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ORIGINAL ARTICLE

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T-cell recovery and evidence of persistent immune activation 12 months after severe COVID-19

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Abstract

Background: T-cell lymphopenia and functional impairment is a hallmark of severe acute coronavirus disease 2019 (COVID-19). How T-cell numbers and function evolve at later timepoints after clinical recovery remains poorly investigated.

Methods: We prospectively enrolled and longitudinally sampled 173 individuals with asymptomatic to critical COVID-19 and analyzed phenotypic and functional characteristics of T cells using flow cytometry, 40-parameter mass cytometry, targeted proteomics, and functional assays.

Results: The extensive T-cell lymphopenia observed particularly in patients with severe COVID-19 during acute infection had recovered 6 months after infection, which was accompanied by a normalization of functional T-cell responses to common viral antigens. We detected persisting $CD4^+$ and $CD8^+$ T-cell activation up to 12 months after infection, in patients with mild and severe COVID-19, as measured by increased HLA-DR and CD38 expression on these cells. Persistent T-cell activation after COVID-19 was independent of administration of a COVID-19 vaccine post-infection. Furthermore, we identified a subgroup of patients with severe COVID-19 that presented with persistently low $CD8^+$ T-cell counts at follow-up and exhibited a distinct phenotype during acute infection consisting of a dysfunctional T-cell response and signs of excessive pro-inflammatory cytokine production.

Abbreviations: COVID-19, coronavirus disease 2019; SARS-CoV-2, severe acute respiratory syndrome coronavirus type 2.

Patrick Taeschler and Sarah Adamo contributed equally.

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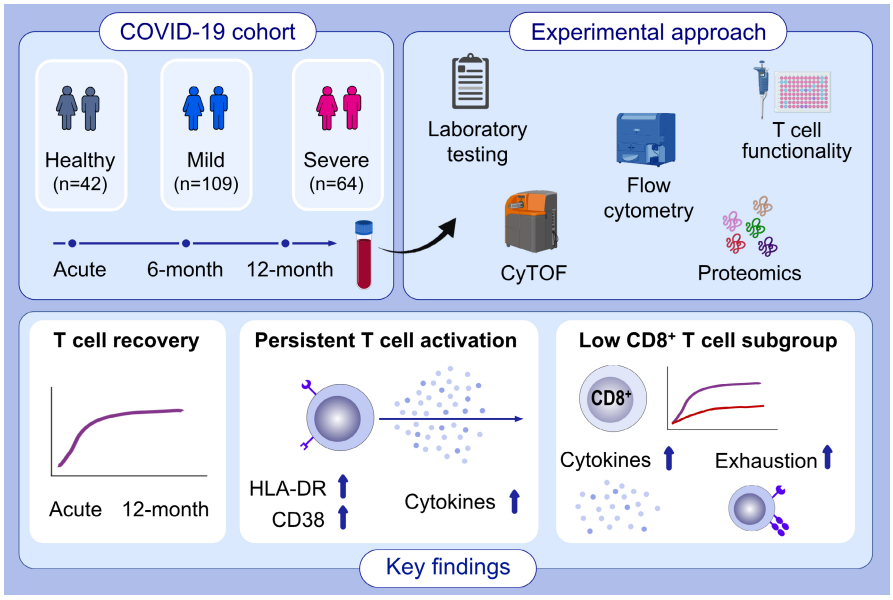
Conclusion: Our study suggests that T-cell numbers and function recover in most patients after COVID-19. However, we find evidence of persistent T-cell activation up to 12 months after infection and describe a subgroup of severe COVID-19 patients with persistently low CD8$^+$ T-cell counts exhibiting a dysregulated immune response during acute infection.

KEYWORDS
COVID-19, follow-up, recovery, SARS-CoV-2, T cells



GRAPHICAL ABSTRACT
In a multicentric cohort of 173 COVID-19 patients followed-up to 1 year we found evidence of functional and numeric T cell recovery. COVID-19 patients showed persistent moderate T cell activation at follow-up timepoints. A subgroup of severe COVID-19 patients exhibited low CD8$^+$ T cell counts at follow-up, coupled to an inflammatory immune signature and T cell exhaustion.

1 | INTRODUCTION

Acute coronavirus disease 2019 (COVID-19), caused by infection with severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is associated with a broad clinical spectrum ranging from asymptomatic infection to severe disease with development of acute respiratory distress syndrome (ARDS)[1–5]. Several studies have identified risk factors for the development of severe disease including advanced age, hypertension, diabetes mellitus, and obesity[6–9]. Furthermore, the development of severe disease has been associated with a dysregulated immune response against SARS-CoV-2[10], including an innate immune response with a persistent inflammatory phenotype and a dysregulated T-cell response[11,12]. The development of virus-specific T cells is a central part of antiviral immunity toward SARS-CoV-2, as virus-specific T cells rapidly eliminate infected cells through cell-mediated mechanisms and support B-cell-mediated production of virus-neutralizing antibodies[8,13,14]. It has been convincingly shown that severe COVID-19 is coupled to delayed virus-specific T-cell response, which in turn is associated with increased and prolonged viral shedding[15,16]. We and others have reported that severe COVID-19 is associated with extensive T-cell lymphopenia, especially in the CD8$^+$ T-cell compartment[9,10,12,17,18]. The T-cell lymphopenia is linked to extensive T-cell apoptosis, activation and exhaustion as well as impaired T-cell function[12]. However, it is unclear whether the T-cell perturbations observed in the acute phase persist, or whether the peripheral T-cell compartment recovers after acute infection. To investigate alterations of the immune system after acute COVID-19, we performed mass cytometry, flow cytometry, targeted proteomics, and functional assays at 6 and 12 months after SARS-CoV-2 infection in a cohort of 173 COVID-19 patients and 42 healthy controls.

2 | RESULTS

2.1 | Study cohort characteristics

We conducted a prospective, observational and cross-sectional study on reverse transcriptase quantitative polymerase chain reaction (rt-qPCR)-positive COVID-19 patients ($n = 173$) recruited at four different centers in the Canton of Zurich, Switzerland (Figure S1). The patients

were stratified into mild ($n = 109$) or severe ($n = 64$) disease based on maximal disease severity according to the world health organization (WHO) classification criteria[19]. After sampling in acute disease, patients were followed up at 6 ($n = 113$) and 12 months ($n = 90$) after infection. 42 individuals with a negative history of SARS-CoV-2 infection and negative serological testing were also included in the study as healthy controls (Figure 1A). Clinical history and routine laboratory parameters including flow cytometry were obtained (Table 1). Comprehensive inflammation marker proteomics based on proximity extension assay technology (Olink®), and in-depth T-cell phenotypic analyses by mass cytometry were performed (Figure 1A). As described[6–9], advanced age and several comorbidities, including hypertension, diabetes mellitus, and heart disease, were associated with severe disease (Table 1). As SARS-CoV-2 vaccines became available, participants were vaccinated with a single- or double-dose regimen, mostly with the mRNA-based COVID-19 vaccines BNT162b2 (Pfizer/BioNTech) or mRNA-1273 (Moderna). At the 12-month follow-up, 63.3% of study participants had been vaccinated (Table 1). Five patients in our control group subsequently were infected with SARS-CoV-2, which allowed for a comparison of their data before infection, during acute infection, and at follow-up.

