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Hyper-reactivity of CD8$^+$ T cells and high expression of IL-3 correlates with occurrence and severity of Long-COVID

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ABSTRACT

Following SARS-CoV-2 infection, some individuals develop Long-COVID-syndrome lasting for more than 3 months. We analyzed blood samples from patients with Long-COVID, controls without persistent symptoms following SARS-CoV-2-infection and non-infected donors without a history of infection. Long-COVID patients showed clear signs of T cell hyper-activation predominantly in the CD8$^+$ T cell subset with a 4-fold higher expression of CD25 and 2-fold more effector-memory T cells. Following polyclonal T cell stimulation, we found a 2-fold stronger upregulation of CD25 and a 7-fold higher release of IL-3 in Long-COVID. Intracellular staining revealed 5-fold more IL-3-expressing CD8$^+$ T cells in Long-COVID, while GM-CSF, IFN-γ and IL-2 were much less upregulated. These changes correlated with the severity of Long-COVID and persisted for up to 18 months after infection. Our data reveal a pronounced and long-lasting CD8$^+$ T cell hyper-activation and hyper-reactivity in Long-COVID and speak for a trial of T cell-immunosuppression in patients with Long-COVID.

1. Introduction

Soon after emergence of the COVID-19 epidemic, it became clear that a considerable proportion of COVID-19 patients suffers from a variety of long-term sequelae of the SARS-CoV-2 infection. More than 200 different symptoms were described including fatigue, muscle weakness, dyspnea and neuro-psychological symptoms [1–4]. According to the WHO, Post-COVID-19 syndrome or Long-COVID is defined as the continuation or development of new symptoms 3 months after the initial SARS-CoV-2 infection, with these symptoms lasting for at least 2 months with no other explanation. The severity of Long-COVID is graded according to a Long-COVID severity score described in the methods section [5].

While some meta-analyses reported a quite high incidence of individuals developing Long-COVID symptoms after infection [2,3,6,7], more controlled studies revealed a frequency of Long-COVID in the range of 5 % of infected adults [8,9]. The initial reports of higher incidences may have also been due to a broader definition of Long-COVID syndrome [10]. More recent virus variants or vaccination may be associated with a lower risk for Long-COVID [11–16]. Risk factors for development of Long-COVID are female sex, age, comorbidities, the severity of acute disease, and obesity [7,17–19]. While Long-COVID is considered as disease entity with large clinical and socioeconomic impact, the pathophysiology of Long-COVID is poorly understood and no targeted treatments are available. Several potential disease mechanisms were described in patients with Long-COVID including autonomic nervous system damage, complement dysregulation, autoimmunity, endothelial dysfunction, occult viral persistence and

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thromboinflammation [20–22]. Long-COVID shows some similarity with the rare chronic fatigue syndrome (ME/CFS) associated with infections [23,24] and autoimmunity and with the fatigue frequently seen in patients with Sjogren's syndrome and rheumatoid arthritis [25], suggesting that altered immune reactions could also contribute to development of Long-COVID.

We have previously described pronounced alterations of immune cell function and phenotype in patients with acute SARS-CoV-2 infection, which was associated with a prominent hypo-reactivity of T cells during acute severe SARS-CoV-2 infections. As immunophenotyping studies with a focus on T cells in clinically well-characterized Long-COVID patients are limited, we wondered whether the immunological changes during acute infection would persist in patients with Long-COVID, or whether Long-COVID would be associated with different immunological changes [26].

To test this hypothesis, we performed a detailed immunophenotyping analysis with focus on T cells and subsets of memory T cells in 68 patients with Long-COVID, which were diagnosed according to WHO definition of Long-COVID. The control group consisted of 23 subjects (non-L-COV) who had a documented COVID-19 infection but did not develop any Long-COVID symptoms, and 69 non-infected subjects who never had a COVID-19 infection (non-infected). All Long-COVID patients were subject to a detailed clinical characterization including clinical symptoms, clinical history, lung function, 6-min walk, echocardiography and psychosomatic evaluation. So far, few immunophenotyping studies with a focus on T cells were performed in clinically well

Table 1
Characteristics of Long-COVID patients.

	Non-infected	Non-Long-COVID	Long-COVID all	Long-COVID Score 1	Long-COVID Score 2	Long-COVID Score 3
Number of patients (n)	69	23	68	11	38	19
Demographics:						
Mean age	26.9	27.5	50.6	51.2	48.6	54.5
(Years. min-max)	(21–82)	(23–45)	(24–72)	(31–61)	(24–64)	(37–72)
Sex (% female)	47 (68 %)	10 (43.5 %)	45 (66 %)	8 (72.7 %)	28 (73.7 %)	9 (47.4 %)
Initial COVID-19 infection:						
Time after infection (months)	-	4.7	13.1	14.8	12.0	14.8
Hospitalization n (%)	–	0	11 (16.2 %)	0 (0 %)	6 (15.8 %)	5 (26.3 %)
ICU n (%)	–	0	5 (7.4 %)	0 (0 %)	2 (5.3 %)	3 (15.8 %)
Ventilation n (%)	–	0	4 (5.9 %)	0 (0 %)	1 (2.6 %)	3 (15.8 %)
Long-COVID symptoms n (%):						
Fatigue	–	0	56 (82.4 %)	5 (45.5 %)	36 (94.7 %)	15 (78.9 %)
Stress dyspnoe	–	0	55 (80.9 %)	8 (72.7 %)	32 (84.2 %)	15 (78.9 %)
Cough	–	0	6 (8.8 %)	0 (0 %)	4 (10.5 %)	2 (10.5 %)
Chest Pain /Tightness	–	0	18 (26.5 %)	2 (18.2 %)	10 (26.3 %)	6 (31.6 %)
Taste	–	0	16 (23.5 %)	1 (9.1 %)	10 (26.3 %)	5 (26.3 %)
Smelling	–	0	16 (23.5 %)	0 (0 %)	11 (28.9 %)	5 (26.3 %)
Poor concentration	–	0	56 (82.4 %)	8 (72.7 %)	32 (84.2 %)	16 (84.2 %)
Affected Sleep	–	0	37 (54.4 %)	3 (27.3 %)	22 (57.9 %)	12 (63 %)
Joint / Muscles pain	–	0	32 (47.1 %)	4 (36.4 %)	19 (50 %)	9 (47.4 %)
Palpitations	–	0	17 (25 %)	1 (9.1 %)	12 (31.6 %)	4 (21.1 %)
Dizziness	–	0	13 (19.1 %)	2 (18.2 %)	7 (18.4 %)	4 (21.1 %)
Headache/Migraine	–	0	15 (22.1 %)	1 (9.1 %)	8 (21.1 %)	6 (31.6 %)
Depression	–	0	28 (41.2 %)	0 (0 %)	18 (47.4 %)	10 (52.6 %)
Comorbidities n (%):						
Smoking	3 (4.3 %)	3 (13.0 %%)	8 (11.8 %)	1 (9.1 %)	2 (5.3 %)	5 (26.3 %)
Cardiovascular disease	0	0	2 (2.9 %)	0 (0 %)	0 (0 %)	2 (10.5 %)
Lung disease	0	0	13 (19.1 %)	3 (27.3 %)	5 (13.2 %)	5 (26.3 %)
Immunosuppression	0	0		0 (0 %)	1 (2.6 %)	2 (10.5 %)
Lung function n (%):						
Restriction	–	–	3 (4.4 %)	0 (0 %)	0 (0 %)	3 (15.8 %)
Impaired Diffusion	–	–	7 (10.3 %)	0 (0 %)	3 (7.9 %)	4 (21.1 %)
FEV1(%)	–	–	92.3	92.9	95.6	85.6
TLCO (%)	–	–	89.2	90.2	91.5	84.3
Echocardiography n (%):						
Impaired LVEF	–	–	3 (4.4 %)	0 (0 %)	1 (2.6 %)	2 (10.5 %)
6-min walking test n (%):						
Distance (% debit)	–	–	87.6 %	90.7 %	91.9 %	77.5 %
Borg-CR10-Scale	–	–	4.5	4.4	4.2	5.3

