-7 serum levels are mainly regulated by its consumption by lymphocytes. In patients with severe disease, we observed higher serum IL-7 levels compared to those with mild disease and healthy controls (Figure 5E), in agreement with a previous report.[46] Furthermore, IL-7 levels inversely correlated with the total number of CD3$^+$, CD4$^+$ and CD8$^+$ T cells (Figure 5F). Taken together, these findings suggest that the lymphopenia in severe COVID-19 could result in a systemic IL-7 elevation, which in turn might contribute to the observed T cell proliferation.

3 | DISCUSSION

T cell lymphopenia and perturbations of T cell homeostasis are very prominent features of severe COVID-19, but their pathogenesis has not yet been fully elucidated. A better understanding of the T cell alterations occurring in severe COVID-19 can provide insight into important disease mechanisms and inform therapeutic strategies. We performed detailed investigations of relative and absolute changes to the peripheral T cell compartment in our well characterized COVID-19 cohort. Our analysis revealed marked T cell loss across naïve and memory cells in the CD4$^+$ and CD8$^+$ T cell compartment in patients with COVID-19 compared to healthy controls. However, certain T cell populations such as cytotoxic, activated and exhausted cells were expanded, especially in severe COVID-19. We were able to identify apoptosis as a possible mechanism driving the lymphopenia and observed signs of T cell reconstitution at later time points from symptom onset.

The occurrence of peripheral lymphopenia has been described in several human acute respiratory viral infections.[47,48] In severe COVID-19, the extent of lymphopenia is closely linked to disease severity and mortality.[32] Since disease severity in COVID-19 is strongly associated with biological age, age-associated decline in lymphocyte counts could in part account for the observed lymphopenia. A recent study, which showed a strong correlation between T cell lymphopenia, diminished SARS-CoV-2-specific T cell responses and severe disease also suggested an association between age and decline in the fraction of naïve CD8$^+$ T cells.[18] Investigating the effect of age in our patient cohort poses a challenge, as this is strongly linked to disease severity. In our healthy control group, however, we saw a correlation between age and CD8$^+$ T cell decline, while this was not the case for CD4$^+$ cells. Despite association with disease severity and male gender, we did not observe CD8$^+$ T cell reduction when comparing males to females among healthy controls, but saw a modest reduction of CD4$^+$ T cells. Given the limited number of subjects, these data must be interpreted with caution, but it is conceivable that age and gender account for pre-existing alterations of the T cell compartment that influence the course of COVID-19 disease.

Within the T cell compartments, we saw a prevalent reduction of naïve T cells, which resulted in increased memory subsets percentages despite the slight contraction in absolute numbers. Whether the loss of naïve populations can be explained by the acquisition of a memory phenotype by virus specific cells or in an antigen independent way remains to be seen.

Another potential explanation for the loss of naïve cells might be selective or preferential death of these subsets. In this study we observed extensive T cell apoptosis in COVID-19, especially among CD8$^+$ T cells, suggesting that apoptosis could be a central mechanism in the immunopathology of severe COVID-19. Apoptosis could be caused by the inflammatory microenvironment generated by SARS-CoV-2, although a direct interaction of the virus with T cells via CD147 or CD26 or perhaps even T cell infection cannot be excluded. In our study, we could not identify a temporal component to the increased apoptosis, but we observed signs of T cell recovery starting at day 20, suggesting that apoptosis might occur relatively early in the disease course.

Lymphocyte redistribution, that is, migration to inflamed tissues, can also be a cause of apparent peripheral lymphopenia. In our study, we found elevated amounts of CXCL9, CXCL10, and CXCL11. These chemokines are known to be produced in response to viral infection and are mainly responsible for T cell and NK cell recruitment to the site of infection. Together with the reduced surface expression of CXCR3, our data could suggest migration of cells expressing higher levels of CXCR3 to the tissue, accounting for their disappearance from the peripheral blood, although downregulation of CXCR3 in response to its ligands or antigen activation cannot be excluded.