2.2 | T-cell recovery at 6 months after infection

As previously described[12], we observed marked peripheral lymphopenia in acute severe COVID-19, involving $CD4^+$ and $CD8^+$ T cells as well as NK cells (Figure 1B). At 6 months after infection, $CD4^+$ T cell, $CD8^+$ T cell, and NK cell counts in the peripheral blood had returned to normal levels in the majority of the patients, and remained stable between 6 and 12 months (Figure 1B). Analysis of five participants initially included in the control group, who were subsequently infected with SARS-CoV-2 ($n = 3$ mild, $n = 2$ severe), confirmed the transient nature of the lymphopenia in severe disease, with normalization of T-cell and NK cell counts (Figure 1C). Of note, the two individuals that eventually developed severe disease had peripheral $CD8^+$ T-cell values in the low range prior to infection (Figure 1C).

In line with previous data[11,20–22], we observed a profound reduction in peripheral eosinophils and basophils in both mild and severe acute COVID-19, which normalized at 6 months after infection and remained stable thereafter (Figure 1D). An analogous pattern was observed in the subgroup of patients sampled before and after infection (Figure 1E).

As previously reported[12], we detected diminished functional responses against multiple viral antigens, including adenovirus, cytomegalovirus (CMV), herpes simplex virus (HSV) 1, HSV-2, and varicella zoster virus (VZV) during acute severe COVID-19 (Figure 1F). We previously hypothesized that this decreased T-cell reactivity was due to a reduction in precursor frequency associated with the observed lymphopenia[12]. Thus, in line with our observation of normalized T-cell counts 6 months after infection, we observed restored functional T-cell responses to most of the tested viral antigens at 6 months (Figure 1G). Taken together, these findings indicate a normalization of T-cell counts and functional responses to common viral antigens by 6 months after acute SARS-CoV-2 infection.

2.3 | Evidence of persistent T-cell activation following SARS-CoV-2 infection

Enhanced T-cell activation with increased surface expression of CD38 and HLA-DR has been shown in acute COVID-19[12,23–25].

Consistently, we also found a markedly increased frequency of $CD38^+$ $HLA\text{-}DR^+$ T cells during acute infection as compared to healthy individuals, which was more pronounced in $CD8^+$ compared to $CD4^+$ cells. (Figure 2A,B). Although frequencies of activated T cells decreased consistently at follow-up, both $CD38^+$ $HLA\text{-}DR^+$ $CD4^+$ and $CD8^+$ T cells remained elevated in COVID-19 patients compared to healthy controls, even at 12 months after infection (Figure 2A,B). This increase was observed for patients with mild and severe disease, with no apparent reduction between the 6-month and 12-month follow-up (Figures 2A,B and S2A,B).

When examining the patients for which sampling before disease onset was available, we also observed elevated frequencies of $CD38^+$ $HLA\text{-}DR^+$ $CD4^+$ T cells 6 months after severe COVID-19, whereas the patients with mild COVID-19 displayed a more diverse pattern (Figure 2C). In contrast, frequencies of $CD38^+$ $HLA\text{-}DR^+$ $CD8^+$ T cells returned to pre-infection levels at 6-month follow-up in this subgroup (Figure 2D).

We next wondered whether a prolonged increase in T-cell activation would be associated with a persistent increase of serum pro-inflammatory markers. Indeed, the amounts of C-reactive protein (CRP), interleukin-6 (IL-6), and tumor necrosis factor α (TNF-α) all showed a positive correlation with the frequency of activated $CD4^+$ and $CD8^+$ T cells at 6 and 12 months after infection (Figure 2E,F). However, this was not the case for interferon γ (IFN-γ), which did not correlate with the level of activated $CD4^+$ or $CD8^+$ T cells post-infection (Figure 2E,F). Collectively, our data showed evidence for low level, persisting T-cell activation, correlating with increased pro-inflammatory cytokine production up to 12 months after infection, affecting both mild and severe COVID-19 patients.

2.4 | Impact of COVID-19 vaccination on T-cell activation and inflammation markers

As SARS-CoV-2 vaccines became available during the follow-up phase of our study, some participants were vaccinated with a single- or double-dose regimen of the available mRNA-based COVID-19 vaccines. In our cohort, 10.6% and 63.3% had received an mRNA-based SARS-CoV-2 vaccine at the 6- and 12-month follow-up, respectively (Table 1). Since vaccination is accompanied by a transient inflammatory reaction[26], we explored the influence of vaccination on T-cell activation and pro-inflammatory cytokine levels. To this end, we grouped recovered COVID-19 patients by vaccination status into (i) unvaccinated at 6 months after infection, (ii) unvaccinated at 12 months after infection, (iii) vaccinated within 30 days of sample

collection, and (iv) vaccinated more than 30 days before sample collection. Interestingly, all groups showed higher T-cell activation than healthy controls (Figure 3A). In CD4$^+$ T cells, we observed no marked difference in T-cell activation between recently vaccinated participants and participants vaccinated more than 30 days prior to sampling (Figure 3A). Conversely, CD8$^+$ T cells showed a markedly increased activation in individuals sampled early after vaccination, which was attenuated at the later time points (Figure 3A).