Statistical differences between Long-COVID (all) and Healthy or Controls were calculated using 2-sided students Test. Statistical differences between Long-COVID Score 1 and Score 2 or Score 3 were calculated using 2-sided students Test.
[a] $p < 0.001$. [b] $p < 0.001$. [c] $p < 0.001$. [d] $p = 0.035$. [e] $p < 0.001$. [f] $p = 0.032$. [g] $p = 0.022$. [h] $p = 0.033$. [i] $p = 0.038$. [j] $p = 0.031$. [k] $p = 0.002$. [l] $p = 0.001$.

characterized Long-COVID patients, as further described in the discussion. We found a clear shift of T cell subsets to a memory and activated phenotype in patients with Long-COVID independent of the age of the Long-COVID-patients. These changes were most pronounced for $CD8^+$ T cells and less pronounced in $CD4^+$ T cells. $CD8^+$ T cells from Long-COVID patients showed a marked hyper-reactivity following polyclonal activation with an enhanced upregulation of CD25 and a higher production of cytokines especially IL-3 known to be associated with autoimmunity and fatigue [27–34]. These phenotypic changes persisted for up to 18 months after initial SARS-CoV-2 infection and suggest that T cells contribute to development of Long-COVID.

2. Results

We performed immunophenotyping of fresh whole blood from patients with Long-COVID (L-COV), controls without Long-COVID (Non-L-COV), and non-infected donors (Non-infected). Long-COVID patients were diagnosed according to established criteria for Long-COVID as

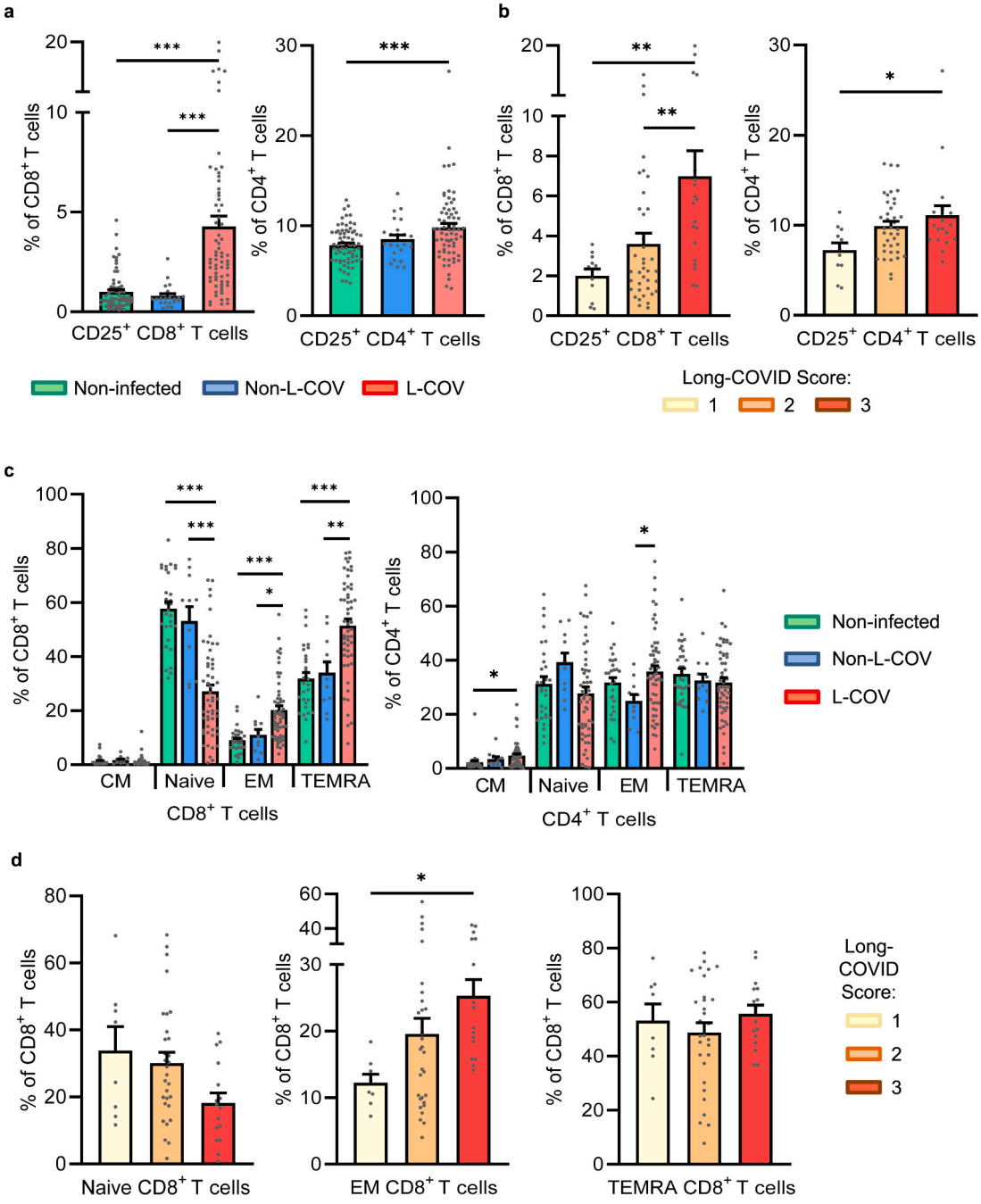

Fig. 1. T cell phenotype of patients with Long-COVID compared to non-Long-COVID controls and non-infected controls.
a, b Percentage of CD25 expressing $CD8^+$ or $CD4^+$ T cells in fresh blood of 69 non-infected controls (Non-infected), 23 non-Long-COVID controls (Non-L-COV) and 68 Long-COVID patients (L-COV). Long-COVID patients were stratified according the severity of symptoms into Long-COVID-Score 1 ($n = 11$), Score 2 ($n = 38$) and Score 3 ($n = 19$). Each sample is represented by one dot and the mean is shown as bar. c, d Percentage of central-memory (CM), naïve (Naïve), effector-memory (EM) and terminal-effector-memory (TEMRA) $CD8^+$ or $CD4^+$ T cells in fresh blood of 30 non-infected controls, 11 non-Long-COVID controls and 54 Long-COVID patients. Long-COVID patients were stratified according the severity of symptoms into Long-COVID-Score 1 ($n = 8$), Score 2 ($n = 30$) and Score 3 ($n = 16$). Each sample is represented by one dot and the mean is shown as bar. One-way ANOVA with Bonferroni's multiple comparison test was used.

defined in the methods section. Controls had a previous SARS-CoV-2 infection without any persistent symptoms, while non-infected controls had no history of SARS-CoV-2 infection.