Based on our data, the changes in T cell populations in COVID-19 might be due to T cell apoptosis, migration, proliferation or differentiation. For example, expansion of T follicular helper cells might result from a combination of differentiation and proliferation, while contraction of Th1 could depend on migration and apoptosis in this population. Although migration and apoptosis are both possible mechanisms, higher T cell counts in the lung do not seem to correlate with disease severity.[49,50] Furthermore, IL-7 elevation in severe disease likely results from global T cell loss, rather than T cell re-distribution or activation-induced downregulation of the IL-7 receptor α subunit.[51,52] Taken together, these findings suggest that apoptosis might be central to the development of lymphopenia in severe COVID-19.

When investigating the T cell function in COVID-19, we found reduced T cell responses to several viral antigens in severe disease. The most likely explanation for this finding is a reduction in the precursor frequency of memory cells specific for common antigens. A similar effect has recently been described for B cells in measles,[53,54] where it has been linked to direct B cell infection and cell death. In

the measle studies, alteration of the B cell repertoire can last for years, whereas we observed an improvement of T cell function in patients sampled later in the disease courses (day 30–40), which occurred in parallel with recovery of T cell counts. This dynamic was present only in patients with severe disease, probably because the more severe lymphopenia had resulted in increased levels of IL-7,

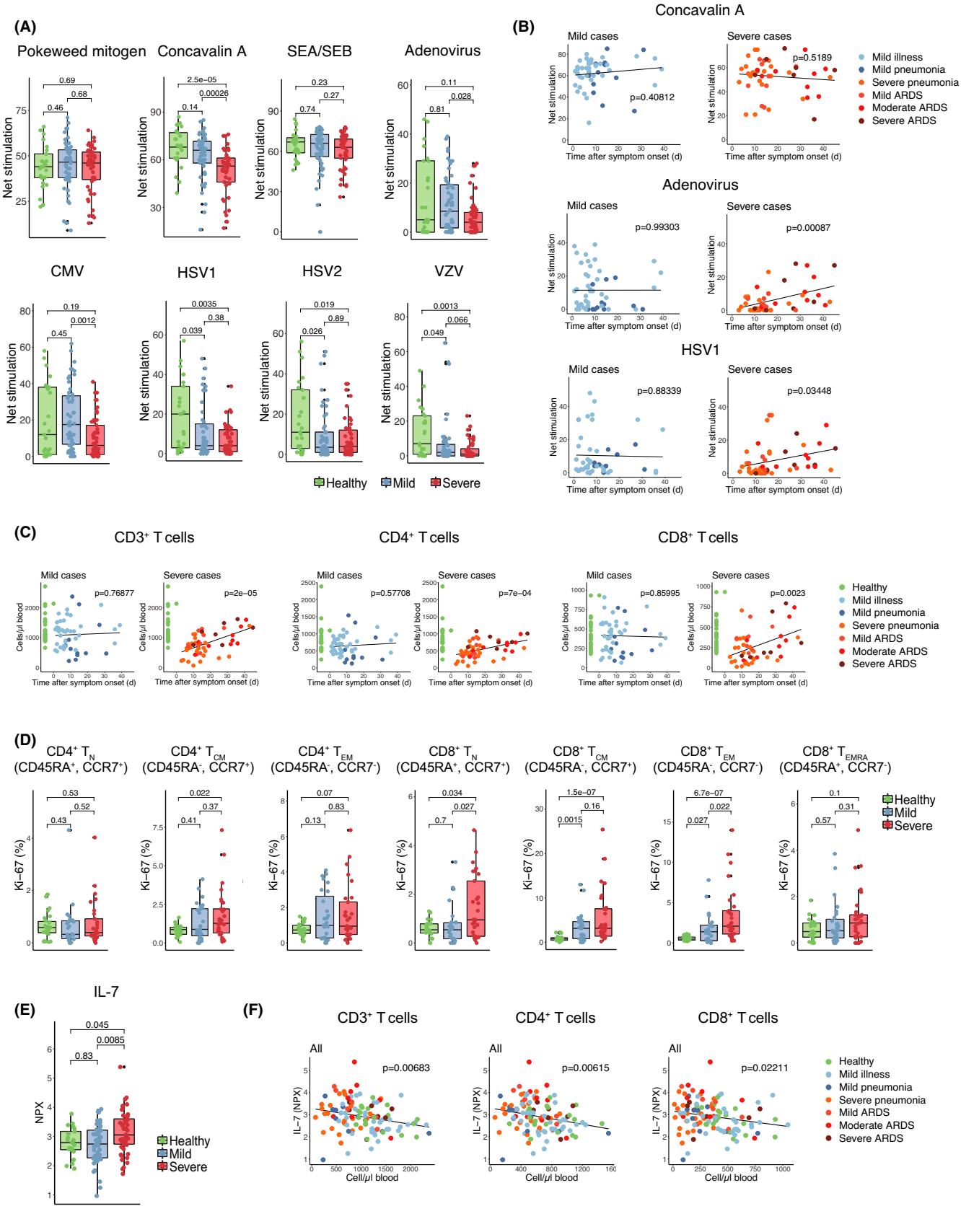


FIGURE 5 Reduced T cell responses toward viral antigens and signs of T cell recovery are evident in severe COVID-19. (A) Net stimulation of $CD3^+$ T cells observed in the presence of mitogens, super antigens and viral antigens shown as individual dots. Box plots display median and interquartile ranges. Indicated *p* values were calculated with a Mann–Whitney–Wilcoxon test and adjusted for multiple comparisons with the Holm method. (B) Linear modeling of blast formation as a function of time after symptom onset in mild and severe COVID-19. (C) $CD3^+$, $CD4^+$, and $CD8^+$ T cell counts are a function of time after symptom onset in patients with mild and severe COVID-19 as shown by a linear model. Counts for healthy subjects are shown for reference but were not included in the model. (D) Percentages of proliferating ($Ki\text{-}67^+$) cells among $CD4^+$ T cell subsets (top) and $CD8^+$ T cell subsets (bottom) identified with mass cytometry in healthy subjects and patients with mild and severe COVID-19 shown as individual dots. Box plots display median and interquartile ranges. Statistical testing was performed as in (A). (E) IL-7 serum levels in healthy subjects and patients with mild and severe COVID-19 measured with the Olink proximity extension assay shown as individual dots. Box plots display median and interquartile range. Statistical testing was performed as in (A). (F) IL-7 serum level is a function of $CD4^+$ T cells, and $CD8^+$ T cells as shown by a linear model

which could in turn trigger lymphopenia-induced IL-7-mediated homeostatic proliferation, as reported for other viral infections.[55]

Understanding the immunological mechanisms underlying severe COVID-19 disease is necessary for risk stratification and the development of interventional therapies, including those aimed at lymphopenia, [56] and protective vaccines. Our study reveals perturbations within $CD4^+$and $CD8^+$ T cell compartments, confirming the association between severe disease, age and T cell lymphopenia, and showing that the lymphopenia is likely partially mediated by a COVID-19 associated apoptotic T cell loss. The observed increased T cell apoptosis in severe disease is closely associated with a highly inflammatory innate immune response. Thus, our study highlights the potential of therapies targeting T cell dysregulation and excessive inflammation in possibly limiting the observed extensive T cell loss associated with severe COVID-19. One promising immunomodulatory therapeutic approach is the use of JAK inhibitors in patients with severe COIVD-19. A recent randomized controlled trial with Baricitinib [57] showed promising effects, especially in patients receiving high-flow oxygen or non-invasive ventilation. Further studies investigating how immunomodulatory therapies such as JAK-inhibitors affect the innate and adaptive immune responses in severe COVID-19 are needed.

4 | METHODS

4.1 | Subjects characteristics

Patients aged 18 years and older with symptomatic, RT-qPCR confirmed SARS-CoV-2 infection were recruited at four different hospitals in the Canton of Zurich, Switzerland, between April 2 and August 19 2020. Both hospitalized patients and outpatients were recruited into the study and all participants gave written informed consent. The study was approved by the Cantonal Ethics Committee of Zurich (BASEC 2016–01440). A more detailed description of the cohort is provided in the Appendix S1.