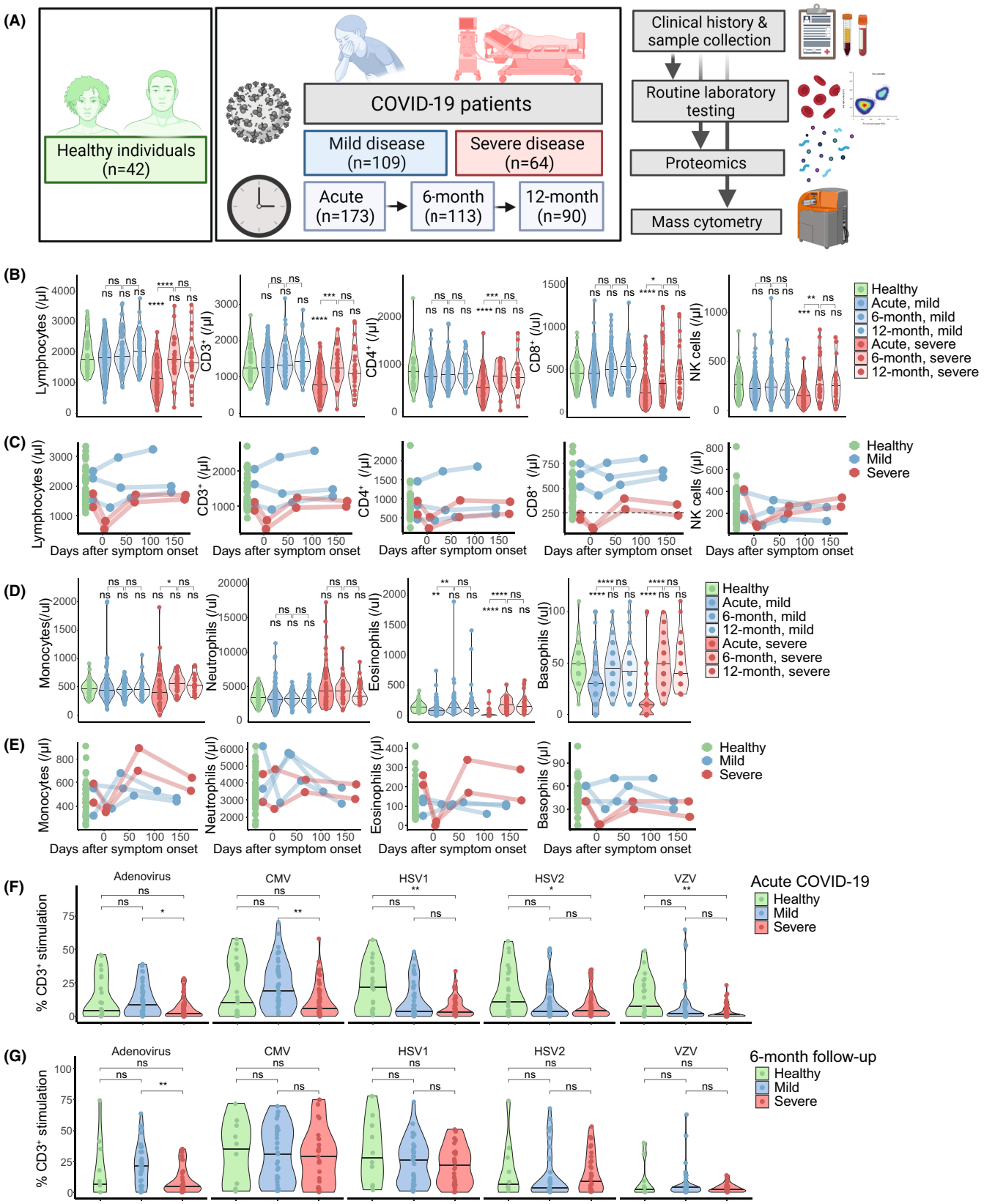


FIGURE 1 Quantitative and functional recovery of T-cell subsets after acute COVID-19. (A) Study overview. (B, C) Counts of peripheral lymphocyte subsets obtained by flow cytometry in healthy controls ($n = 41$), during acute COVID-19 ($n = 167$), at 6-month ($n = 111$) and at 12-month follow-up ($n = 90$) (B), or in individuals that were sampled prior and after SARS-CoV-2 infection (C; $n = 5$). (D, E) Peripheral leukocyte counts obtained by complete blood count, in the whole study cohort (healthy, $n = 37$; acute, $n = 153$; 6-months, $n = 111$; 12-month, $n = 90$) (D), or in patients with pre-infection samples (E; $n = 5$). (F, G) Functional T-cell responses as assessed by $CD3^+$ T-cell stimulation in FASCIA, after stimulation with the indicated viral antigen, during acute COVID-19 (F; healthy, $n = 25$, mild, $n = 46$; severe, $n = 58$) and at 6-month follow-up (G; healthy, $n = 10$; mild, $n = 33$; severe, $n = 30$). All *p*-values were obtained by Mann–Whitney *U*-tests and adjusted for multiple comparisons by the Holm method. *p*-values without brackets (B, D) indicate comparison to healthy controls. Horizontal bars in violin plots represent medians. *ns, non-significant; *, p < .05; **, p < .01; ***, p < .001; ****, p < .0001. CMV, cytomegalovirus; HSV, herpes simplex virus; VZV, varizella zoster virus*

Accordingly, starting 5 days after vaccination, we observed a decline of activated $CD8^+$ T cells, which was less apparent for activated $CD4^+$ T cells (Figure 3B). Regarding the effect of COVID-19 vaccination on inflammation markers, we did not observe consistent changes in vaccinated individuals, which was possibly due to the short duration and low level of systemic inflammation after COVID-19 vaccination (Figure 3C). However, a discrete negative association with time after vaccination was observed for TNF-α, but not for other inflammation markers (Figure 3D). Taken together, we found evidence of transient $CD8^+$ T-cell activation following mRNA-based vaccination in recovered COVID-19 patients, whereas $CD4^+$ T cells and systemic inflammatory markers remained largely unaffected.

2.5 | Persistently low $CD8^+$ T cells in subgroup of severe COVID-19 patients

T-cell lymphopenia is a well-described feature of severe acute COVID-19[9,10,12,17,18], but whether it is elicited by acute SARS-CoV-2 infection or rather a pre-existing risk factor for severe COVID-19 remains unclear. In the whole cohort, recovery of peripheral $CD4^+$ and $CD8^+$ T-cell counts was observed already at the 6-month follow-up (Figure 4A). However, we identified a subgroup of patients ($n = 10$) with severe COVID-19 that presented with $CD8^+$ T-cell counts <250/μl at 6-month follow-up, which was maintained at 12 months after infection (Figures 4A and S3A). This CD8-low subgroup exhibited markedly lower $CD8^+$ T-cell counts during acute infection compared to other patients with severe disease (Figure 4A). To investigate the characteristics of the CD8-low subgroup during acute COVID-19, we used a multivariate analysis comprising 130 parameters, including routine laboratory parameters, a comprehensive inflammation proteomics panel and demographic parameters (Tables 2 and S1). This analysis allowed for a separation of severe COVID-19 patients and healthy individuals, while mild COVID-19 patients showed an intermediate phenotype (Figure 4B). Patients in the CD8-low subgroup trended toward more pronounced perturbations, which reflected marked differences in numerous parameters when compared to severe COVID-19 patients with $CD8^+$ T-cell counts >250/μl (CD8-high) at 6 months after infection (Figures 4C and S3B). The patients in the CD8-low subgroup were almost exclusively male, in contrast to the more balanced sex distribution observed in the CD8-high subgroup or in patients with mild disease (Figure 4D). Furthermore, the patients in the CD8-low subgroup were older and presented with decreased peripheral NK cells and monocytes, as well as increased plasmablasts during acute COVID-19 (Figure 4E). This was accompanied by increased levels of several inflammation markers, including CRP, TNF-α, soluble IL-2Rα, and CXCL9 (Figure 4F). At 6 months after acute infection, we observed a normalization of inflammation markers and an increased monocyte count in the CD8-low subgroup (Figure 4G). Collectively, our data provide evidence of a subgroup of patients with low $CD8^+$ T-cell counts after recovery from severe COVID-19, encompassing elderly, predominantly male individuals exhibiting an accentuated pro-inflammatory immunological profile during acute infection. These features could possibly be linked to a pre-existing $CD8^+$ T-cell lymphopenia that predisposes to the development of severe disease.

2.6 | Persistently low $CD8^+$ T cells are associated with increased $CD8^+$ T-cell exhaustion

Next, we investigated whether the reduced $CD8^+$ T-cell counts in the CD8-low subgroup were accompanied by T-cell dysfunction during acute COVID-19. We took advantage of an extended CyTOF panel for T-cell phenotyping that was performed in a subgroup of included patients during acute disease and 6-month follow-up ($n = 36$ and $n = 46$, respectively). The CD8-low subgroup showed no differences during acute infection within the $CD4^+$ T-cell compartment in terms of cell proliferation, activation, exhaustion, and apoptosis, as compared to other COVID-19 patients (Figure 5A). On the contrary, we observed elevated frequencies of proliferating, activated and exhausted cells within the $CD8^+$ T-cell compartment of CD8-low patients during acute disease (Figure 5A). Correlation analyses with serum proteomics, flow cytometry data, and age in patients with mild or severe acute COVID-19 ($n = 42$) revealed an association of age and various pro-inflammatory markers with $CD8^+$ T-cell activation, exhaustion, and apoptosis (Figure 5B). Conversely, age and pro-inflammatory markers negatively correlated with naive $CD8^+$ T cells (Figure 5B). Furthermore, $CD3^+$ and $CD8^+$ T-cell counts correlated negatively with exhausted $CD8^+$ and $CD4^+$ T cells (Figure 5B).