Characteristics of Long-COVID patients are shown in Table 1 and reveal typical clinical symptoms like fatigue, stress dyspnea, chest pain, poor concentration, impaired sleep and depression. Patients were stratified according to the severity of their symptoms using a Long-COVID-Score ranging from 1 to 3. A score of 3 was associated with more severe initial SARS-CoV-2 infection as seen by a higher frequency of hospitalization and ventilation and with more comorbidities (Table 1), as already described in the literature [2,35].

Only in a small fraction of the patients, objective investigational findings became evident with impaired lung function consisting of restriction and impaired diffusing capacity as well as reduced left-ventricular ejection fraction and impaired performance in the 6-min walk. These changes were more prominent in patients with a Long-COVID-Score of 3.

2.1. $CD8^+$ T cells are more activated and show a memory phenotype in patients with Long-COVID

Total numbers of $CD4^+$ and $CD8^+$ T cells were almost identical in

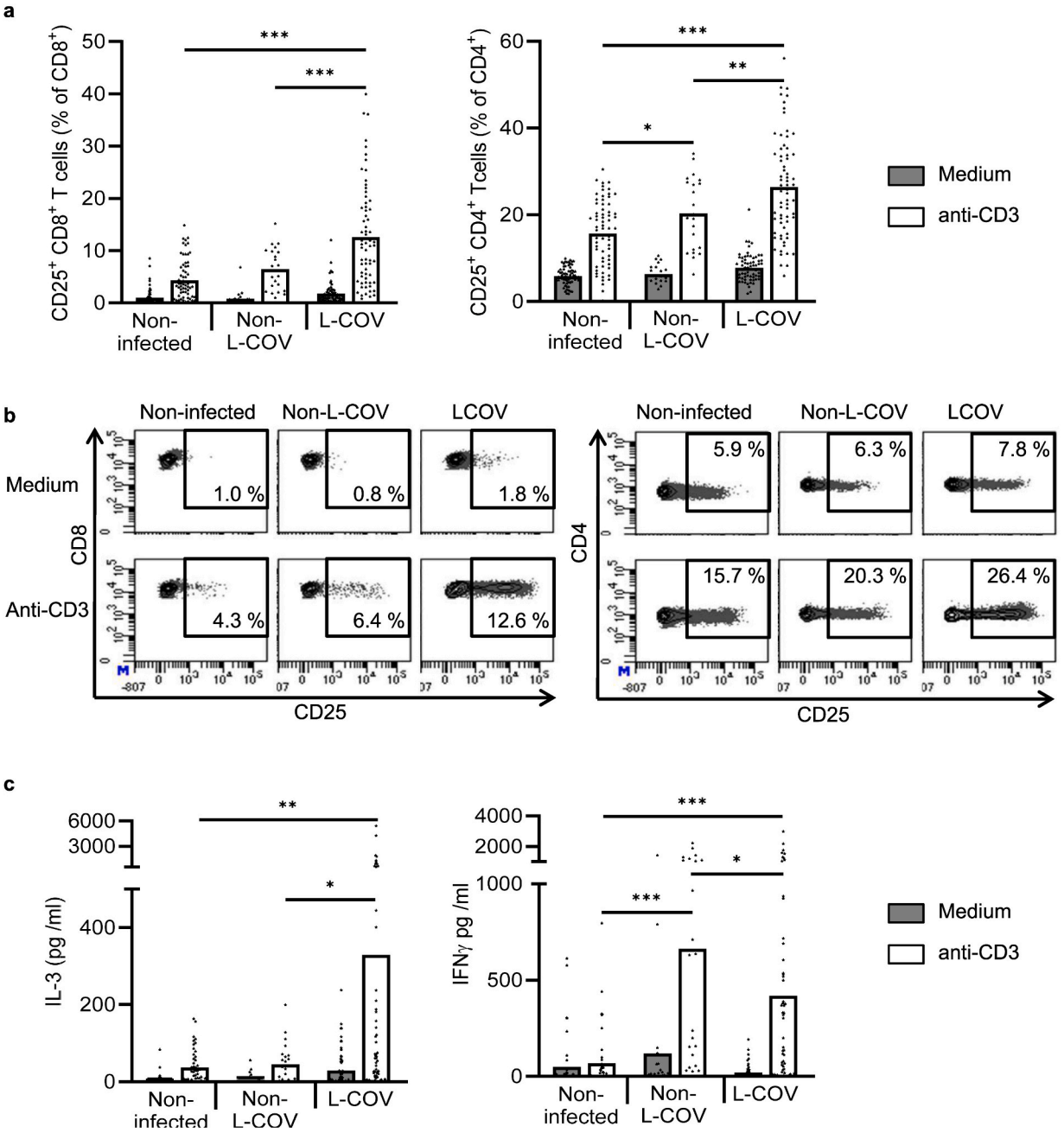

Fig. 2. T cell reactivity of Long-COVID patients compared to non-Long-COVID controls and non-infected controls.
a-c Whole-blood samples from 69 non-infected controls (Non-infected), 23 non-Long-COVID controls (Non-L-COV) and 68 patients with Long-COVID (L-COV) were cultured with or without immobilized anti-CD3 antibodies for 24 h. a, b Expression of CD25 was quantified by flow cytometry on $CD8^+$ and $CD4^+$ T cells. Each sample is represented by one dot, and the mean is shown as bar. Representative dot plots are shown. c Concentrations of IL-3 and IFN-γ were measured in the culture supernatant by ELISA from 48 non-infected controls, 23 non-Long-COVID controls and 68 patients with Long-COVID. Each sample is represented by one dot, and the mean is shown as bar. One-way ANOVA with Bonferroni's multiple comparison test was used.

non-infected controls and Long-COVID patients (Suppl. Fig. 1a). Controls showed somewhat higher numbers of T cells most likely due to the shorter interval between SARS-CoV-2 infection and blood analysis. Immunophenotyping revealed a highly significant and 7-fold higher frequency of activated $CD8^+$ T cells in patients with Long-COVID as shown by surface expression of CD25 (Fig. 1a). The percentage of activated $CD8^+$ T cells also strongly correlated with the Long-COVID-Score (Fig. 1b). Among $CD4^+$ T cells, we found only a minor expansion of $CD25^+$ cells and no clear correlation with the Long-COVID-Score (Fig. 1a, b). However, one has to consider that in $CD4^+$ T cells expression of CD25 is not specific for activated cells, but also present on regulatory cells [36]. In Long-COVID-patients we did not detect significant age-dependent differences in the frequency of $CD8^+$ or $CD4^+$ T cells expressing CD25 (Suppl. Fig. 1b), while in uninfected controls older individuals showed a somewhat higher frequency of CD25-expressing T cells in accordance with the literature [37].