4.2 | Flow cytometry

For quantification of the main T cell subsets, blood samples were processed in the accredited routine immunology laboratory at University Hospital Zurich. Flow cytometry staining, assessment and analysis was done as established,[58,59] using the reagents and methodology detailed in the Appendix S1.

4.3 | Mass cytometry analysis

Samples were pre-processed as described in the Appendix S1 and mass cytometry analysis was performed as previously described.[17,60–67] A detailed description of the procedure is provided in the Appendix S1.

4.4 | Flow cytometric assay for specific cell-mediated immune responses in whole blood

Venous blood collected in sodium heparin tubes was diluted with RPMI 1,640 medium supplemented with 10% FBS, 100 IU/mL penicillin and 100 IU/mL streptomycin (all from Gibco). Blood cells were stimulated with pokeweed mitogen, Concanavalin A, *Staphylococcus* enterotoxins A and B, or antigens from varicella zoster virus (VZV), adenovirus, cytomegalovirus (CMV), herpes simplex virus 1 (HSV1), or herpes simplex virus 2 (HSV2) or left unstimulated for 7 days. Cells were then stained with live/dead fixable Aqua stain (Thermo Fisher, catalog number L34957) and with Cyto-stat tetrachrome (containing FITC anti-CD45, PECy5 anti-CD3, PE anti-CD4, and ECD anti-CD8; Beckman Coulter, catalog number 660713). Data were acquired on a Navios flow cytometer and analyzed with Kaluza analysis software. Net stimulation was calculated by subtracting the percentage of $CD3^+$ blasts over all lymphocytes in the unstimulated sample from their counterparts in stimulated samples.

4.5 | Cytokine measurements

Serum was collected in BD vacutainer clot activator tubes (Becton Dickinson). The samples were processed in an accredited immunology laboratory at the Department of Immunology of University Hospital Zurich. IFN-γ and TNF-α were quantified using an ELISA (R&D Systems), as previously established.[68]

4.6 | Proteomics analyses

Heat-inactivated plasma samples were analyzed using the Olink® Proteomics 92-plex inflammation immunoassay. A brief description of the method is provided in the Appendix S1.

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CONFLICT OF INTEREST

Dr. Adamo reports grants from Swiss Academy of Medical Sciences, during the conduct of the study. Dr. Chevrier has nothing to disclose. Dr. Cervia reports grants from Swiss Academy of Medical Sciences, during the conduct of the study. Dr. Zurbuchen reports grants from Swiss Academy of Medical Sciences, during the conduct of the study. Dr. Raeber reports grants from Young Talents in Clinical Research Fellowship by the Swiss Academy of Medical Sciences and Bangerter Foundation, during the conduct of the study. Dr. Yang has nothing to disclose. Dr. Sivapatham has nothing to disclose. Dr. Jacobs has nothing to disclose. Dr. Baechli has nothing to disclose. Dr. Rudiger has nothing to disclose. Dr. Stüssi-Helbling has nothing to disclose. Dr. Huber has nothing to disclose. Dr. Schaer has nothing to disclose. Dr. Bodenmiller reports grants from Swiss National Science Foundation, grants from Pandemic Fund of the University of Zurich, during the conduct of the study. Dr. Boyman reports grants from Swiss National Science Foundation, grants from Clinical Research Priority Program of the University of Zurich, from Innovation grant of University Hospital Zurich, during the conduct of the study. Dr. Nilsson reports grants from Swiss National Science Foundation, during the conduct of the study.

AUTHOR CONTRIBUTION

SA contributed to study design, patient recruitment, data collection, data analysis, and data interpretation.SC contributed to study design, data collection, data analysis, and data interpretation. CC and YZ contributed to patient recruitment, data collection and data analysis. MER, EB, AR, MS-H, LCH, and DJS contributed to patient recruitment and clinical management. SS, AJ, and SC developed the CyTOF antibody panel and performed the CyTOF experiments. SA and JN wrote the manuscript with contribution by OB and BB. JN, OB, and BB contributed to study conception and design, data analysis and data interpretation. All authors reviewed and approved the final version of the manuscript.

ORCID

Onur Boyman https://orcid.org/0000-0001-8279-5545

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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