The phenotypic changes of $CD8^+$ T cells observed in the CD8-low subgroup were no longer significant at the 6-month follow-up apart from persistently elevated levels of exhausted $CD8^+$ T cells (Figure 5C). Interestingly, we also detected increased frequencies of regulatory T cells and exhausted $CD4^+$ T cells in the CD8-low subgroup 6 months after acute infection (Figure 5C). In summary, we found evidence of a dysfunctional $CD8^+$ T-cell response in the

TABLE 1 COVID-19 study cohort characteristics

		Healthy controls	Acute COVID-19		6-month follow-up		12-month follow-up	
Disease severity		-	**Mild**	**Severe**	**Mild**	**Severe**	**Mild**	**Severe**
	n	42	109 (62.6%)	64 (36.7%)	76 (67.2%)	37 (32.7%)	64 (71.1%)	26 (28.9%)
Demographics	Age	32 (28-52)	34 (28-52) ns	67 (57-78) ****	36 (29-53) ns	64 (57-73) ****	35 (28-45) ns	65 (53-72)****
	Days PSO	-	10 (7-16)	13 (9-25)	194 (185-203)	210 (194-224)	375 (367-387)	386 (368-397)
	Sex (female)	24 (57.1%)	54 (49.5%)	27 (42.8%)	39 (51.3%)	14 (37.8%)	34 (53.1%)	8 (30.8%)
Covid vaccination	Total	0 (0%)	0 (0%)	0 (0%)	9 (11.8%)	3 (8.1%)	43 (67.1%)	14 (53.8%)
	One shot	0 (0%)	0 (0%)	0 (0%)	5 (55.5%)	0 (0%)	27 (62.8%)	7 (50.0%)
	Biontech/Pfizer	0 (0%)	0 (0%)	0 (0%)	6 (66.7%)	1 (33.3%)	13 (30.2%)	8(57.1%)
	Moderna	0 (0%)	0 (0%)	0 (0%)	3 (33.3%)	2 (66.7%)	28 (65.1%)	5 (35.7%)
	Unknown/other	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	2 (4.7%)	1 (7.1%)
Laboratory parameters	Lymphocytes (G/L)	1.8 (1.48-2.33)	1.81 (1.31-2.17) ns	0.71 (0.55-1.09) ****	1.95 (1.66-2.41) ns	1.76 (1.48-2.26) ns	2.3 (1.82-2.69) ns	1.67 (1.43-2.19) ns
	CRP (mg/L)	0.6 (0.4-1.6)	1.3 (0.1-4.9) **	58 (31-117) ****	0.6 (0.6-1.3) ns	2.2 (1.7-5.1) **	1 (0.6-2.22)**	1.6 (1-3.3)***
	TNF-α (ng/L)	8.1 (6.38-10.0)	9.8 (7.6-12.0) *	16.5 (13.8-20.6) ****	9.05 (6.88-10.90) ns	11.6 (9.3-14.6) ****	8.55 (6.92-10.8) ns	9.9 (8.7-13.6) **
	IL-6 (ng/L)	0.45 (0-1.12)	1.3 (0.1-4.9) **	20.3 (7.6-57.3) ****	0.9 (0-2.1) *	1.7 (0.2-5.4) **	1.25 (0.4-2.4)**	1.7 (0.2-3.1)**
	Anti-S1 IgA (OD ratio)	0.33 (0.25-0.46)	1.77 (0.7-4.8) ****	6.34 (2.4-10.0) ****	2.5 (1.6-5.0)) ****	5.1 (3.1-7.4) ****	8.4 (3.0-10.0)****	7.4 (4.2-8.4)****
	Anti-S1 IgG (OD ratio)	0.2 (0.17-0.25)	0.61 (0.3-2.2) ****	4.6 (0.3-9.3) ****	2.7 (1.3-5.7) ****	7.3 (5.2-8.6) ****	10.0 (4.0-10.0)****	9.2 (7.1-11.3)****
Comorbidities:	Hypertension	5 (11.9%)	13 (11.9%), ns	36 (57.8%), ***	7 (9.2%), ns	20 (54.1%), **	6 (9.4%), ns	14 (53.8%), **
	Diabetes	2 (4.7%)	6 (5.5%), ns	19 (29.7%), *	4 (5.3%), ns	12 (32.4%), **	4 (6.3%), ns	6 (23.1%), ns
	Heart disease	1 (2.4%)	6 (5.5%), ns	24 (37.5%), ***	2 (2.6%), ns	15 (40.5%), ***	2 (3.1%), ns	9 (34.6%), **
	Lung disease	6 (14.2%)	10 (9.2%), ns	12 (18.8%), *	5 (6.5%), ns	11 (29.7%), ns	5 (7.8%), ns	9 (34.6%), ns
	Malignancy	1 (2.4%)	3 (2.8%), ns	7 (10.9%), ***	3 (3.9%), ns	4 (10.8%), ns	0 (0%), ns	5 (19.2%), ns
	Kidney disease	0 (0%)	8 (7.3%), ns	16 (25.0%), **	3 (3.9%), ns	9 (24.3%), **	3 (4.7%), ns	8 (30.7%), ***

Note: For continuous variables, medians and interquartile ranges (in parentheses) are shown, with *p*-values obtained by Mann–Whitney *U*-test, compared to healthy individuals. For categorical variables, numbers of individuals and percentage of corresponding subgroup (in parentheses) are shown, with *p*-values calculated by Fisher's exact test compared to healthy individuals. ns, non-significant; *, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$. PSO, post symptom onset; OD, optical density; S1, SARS-CoV-2 spike subunit S1.