Subgrouping of T cells into naïve, central-memory, effector-memory, and terminal-effector cells according to their expression of CCR7 and CD45RA revealed an about 2-fold increased fraction of effector-memory and terminal-effector $CD8^+$ T cells and a decreased fraction of naïve $CD8^+$ T cells in Long-COVID patients (Fig. 1c). The expansion of effector-memory $CD8^+$ T cells was most pronounced in patients with a Long-COVID-Score of 3 (Fig. 1d). No significant age-dependent differences were detectable in the fraction of naïve, effector-memory and terminal-effector $CD8^+$ T cells in Long-COVID-patients or controls (Suppl. Fig. 1c). For $CD4^+$ T cells these changes were absent or less clear (Fig. 1c and Suppl. Fig. 1d).

2.2. *T cells from patients with Long-COVID are more susceptible to activation and release larger amounts of cytokines*

Fresh whole blood was incubated with immobilized anti-CD3 antibodies for 24 h to induce polyclonal activation of T cells. Upregulation of CD25 was detected by flow cytometry and the percentage of $CD25^+$ T cells was analyzed. $CD8^+$ T cells from patients with Long-COVID showed a more pronounced upregulation of CD25 after polyclonal stimulation. CD25 expression was induced on 12,6 % of the $CD8^+$ T cells from Long-COVID while only 4.0 % and 6.4 % of the $CD8^+$ T cells from non-infected controls and non-Long-COVID controls upregulated CD25 (Fig. 2a, b). Similar but less pronounced changes were seen for $CD4^+$ T cells (Fig. 2a, b). No significant age-dependent differences were detectable in the upregulation of CD25 after polyclonal activation of $CD8^+$ or $CD4^+$ T cells in Long-COVID-patients or controls (Suppl. Fig. 2a).

We also quantified the release of T cell derived cytokines in the supernatant of whole blood stimulated with immobilized anti-CD3 antibodies, choosing cytokines that were previously found to be altered in patients with acute COVID-19 infection [26]. In contrast to non-infected controls and non-Long-COVID controls, we detected high amounts of IL-3 in the supernatant of whole blood from Long-COVID patients (Fig. 2c). The release of IFN-γ was increased in both, non-Long-COVID controls and Long-COVID patients, indicating that an increased release of IFN-γ is more a consequence of a recent SARS-CoV-2 infection and not

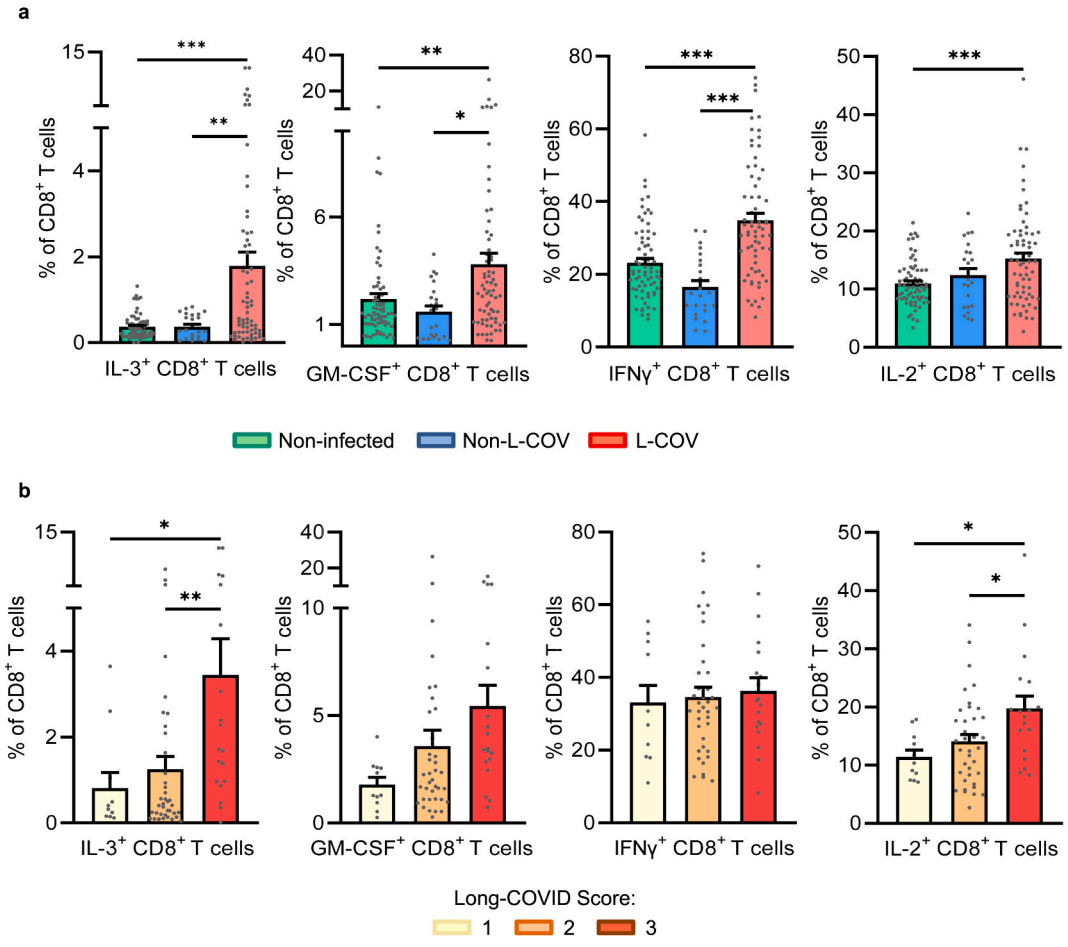

Fig. 3. Intracellular cytokine expression of $CD8^+$ T cells in patients with Long-COVID compared to non-Long-COVID controls and non-infected controls.
a-b PBMCs from 69 non-infected controls (Non-infected), 23 non-Long-COVID controls (Non-L-COV) and 68 patients with Long-COVID (L-COV) were activated with PMA/Ionomycin in the presence of brefeldin A for 3 h. Long-COVID patients were stratified according the severity of symptoms into Long-COVID-Score 1 (n = 11), Score 2 (n = 38) and Score 3 (n = 19). Intracellular cytokine expression by $CD8^+$ T cells was analyzed by flow cytometry. Each sample is represented by one dot, and the mean is shown as bar. One-way ANOVA with Bonferroni's multiple comparison test was used.

necessarily associated with development of Long-COVID (Fig. 2c). No significant age-dependent differences were detectable for the release of IL-3 from T cells in Long-COVID-patients or controls (Suppl. Fig. 2b).

2.3. Intracellular staining shows increased cytokine expression in CD8$^+$ effector-memory T cells

To find out which subsets of T cells are responsible for the increased release of cytokines in Long-COVID, we performed intracellular cytokine staining after stimulation with PMA/Ionomycin in the presence of brefeldin-A for 3 h.

We found an about 5-fold higher percentage of CD8$^+$ T cells expressing IL-3 in Long-COVID patients compared to non-infected controls and non-Long-COVID controls (Fig. 3a), while expression of GM-CSF, IFN-γ and IL-2 was much less upregulated in Long-COVID patients. Intracellular staining of IL-3 and IL-2 strongly correlated with the Long-COVID-Score (Fig. 3b). The majority of patients with a Long-COVID-Score of 3 had clearly elevated counts of IL-3$^+$ CD8$^+$ T cells. Again, no significant age-dependent differences were detectable for the intracellular IL-3 staining in CD8$^+$ T cells in Long-COVID-patients or controls (Suppl. Fig. 3a). CD4$^+$ T cells did not show a convincing overexpression of cytokines in Long-COVID (Suppl. Fig. 3b, c), indicating that hyper-activity and hyper-reactivity in patients with Long-COVID is largely restricted to CD8$^+$ T cells.

To more specifically identify the subset of IL-3 producing CD8$^+$ T cells in patients with Long-COVID we further characterized these T cells according to their memory status. Expression of IL-3 was predominately found in effector-memory (EM) and to lower degree in terminal-effector-memory (TEMRA) cells of patients with Long-COVID as shown in the dot plots and the statistical analysis (Fig. 4a, b). In contrast, almost no IL-3 expression was seen in central-memory and naïve CD8$^+$ T cells. Non-infected controls showed a much lower IL-3 expression in effector-memory CD8$^+$ T cells. CD4$^+$ T cell memory subsets showed no differences in IL-3 expression between Long-COVID and non-infected controls (Fig. 4b).

Thus, Long-COVID patients did not only show an increased frequency of EM and TEMRA CD8$^+$ T cells (Fig. 1c) but also an increased percentage of EM and TEMRA CD8$^+$ T cells expressing IL-3.

2.4. Phenotypic changes of CD8$^+$ T cells persist for up to 18 months after SARS-Cov-2 infection in patients with Long-COVID

To find out whether the above described phenotypic changes gradually disappear with a longer duration of Long-COVID, we stratified our

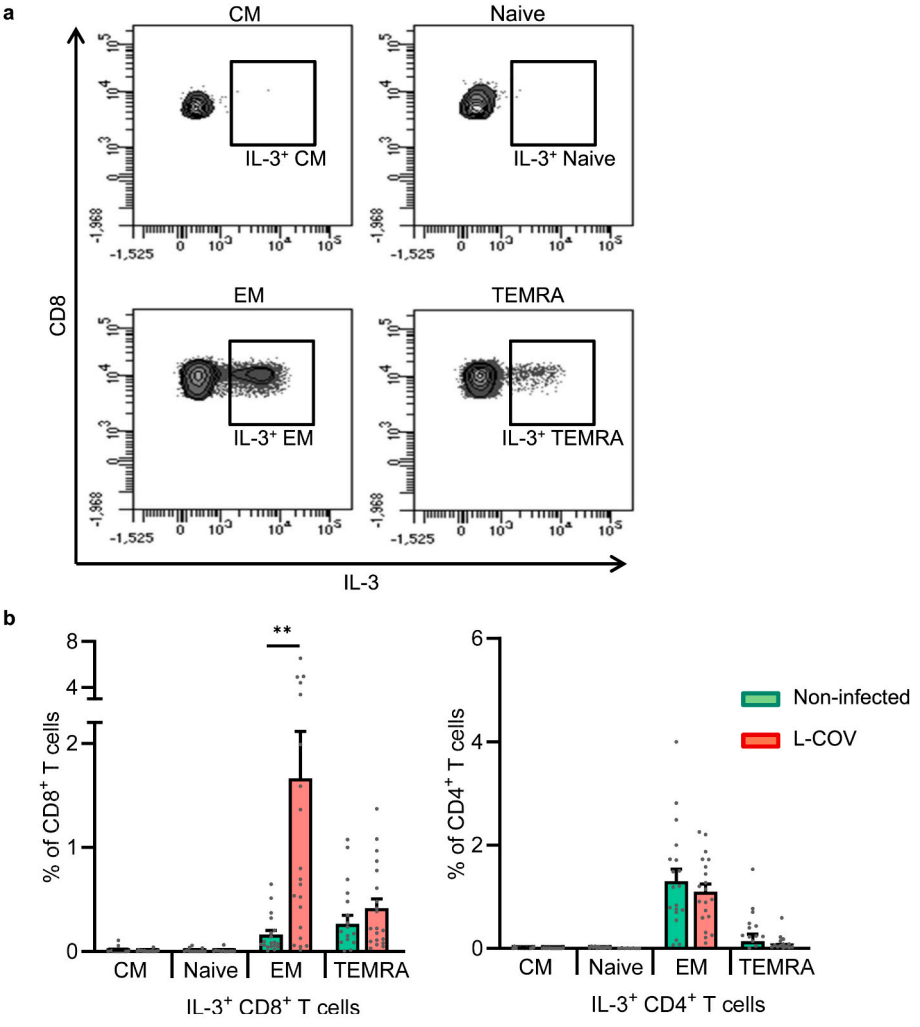

Fig. 4. Long-COVID patients show elevated IL-3 expression by CD8$^+$ memory T cells compared to non-infected controls.
a-b PBMCs from 19 non-infected controls (Non-infected) and 20 patients with Long-COVID (L-COV) were activated with PMA/Ionomycin in the presence of brefeldin A for 3 h. Intracellular IL-3 expression by central-memory (CM), naïve (Naïve), effector-memory (EM) and terminal-effector-memory (TEMRA) CD8$^+$ and CD4$^+$ T cells was analyzed by flow cytometry. a Representative dot plots for CD8$^+$ T cells. b Each sample is represented by one dot, and the mean is shown as bar. Two-tailed student's *t*-test was used to calculate the significance between patients with Long-COVID and non-infected controls.

cohort into patients with symptoms <12 months after infection, 12 to 18 months after infection and more than 18 months after infection. All non-Long-COVID controls were analyzed <12 months after infection and are also included in the analysis. As shown in Fig. 5a, the high fraction of CD25$^+$ CD8$^+$ T cells did not disappear in patients with a longer duration of Long-COVID. Regarding the memory status of CD8$^+$ T cells, there was even some increase of effector-memory and TEMRA cells over time and a concomitant decrease of naïve CD8$^+$ T cells (Fig. 5b). IL-3 expression and the percentage of IL-3$^+$ CD8$^+$T cells remained high more than 18 months after infection in patients with Long-COVID (Fig. 5c, d). Overall, these data suggest that CD8$^+$ T cell hyper-activation and hyper-reactivity is persistent in Long-COVID patients.