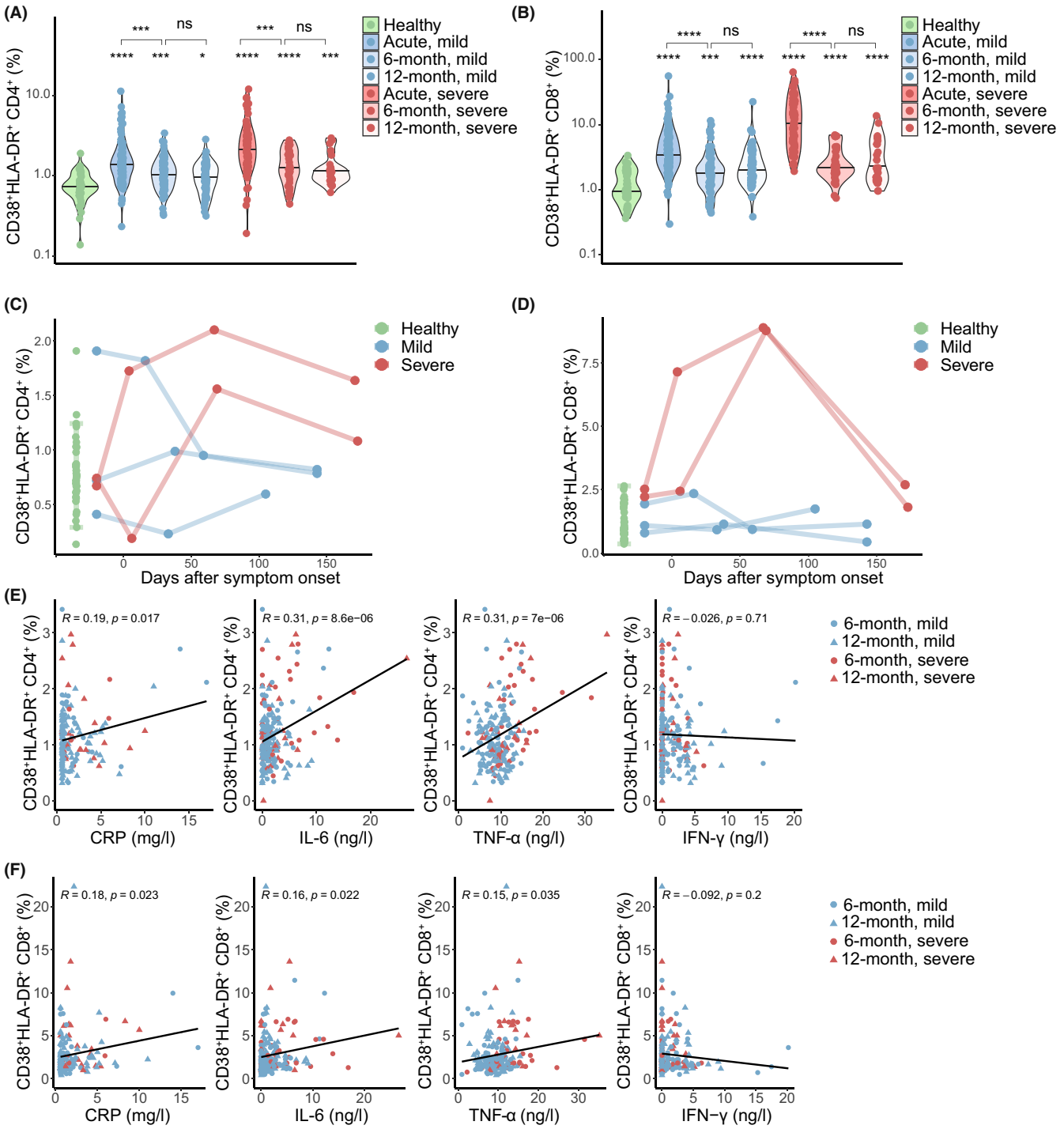


FIGURE 2 Persistent T-cell activation 12 months after acute COVID-19. (A–D) Frequency of activated ($CD38^+HLA\text{-}DR^+$) $CD4^+$ (A, C) and $CD8^+$ (B, D) T cells in the whole study cohort (healthy, $n = 41$; acute, $n = 167$; 6 months, $n = 111$; 12 months, $n = 90$) (A, B), or in individuals that were sampled prior to SARS-CoV-2 infection (C, D; $n = 5$). p-values in (A and B) were obtained by Mann–Whitney U-tests and adjusted for multiple comparisons by the Holmes method. p-values without brackets indicate comparison to healthy controls. Horizontal lines in violin plots indicate medians. (E, F) Correlation of activated ($CD38^+HLA\text{-}DR^+$) $CD4^+$ (E) and $CD8^+$ T (F) cells with inflammation markers, that is, CRP ($n = 151$), IL-6 ($n = 201$), TNF-α ($n = 201$), and IFN-γ ($n = 201$), at 6-month or 12-month follow-up. Regression lines represent simple linear regression models, with Pearson's correlation coefficient calculated for all observations

CD8-low subgroup during severe acute COVID-19. Furthermore, older age, low naive $CD8^+$ T cells and increased $CD8^+$ T-cell activation, exhaustion and apoptosis were linked to signs of extensively elevated systemic inflammation during acute COVID-19.

3 | DISCUSSION

Acute COVID-19 has been associated with peripheral T-cell lymphopenia, and the extent of lymphopenia strongly correlated with disease

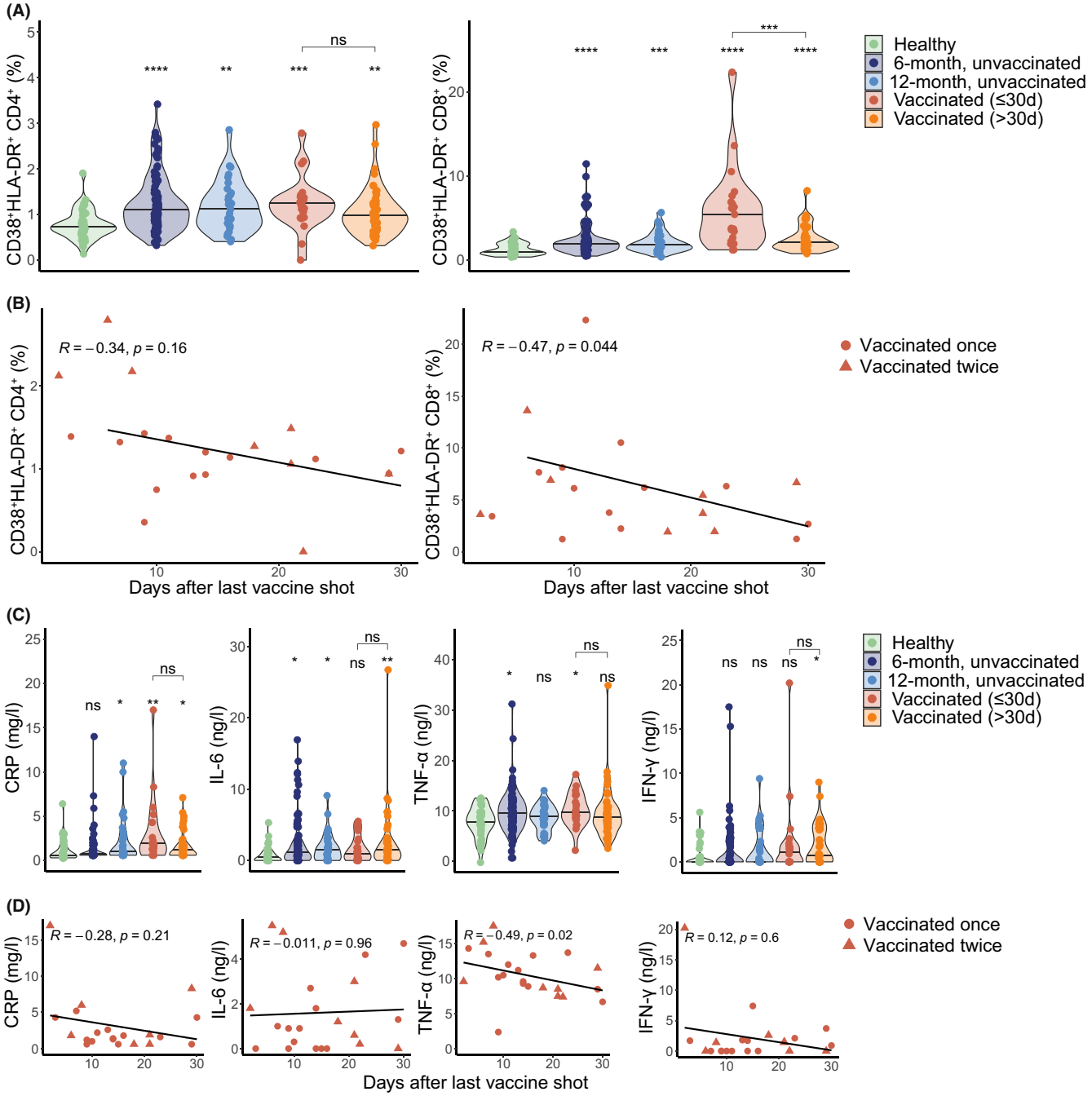


FIGURE 3 Immune activation following mRNA-based COVID-19 vaccination. (A) Frequency of activated ($CD38^+HLA\text{-}DR^+$) $CD4^+$ and $CD8^+$ T cells in healthy controls ($n = 41$) and followed up COVID-19 patients grouped according to their vaccination status, that is, unvaccinated (6 months, $n = 100$; 12 months, $n = 33$) or vaccinated (≤30d before sampling, $n = 21$; >30d before sampling, $n = 47$). (B) Temporal association of activated $CD4^+$ and $CD8^+$ T cells following vaccination, in followed up COVID-19 patients that were vaccinated within 30 days prior to sampling ($n = 21$). Regression lines represent simple linear regression models, starting from 5 days after the last vaccine shot, with Pearson's correlation coefficient calculated for $n = 19$ observations. (C) Inflammation markers, that is, CRP ($n = 188$), IL-6 ($n = 243$), TNF-α ($n = 243$), and IFN-γ ($n = 243$), in healthy controls or followed up COVID-19 patients grouped according to their vaccination status and sampling timepoint. (D) Temporal trajectories of inflammation markers following mRNA vaccination in followed up COVID-19 patients that were vaccinated within 30 days prior to sampling ($n = 21$). Regression lines represent simple linear regression models, with Pearson's correlation coefficient calculated for all observations. Horizontal bars in violin plots represent medians. *p*-values in (A and C) were calculated using Mann–Whitney *U*-tests, and adjusted for multiple comparison using the Holm method. *p*-values without brackets represent comparisons to healthy controls. *ns, non-significant; *, p < .05; **, p < .01; ***, p < .001; ****, p < .0001*

severity[9,10,12,17,18]. We and others have shown evidence of increased T-cell apoptosis, especially affecting $CD8^+$ T cells in severe acute COVID-19[12,17]. However, it has been unclear whether these immune disruptions persist after recovery from acute COVID-19. In the current study, we present the follow-up of a large COVID-19 cohort over a period of up to 12 months to further decipher the phenotypic