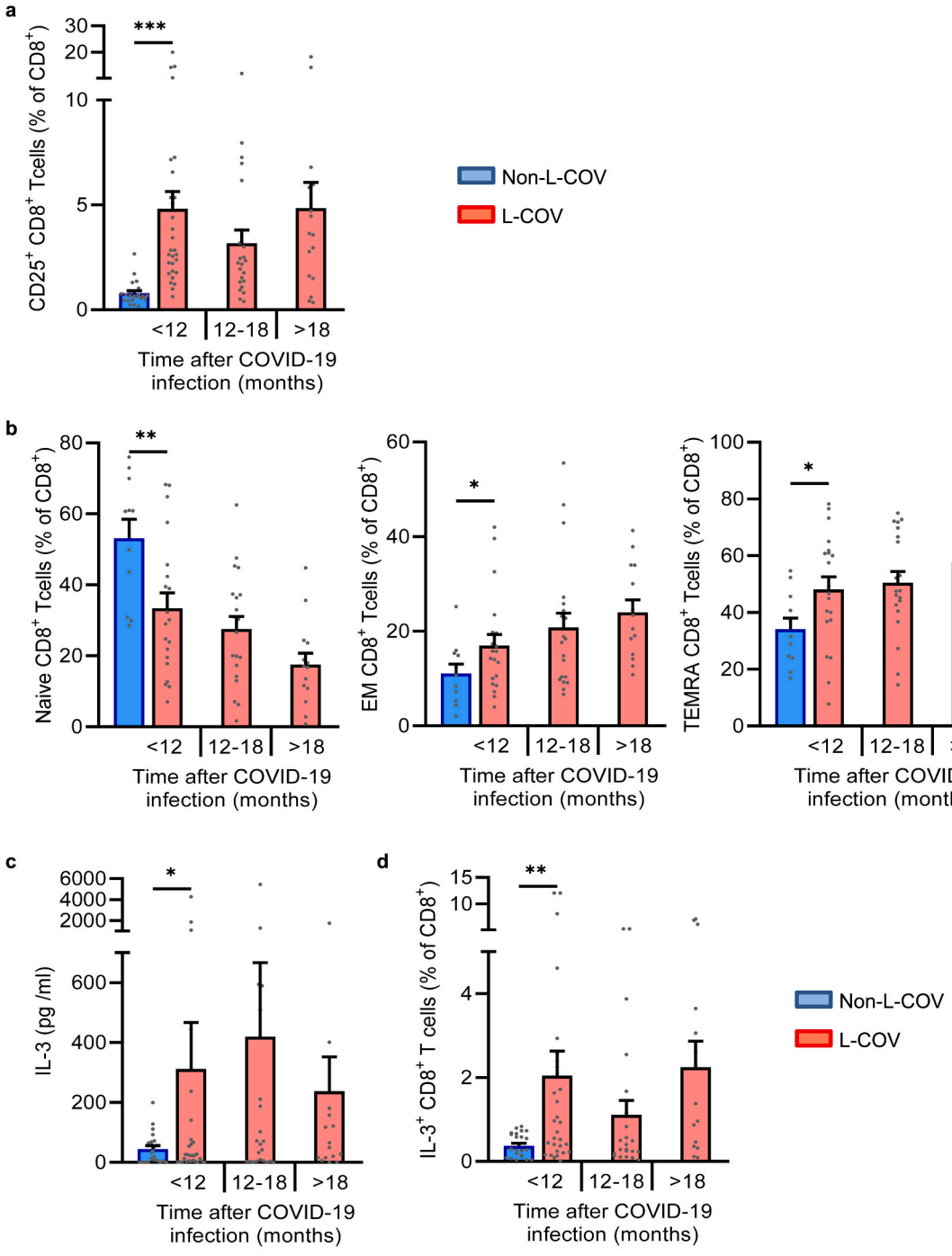

Fig. 5. Persisting T cell hyper-activation of Long-COVID patients for up to 18 months after initial SARS-CoV-2 infection.
a-d Peripheral blood from 23 non-Long-COVID controls (Non-L-COV) and 68 patients with Long-COVID (L-COV) was analyzed. Non-Long-COVID controls and patients with Long-COVID were stratified according to the time after the initial SARS-CoV-2 infection in <12 months (Controls $n = 23$, L-COV n = 30), 12–18 months (L-COV $n = 22$), >18 months (L-COV n = 16). a Percentage of CD25 expressing CD8$^+$ T cells. b Percentage of central memory (CM), naïve (Naïve), effector-memory (EM) and terminal-effector-memory (TEMRA) CD8$^+$ T cells. c Concentration of IL-3 in the supernatant of whole-blood samples cultured with immobilized anti-CD3-antibodies for 24 h. d Intracellular expression of IL-3 by CD8$^+$ T cells. Each sample is represented by one dot, and the mean is shown as bar. One-tailed Welch's t-test was used to calculate the significance between patients with Long-COVID patients and non-Long-COVID controls.

3. Discussion

In our study we analyzed T cell numbers and functions in patients with Long-COVID, controls without Long-COVID and non-infected controls. A pronounced T cell hypo-reactivity and reduced expression of IL-3 was found in patients with severe acute SARS-CoV-2 infection [26,38]. Interestingly, the opposite was the case as we detected a marked hyper-reactivity of T cells in Long-COVID. Hyper-activation was evident by a higher percentage of $CD8^+$ T cells expressing the activation marker CD25, a stronger upregulation of CD25 after polyclonal stimulation, a stronger release of cytokines especially IL-3 and a higher fraction of memory T cells. These changes were more pronounced in patients with a higher Long-COVID severity score and persisted for up to 18 months. More specifically, effector-memory and terminal-effector-memory $CD8^+$ T cells were expanded in Long-COVID and expressed more IL-3. Most of the IL-3 was expressed by effector-memory T cells in patients with Long-COVID. Moreover, the hyper-reactivity was much more pronounced in $CD8^+$ T cells compared to $CD4^+$ T cells. To find out whether age has an impact on T cell hyper-activation and hyper-reactivity of Long-COVID patients and to correct for age differences between individuals with and without Long-COVID in our study, we stratified Long-COVID patients according to their age. T cell hyper-activation and hyper-reactivity was equally pronounced in younger and older Long-COVID patients, excluding age as a confounding factor in our study.

A pronounced T cell hyper-reactivity was seen even 18 months after initial SARS-CoV-2 infection with little decrease over time suggesting that counter regulatory mechanisms leading to contraction of immune responses were insufficient in Long-COVID. The reason for this persisting T cell hyper-reactivity is currently unknown. Some possible explanations include persisting viral infections or persistence of viral RNA and antigen fragments [39–41]. In our study we analyzed T cell responses after polyclonal but not SARS-CoV-2-specific stimulation. The high frequency of activated T cells in Long-COVID patients under basal conditions and even more after polyclonal activation suggests that the T cell hyper-reactivity is not restricted to SARS-CoV-2, but may include multiple antigens. During the primary SARS-CoV2 infection, expansion and activation of non-SARS-CoV2 specific T cells could be caused by a strong bystander T cell activation [42] and a failure to eliminate these cells later on. This may not only apply to T cells but also to humeral immune responses, which is evident by increased levels of autoantibodies and SARS-CoV2 specific immunoglobulin levels in patients with Long-COVID [43–46].

Some T cell alterations have already been described in patients with Long-COVID including more IL-2 and IL-6 producing T cells [43], reduced naïve T cell subsets [47,48], altered regulatory T cells [49], T cell senescence [50], a more rapid decline of SARS-CoV-2 specific memory $CD8^+$ T-cells [51] and increased serum levels of pro-inflammatory and proangiogenic cytokines and chemokines [47,48,52–56]. However, in most studies, patients were only stratified by the severity of the initial SARS-CoV-2 infection and not by the severity or duration of their Long-COVID symptoms. None of the study analyzed expression of IL-3, which best distinguished Long-COVID from controls in our patient cohort.

Other studies analyzed changes in T cell phenotype following acute SARS-CoV-2 infection without a special focus on development of Long-COVID. One study described a persistent activation of $CD4^+$ and $CD8^+$ T cells, based on expression of HLA-DR, CD38, Ki67, and granzyme B, as well as elevated plasma cytokine levels; however, these changes were not associated with development of Long-COVID [57]. A further study showed exhausted $CD4^+$ and $CD8^+$ T cells and a high proportion of terminal-effector-memory $CD8^+$ T cells together with a decrease of naïve T cells and augmented granzyme B and IFN-γ production in T cells following acute SARS-CoV-2 infection. The study concluded that development of Long-COVID is independent of the severity of the acute infection, however 61–81 % of the individuals were described to develop Long-COVID in this study, which is difficult to reconcile with most other epidemiological data [58].

Expansion of effector-memory and terminal-effector-memory T cells is a hallmark of various autoimmune diseases. For example, increased frequencies of effector-memory and terminal-effector-memory T cells were found in blood and tissue of patients with systemic lupus erythematodes [59,60] and other autoimmune diseases. In addition, more $CD8^+$ than $CD4^+$ T cells are present in inflamed tissue of patients with autoimmunity e.g. renal tissue in SLE [61,62], pointing also to an important role of $CD8^+$ T cells in autoimmunity.

Among the cytokines measured, the differences between controls and Long-COVID patients were most pronounced for IL-3 as seen by the release of IL-3 in the supernatant and by intracellular cytokine staining of $CD8^+$ T cells. For IFN-γ the data were somewhat inconsistent as there was a somewhat decreased release of IFN-γ and a somewhat increased frequency of IFN-γ^+ $CD8^+$ T cells. In recent years, IL-3 was found important not only for helminth and parasite infection [63,64] but also for development of autoimmunity, inflammation and fibrosis. This has been shown in a number of animal models as well as in patients [27–33]. The link of IL-3 with fibrosis fits to the presence of restrictive changes in lung function tests. Apart from organ specific manifestations, which are not present in Long-COVID, many of these autoimmune diseases are associated with general clinical symptoms like fatigue, stress dyspnea and neuro-psychological symptoms suggesting that chronic T cell activation and T cell hyper-reactivity with release of cytokines and downstream effects on innate immune cells could be responsible for general, organ-independent symptoms of autoimmunity and Long-COVID [65]. Of interest, in a clinical study 87 % of patients treated with recombinant human IL-3 developed fatigue as major side effect [34].

Overall, our data strongly suggest an involvement of $CD8^+$ T cells in the development of Long-COVID. The increased release of IL-3 and other T cell-derived cytokines by hyper-activated and hyper-reactive T cells may cause clinical symptoms of Long-COVID similar to the chronic fatigue syndrome in patients with autoimmunity and after other viral infections. Our data suggest that interference with T cell-derived effector molecules, especially IL-3 or treatment with well tolerated T cell-directed immunosuppressive agents like low dose rapamycin (clinical trial number NCT06257420) and intravenous immunoglobulins [66] may be of benefit for patients with Long-COVID.

4. Materials and methods

4.1. Study subjects and sampling

A total of 68 adult patients diagnosed with Long-COVID syndrome, 23 controls with documented SARS-CoV-2 infection without Long-COVID symptoms and 69 non-infected controls (Non-infected) without any history of SARS-CoV-2 infection were enrolled at a unit specialized for Long-COVID at the Klinik Donaustauf, Germany, from Nov 2021 to July 2022. This study was approved by the Research Ethics Committee from the University Hospital Regensburg (Study and Approval Number: 20–1785-101). Informed consent was obtained from all participants.

Long-COVID syndrome was defined as a condition that occurs in individuals with a history of confirmed SARS-CoV-2 infection, ≥3 months from the onset of infection with symptoms that last for at least 2 months and cannot be explained by an alternative diagnosis (according to WHO definition). Symptoms could either be new onset, following initial recovery from an acute SARS-CoV-2 infection, or persist from the initial illness.

Patients in the non-Long-COVID control group had a history of confirmed SARS-CoV-2 infection but no clinical symptoms. The Long-COVID cohort was classified based on self-assessment of the severity of their illness using the Long-COVID severity score as follows [5]: 0 = no limitations in everyday life and no symptoms, pain, depression or anxiety related to the infection, 1 = negligible limitations in everyday life, performance of all usual duties/activities, however with persistent

symptoms, pain, depression or anxiety related to the infection, 2 = limitations in everyday life with occasionally need to avoid or reduce usual duties/activities or to spread these over time due to symptoms, pain, depression or anxiety related to the infection. Patients are able to perform all activities without any assistance. 3 = limitations in everyday life with inability to perform all usual duties/activities due to symptoms, pain, depression or anxiety related to the infection. Patients are able to take care of themselves without any assistance. 4 = severe limitations in everyday life with inability to take care of themselves and dependence on nursing care and/or assistance from other persons due to symptoms, pain, depression or anxiety related to the infection.