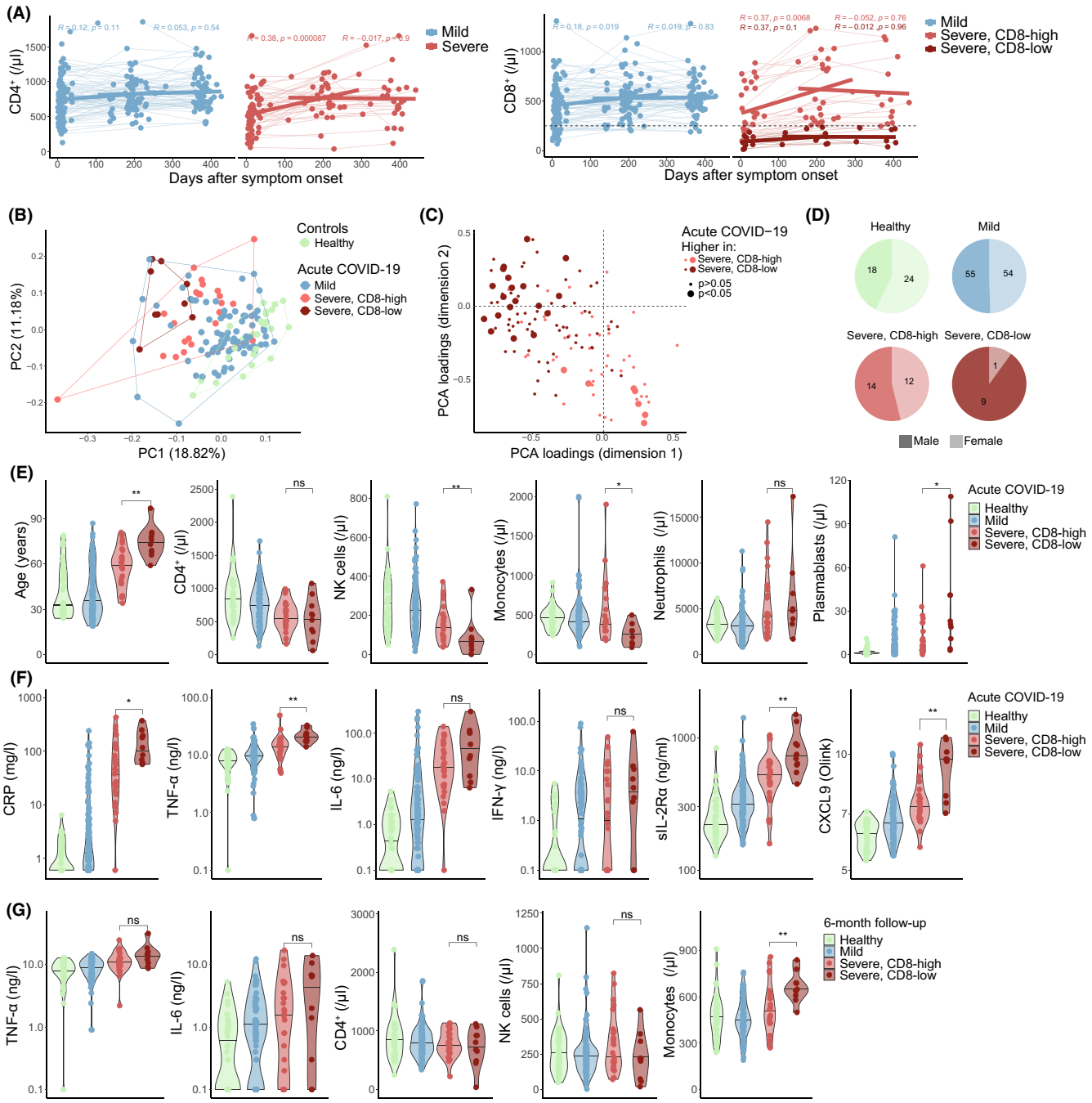


FIGURE 4 Persistent peripheral CD8$^+$ T-cell lymphopenia in a subgroup recovering from severe COVID-19. (A) Temporal trajectories of peripheral CD4$^+$ and CD8$^+$ T-cell counts in COVID-19 patients during acute infection ($n = 167$), and at 6-month ($n = 111$) and 12-month ($n = 90$) follow-up, separating patients with mild vs. severe COVID-19. Regression lines indicate separate simple linear regression models, with Pearson's correlation coefficient R. The CD8-low subgroup ($n = 10$) was defined as patients with severe disease that presented with CD8$^+$ counts below 250/µl at the 6-month follow-up. (B) Principal component analysis (PCA) including 131 parameters during acute COVID-19 (Table S1). Each dot represents an individual study participant, including healthy controls ($n = 27$) and acute COVID-19 patients ($n = 127$). (C) Loadings of PCA depicted in (B), with each parameter shown as an individual dot. Colors indicate group of participants with higher mean for each parameter. Dot sizes indicate p-values of the difference, as calculated by Mann–Whitney U-test (Table S1 and Figure S3B) (D) Sex distribution in healthy individuals ($n = 42$), and mild ($n = 109$) and severe ($n = 36$) COVID-19 patients, dividing severe COVID-19 patients into subgroups based on CD8$^+$ T-cell counts at 6-month follow-up. (E, F) Selected parameters, of PCA in (B, C), comparing severe acute COVID-19 patients of CD8-high and CD8-low subgroups. (G) Selected inflammation markers and peripheral leukocyte counts in healthy controls and COVID-19 patients at 6-month follow-up, comparing severe COVID-19 patients of CD8-high ($n = 26$) and CD8-low ($n = 10$) subgroups. p-values in (E–G) were calculated using Mann–Whitney U-test. Horizontal lines in violin plots indicate medians. *ns*, *non-significant*; *, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$

TABLE 2 Patient characteristics of CD8-high and CD8-low severe acute COVID-19 patients

Subgroup		Severe acute COVID-19		
		CD8-high	CD8-low	*p*-value
$CD8^+$ count at 6-month follow-up		>250/μl	<250/μl	
n		26 (72.2%)	10 (27.8%)	-
Demographics	Age	60 (50-65)	75 (68-80)	.0018
	Days PSO	16 (9-35)	13 (10-21)	.61
	Sex (female)	12 (46.2%)	1 (10.0%)	.06
Laboratory parameters	Lymphocytes (G/L)	0.9 (0.6-1.4)	0.4 (0.3-0.5)	.033
	CRP (mg/L)	41 (16-104)	101 (63-191)	.019
	TNFalpha (ng/L)	14.5 (10.8-17.2)	21.5 (17.8-23.7)	.0024
	IL-6 (ng/L)	17.9 (7.4-52.4)	44.6 (15.6-102)	.12
	Anti-S1 IgA (OD ratio)	5.7 (1.6-9.9)	9.2 (3.3-10.9)	.35
	Anti-S1 IgG (OD ratio)	2.6 (0.4-10.2)	5.1 (0.4-8.6)	.6
Comorbidities	Hypertension	12 (46.1%)	8 (80.0%)	.13
	Diabetes	9 (25.0%)	3 (30.0%)	1
	Heart disease	6 (23.1%)	8 (80.0%)	.005
	Lung disease	6 (23.1%)	3 (30.0%)	.69
	Malignancy	1 (3.8%)	0 (0%)	1
	Kidney disease	4 (15.3%)	4 (40.0%)	.18

Note: For continuous variables, medians and interquartile ranges (in parentheses) are shown, with *p*-values obtained by Mann–Whitney *U*-test. For categorical variables, numbers of individuals and percentage of corresponding subgroup (in parentheses) are shown, with *p*-values obtained by Fisher's exact test. OD, optical density; S1, SARS-CoV-2 spike subunit S1.

and functional alterations in T cells. By using high-dimensional mass cytometry, functional assays, and routine laboratory testing, we conclusively show (i) a functional and numeric recovery of peripheral leukocyte compartments 6 months after acute COVID-19, (ii) the persistence of moderate T-cell activation for up to 1 year after SARS-CoV-2 infection, and (iii) persistently low $CD8^+$ T-cell counts in a subgroup of patients at follow-up, coupled to excessive inflammation during acute COVID-19, suggesting that pre-existing $CD8^+$ T-cell lymphopenia could be a risk factor for severe COVID-19. These results argue against a persistent damage to the T-cell compartment and memory T-cell responses upon COVID-19 infection, in contrast to what has been shown for measles virus infection, where a broad depletion of memory cells is observed, leading to loss of previously acquired adaptive immunity[27,28] and increased susceptibility to subsequent infections[29]. In contrast, the correlation of T-cell lymphopenia with severe disease and the transient nature of the observed T-cell depletion are in line with previous studies on the related human coronaviruses severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome-related coronavirus (MERS-CoV)[30,31]. Although we observed a normalization of peripheral immune cell counts 6 months after acute infection, a slight but consistent increase in activated $CD4^+$ and $CD8^+$ T cells was evident in both patients with mild and severe COVID-19 at 6- and 12-month follow-up. T-cell activation was positively associated with persistently increased levels of several pro-inflammatory markers, including CRP, IL-6, and TNF-α. Since a substantial part of our patient cohort received an mRNA-based COVID-19 vaccine during the follow-up period, we investigated whether persisting T-cell activation was associated with vaccination. Our assay was sensitive enough to detect transient activation of peripheral $CD8^+$ T cells following vaccination. However, the immune-stimulating effect of vaccination could not explain the immune activation observed in recovered COVID-19 patients, as immune activation was similar in vaccinated and non-vaccinated individuals. Thus, the cause of the increased immune activation observed in our study remains unclear, but it could be associated with residual tissue damage or persisting SARS-CoV-2 antigen, resulting in ongoing T- and B-cell immune responses[32,33]. Ongoing immune activation detected up to 12 months after mild and severe COVID-19 could potentially be related to long-term post-viral symptoms[34,35], termed post-acute COVID-19 syndrome or long-COVID[36,37], our study did, however, not investigate this aspect. Moreover, potential demographic confounders, such as age and general health status, could account for differences between patient groups and healthy controls. Further studies are needed to determine whether there is a correlation between persistently increased T-cell activation and the extent and duration of long-COVID symptoms.

It has been speculated that pre-existing deficiencies in the T-cell compartment, including reduced naive T-cell counts or increased low-avidity $CD4^+$ T cells, are associated with an increased risk of severe COVID-19[38]. Furthermore, previous studies have shown a clear association between severe disease and delayed SARS-CoV-2 specific T-cell responses[15,39], which could allow prolonged viral replication and dissemination. Coupled to large amounts of viral antigen[40], this delayed response could result in excessive