Fresh whole blood was available from 68 non-infected controls (Non-infected) without a previous history of SARS-CoV-2 infection, 23 controls (Non-L-COV) without persistent symptoms following SARS-CoV-2 infection and 68 Long-COVID (L-COV) patients for immediate immunophenotyping by flow cytometry with at least one antibody panel and for the whole blood stimulation assay. Peripheral blood mononuclear cells (PBMCs) were prepared from 69 non-infected controls (Non-infected), 23 controls (Non-L-COV) and 68 Long-COVID patients (L-COV). For subgroup analysis the Long-COVID patients were stratified according the Long-COVID-Score into the groups "1" (11 patients), "2" (38 patients) and "3" (19 patients). Further antibody panels were used in subsets of patients with sample numbers for each readout provided in the figure legends, as for some read-outs not enough material was available.

4.2. Whole blood stimulation assay

FACS-tubes (BD Bioscience) were pre-coated with 300 μl RPMI-1640 (Gibco, Karlsruhe, Germany) containing 5 μg/ml anti-CD3 antibodies (OKT-3, eBioscience, San Diego, USA) or just with 300 μl RPMI-1640 medium at 37 °C for 4 h and washed twice with 4 ml 0,9 % NaCl. After the last washing step 300 μl RPMI 1640 containing 1 % penicillin/streptomycin and 10 % heat-inactivated FCS was added, tubes were sealed and stored frozen by −20 °C up to 4 weeks until use. For T cell activation assays, pre-coated FACS-tubes were thawed by room temperature and 100 μl medium were removed. 100 μl of heparinized blood were added to pre-coated tubes and incubated at 37 °C in 5 % CO_2 for 24 h. The supernatants were recovered and released cytokines analyzed by ELISA. Cells were analyzed by multi-parametric flow cytometry as described below.

4.3. Flow cytometry

Heparin-anticoagulated fresh blood samples (100 μl) or cells after whole blood stimulation were incubated with various panels of the following directly labeled monoclonal antibodies for 20 min by 4 °C: anti-CD3 FITC (clone: OKT3, BioLegend, San Diego, CA), anti-CD3 (clone: SK7, BioLegend), anti-CD4 PerCP-Cyanine5.5 and APC (clone: RPA-T4, BioLegend), anti-CD4 BD Horizon V500 (clone: RPA-T4, BD Bioscience), anti-CD8a APC-Cyanine7 and PerCP-Cyanine5.5 (clone: RPA-T8, BioLegend), anti-CD16 Pacific Blue (clone: 3G8, BioLegend), anti-CD25 APC (clone: BC96, BioLegend), anti-CD45RA Brilliant Violett 510 (clone: HI100, BioLegend), anti-CD197/CCR7 APC (clone: 7–239, BioLegend).

Subsequently, samples were treated with FACS Lysing Solution (BD Bioscience) for 10 min, washed with 0,9 % NaCl, centrifuged, resuspended in 0,9 % NaCl together with FACS counting beads (Invitrogen, Darmstadt, Germany) and acquired with a BD FACSCanto II flow cytometer and analyzed with FACSDIVA 8 software (BD Biosciences). The gating strategy for key cell populations is shown in Suppl. Fig. 4.

4.4. Preparation of PBMCs

Peripheral blood mononuclear cells (PBMCs) were prepared by Ficoll-Paque density gradient centrifugation from heparin-anticoagulated fresh blood samples. For cryopreservation, PBMCs were resuspended in fetal calf serum (FCS) with 10 % dimethylsulfoxide (DMSO) at a concentration of 1–2 × 10^6 cells / ml, frozen at −80 °C for 2–3 days and then transferred into liquid nitrogen. The cells were thawed in a 37 °C water bath and washed 3 times with medium. The viability was controlled by Trypan-blue staining and was 90–95 %.

4.5. Intracellular cytokine staining

For intracellular staining of cytokines 500.000 PBMC/well were cultured in 200 μl RPMI 1640 medium (Gibco, Karlsruhe, Germany) containing 1 % penicillin/streptomycin (Gibco, Karlsruhe, Germany), 10 % fetal calf serum, 1 % L-glutamine (Gibco, Karlsruhe, Germany) and Phorbol-12-myristat-13-acetat (PMA, 10 ng/ml), ionomycin (1 μg/ml) and brefeldin A (5 μg/ml) (eBioscience) for 3 h by 37 °C. After extracellular staining anti-CD3-APC-Cy7 (clone: SK7, BioLegend), anti-CD4-APC (clone: RPA-T4, BioLegend), anti-CD8a-PerCP-Cy5.5 (clone: RPA-T8, BioLegend) cells were treated with Fix-Perm and Perm-Wash solutions (BD Biosciences) incubated and stained intracellularly with the following antibodies: anti-IL-2 PECy7 (clone: MQ1-17H12, BioLegend), anti-IL-3 PE (clone: clone-13, AG Mack) or mouse IgG1, κ Isotype Ctrl PE (clone: MOPC-21, BioLegend), anti-GM-CSF Pacific Blue (clone: BVD2-21C11, BioLegend), anti-IFN-γ FITC (clone: 4S·B3, BioLegend).

The gating strategy for key cell populations is shown in Suppl. Fig. 4.

4.6. Enzyme Linked Immuno Sorbent Assay (ELISA)

Concentration of IFN-γ in whole blood assay supernatants was measured by ELISA, according to manufacturer's protocol (DuoSet Elisa, R&D Systems, Abington, UK). For measurement of IL-3, ELISA plates (NUNC Maxisorb, Thermofisher Scientific, Waltham, USA) were coated with a capture anti-IL-3 antibody (Clone P8C11, AG Mack; 5 μg/ml in PBS) in 100 μl / well at room temperature (RT) overnight. Plates were washed 3 times with PBS / 0.05 % Tween-20, blocked with PBS containing 1 % bovine serum albumin (BSA) at RT for 1 h and washed again with PBS. Samples where preincubated with 100 μg/ml mouse IgG1, kappa isotype control antibody (MOPC21, BioXCell, Lebanon, USA) for 1 h by RT and added to the plates for 2 h by RT (100 μl / well, diluted in PBS / 1 % BSA). Recombinant human IL-3 (BioLegend) was diluted from 7.8 to 500 pg/ml in PBS / 1 % BSA and served as standard. After washing plates were incubated with 400 ng/ml HRP-labeled detection anti-IL-3 antibody (Clone 13, AG Mack) (100 μl/well) for 1.5 h at RT and color reaction was performed with TMB Substrate Solution (BioLegend) according to manufacturer's protocol.

4.7. Statistical analysis

Statistical analysis was performed using the GraphPad Prism 8 Software. Statistical differences between more than two different cell stimulations or patient cohorts were calculated using one-way ANOVA with Bonferroni's multiple comparison as indicated in the figures. For two group comparisons, 2-tailed unpaired *t*-test was used as indicated in the figs. *P* values less than 0.05 were considered significant and marked with one asterisk or with two asterisks if less than 0.01. P values less than 0.001 were marked with three asterisks.

CRediT authorship contribution statement

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Declaration of competing interest

The authors declare no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2025.110502.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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