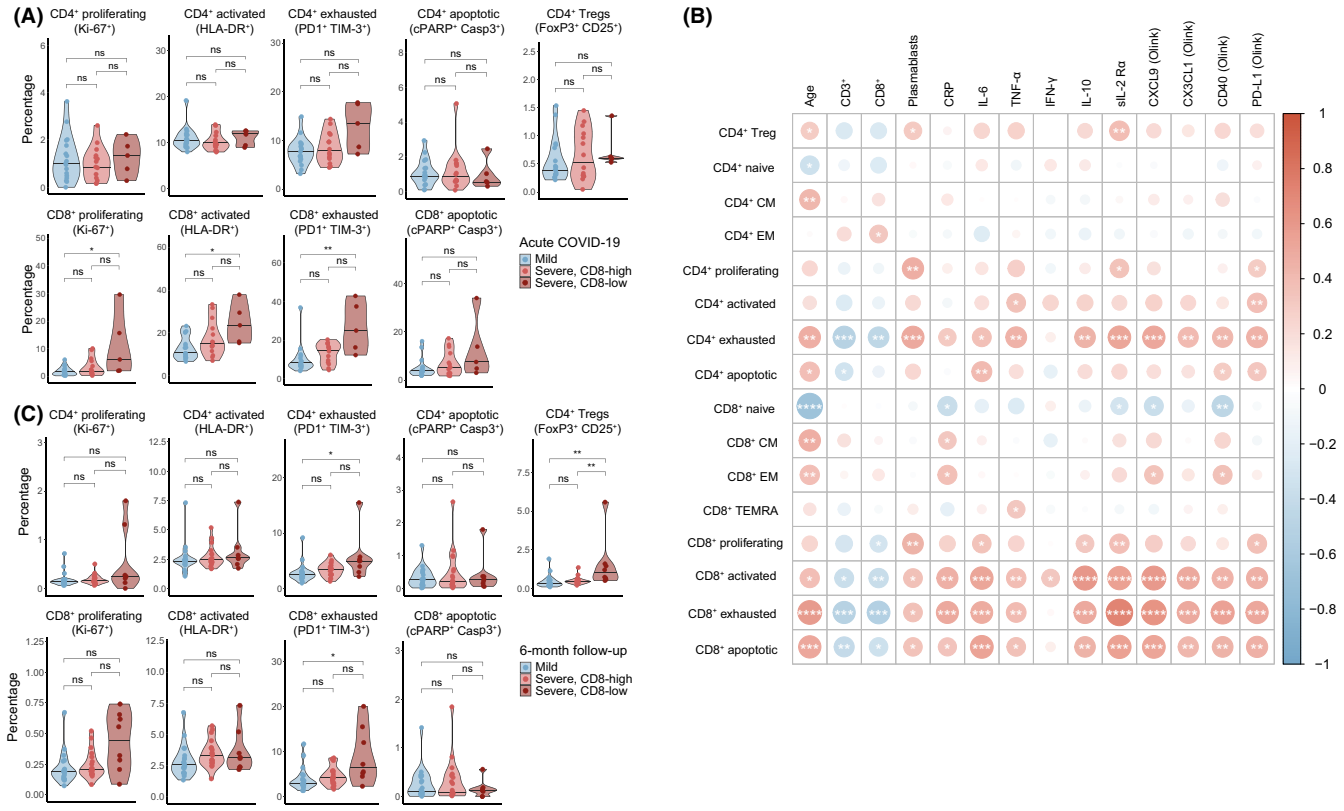


FIGURE 5 Phenotypic perturbations of CD8$^+$ T cells in the CD8-low subgroup of severe COVID-19 patients. (A) Frequencies of CD4$^+$ regulatory cells as well as proliferating, activated, exhausted and apoptotic CD4$^+$ and CD8$^+$ T cells, as obtained by mass cytometry, comparing mild ($n = 17$) to severe CD8-high ($n = 14$) and severe CD8-low ($n = 5$) acute COVID-19 patients. (B) Correlation matrix of T-cell phenotypes (vertical axis) with age, routine laboratory testing and serum proteomics (horizontal axis), including data from 15 mild and 27 severe acute COVID-19 patients. Dot sizes and colors correspond to Spearman's correlation coefficient, with significance indicated by asterisks. (C) Frequencies of CD4$^+$ regulatory cells as well as proliferating, activated, exhausted and apoptotic CD4$^+$ and CD8$^+$ T cells, as obtained by mass cytometry, comparing mild ($n = 19$) to severe CD8-high ($n = 19$) and severe CD8-low ($n = 8$) COVID-19 patients at 6 months follow-up. *p*-values in (A and C) were obtained using Mann–Whitney *U*-tests and adjusted for multiple comparisons using the Holm method. Horizontal lines in violin plots indicate medians. *ns*, *non-significant*; *, $p < .05$; **, $p < .01$; ***, $p < .001$; ****, $p < .0001$. *reg*, *regulatory*; *CM*, *central memory*; *EM*, *effector memory*; *TEMRA*, *terminally differentiated EM*

activation of the innate immune system, leading to systemic inflammation, ARDS, and organ failure[15]. In our cohort, a subgroup of severe COVID-19 patients had persistently low CD8$^+$ T-cell counts up to 12 months after acute infection. If the reduction pre-dated the SARS-CoV-2 infection, a proportionally reduced CD8$^+$ naive T-cell repertoire could partially account for a delay in building an efficient virus-specific T-cell response. Persistent CD8$^+$ T-cell lymphopenia was strongly associated with male sex, advanced age, increased inflammation, and CD8$^+$ T-cell exhaustion during acute COVID-19. Increased levels of exhausted CD8$^+$ T cells have been previously associated with an aging immune system[41]. We only included very few patients with samples available prior to SARS-CoV-2 infection, precluding definite conclusions. However, the two patients that subsequently developed severe disease in this subgroup both had peripheral CD8$^+$ T-cell counts in the low range before COVID-19. Thus, our data suggest that, at least in a subgroup of patients, a pre-existing deficiency in CD8$^+$ T-cell immunity could be associated with the development of an inflammatory phenotype and with severe COVID-19. Alternatively, it is conceivable that the apoptosis observed in severe COVID-19 could lead to persistently low CD8$^+$ T-cell counts in the peripheral blood of a subgroup of patients, although we did not observe an increased frequency of apoptotic cells in this specific subgroup. Further studies are needed to investigate how decreased CD8$^+$ T-cell counts are related to the breadth of the T-cell receptor repertoire in CD8-low individuals, and how this affects the development and quality of SARS-CoV-2-specific T- and B-cell immunity.

Our study has several limitations related to the observational study design, loss to follow-up, as well as the heterogeneity of the study population, including COVID-19 disease course, drug treatment, and comorbidities. In summary, our study presents novel insights into the dynamics of T-cell perturbations following SARS-CoV-2 infection, including evidence of T-cell recovery at 6 months after infection in the majority of patients, persisting immune activation as well as the identification of a CD8 low subgroup with a distinct severe disease phenotype.

4 | METHODS

4.1 | Cohort recruitment

Following written informed consent, adult individuals were recruited for blood sampling between April 2020 and May 2021. All experiments conducted in this study were approved by the authorities of the Canton of Zurich, Switzerland (BASEC #2016-01440). 173 patients with RT-qPCR-confirmed SARS-CoV-2 infection were recruited during acute COVID-19 at four different hospitals in the Canton of Zurich, that is, University hospital Zurich (n = 110), City Hospital Triemli (n = 34), Limmattal Hospital (n = 15), and Uster Hospital (n = 14) between March 2020 and March 2021. Follow-up visits were conducted at 6 months (n = 113) and 12 months (n = 90) after recovery (Figure S1). Clinical history was obtained, and blood samples were collected at each sampling time point. Maximum disease severity was classified according to the world health organization (WHO) criteria into mild COVID-19, including asymptomatic (n = 4), mild illness (n = 93) and mild pneumonia (n = 12), and severe COVID-19, including severe pneumonia (n = 29) and ARDS (n = 35) (Table 1). According to the $CD8^+$ T-cell count, severe COVID-19 patients were further divided at 6-month follow-up into a CD8-low (<250/ul, n = 10) and a CD8-high (>250/ul, n = 26) subgroup (Table 2). 42 participants with negative serology and history for SARS-CoV-2 were included as healthy controls. Five healthy controls were infected with SARS-CoV-2 after recruitment and subsequently included in the COVID-19 patient cohort.

4.2 | Immunoassays

Serum was collected with BD Vacutainer CAT serum tubes (Becton Dickinson). Immunoglobulin subsets, semi-quantitative anti-SARS-CoV-2 Spike S1 IgG and IgA and cytokines, including interleukin (IL) 1β, Il-2, IL-5, IL-6, IL-8, IL-10, IL-12, interferon (IFN) γ, tumor necrosis factor (TNF) α, and soluble IL-2 receptor (sIL-2 R) α were measured in accredited laboratories at the University Hospital Zurich, as previously described[8,12].

4.3 | Flow cytometry

Quantification of the main lymphocyte subsets was obtained by accredited laboratories at University Hospital Zurich, as previously established[12]. A more comprehensive description of reagents and methodology is available in Table S2.

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

Clinically validated functional T-cell response assays were conducted as described previously[12,42]. Briefly, whole blood cells were stimulated with mitogens/superantigens (pokeweed mitogen, Concanavalin A, Staphylococcus enterotoxin A), or viral antigens (adenovirus, VZV, HSV-1, HSV-2, or CMV) for 7 days. As a read-out, the difference of $CD3^+$ blast frequency compared to unstimulated samples was assessed by flow cytometry.

4.5 | Serum proteomics

Serum samples were analyzed by commercially available proximity extension assay-based technology (Olink® Proteomics) in a 92-marker inflammation panel, as previously described[11]. All reported samples passed the quality control, and six markers were excluded because more than half of samples did not exceed the detection limit.

4.6 | Mass cytometry

40-parametric mass cytometric analysis was performed using the same antibody panel (Table S3) and methodology as previously described for this cohort[12]. A comprehensive description of the computational pipeline used for data pre-processing is available in Crowell et al [43].

4.7 | Statistics

All statistical analyses were performed using R (version 4.1.0) and RStudio (1.4.1717). Unless specified differently, between group comparison was performed using unpaired, non-parametric testing (Mann–Whitney U). As indicated, p-values were adjusted for multiple comparisons using the Holm method for tests shown in the same plot. Principal component analyses (PCA) were performed with scaled, centered variables, and variable coordinates were used to illustrate loadings. Correlations of numeric variables are shown as simple linear regression models and quantified with Spearman's or Pearson's rank correlation as annotated. For statistical analysis and illustration, various packages were used, including *stats (4.2.0)*, *factoextra (1.0.7)*, *ggplot2 (3.3.5)*, *ggfortify (0.4.12)*, and *corrplot (0.90)*.

AUTHOR CONTRIBUTIONS

PT and SA contributed to study design, patient recruitment, data collection, data analysis, and data interpretation. YD contributed to data analysis and interpretation. CC and YZ contributed to study design, patient recruitment, and data collection. SC and BB contributed to the CyTOF data collection and analysis. MER, SH, EB, AR, MS-H, and LCH contributed to patient recruitment and clinical management. JN wrote the manuscript, with contributions from PT, SA, and OB. 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.

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CONFLICT OF INTEREST

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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