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Post-COVID-19 sequelae are associated with sustained SARS-CoV-2-specific $CD4^+$ immune responses

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ABSTRACT

Background: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has led to widespread post-acute sequelae of COVID-19 (PASC), affecting multiple body systems. Despite its prevalence, PASC's pathogenesis remains unclear, with hypotheses suggesting viral persistence, immune activation, and autoimmune responses among the pathogenetic mechanism. This study aimed to evaluate T cell memory response in PASC patients, one year post-hospital discharge and correlate it with clinical parameters to identify a potential PASC-associated fingerprint.

Methods: Peripheral blood mononuclear cells (PBMCs) from PASC patients and healthy controls (HC) were stimulated with a pool of spike peptides. $CD4^+$ and $CD8^+$ T cell responses were evaluated by flow cytometry using the activation-induced markers assay (AIM).

Results: Findings showed significant activation of the $CD4^+$ T cell compartment, with a higher proportion of responders among PASC patients. Central memory (CM) T cells expressing pro-inflammatory cytokines were more prevalent in *responders*. Clinical correlations revealed higher SARS-CoV-2-specific T cell responses in patients with reduced diffuse lung capacity for carbon monoxide (DLCO) and residual symptoms.

Conclusion: These immune changes, especially in CM T cells, could play a pivotal role in PASC's development and persistence, impacting patients' daily lives.

1. Introduction

The population worldwide has experienced increased mortality and morbidity due to the pandemic caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection. Among patients who recovered from COronaVIrus Disease-2019 (COVID-19), unfortunately,

Abbreviations: (PASC), Post-acute sequelae of COVID-19; (COVID-19), COronaVIrus Disease-2019; (SARS-CoV-2), Severe acute respiratory syndrome coronavirus 2; (AIM) assay, Activation induced marker; (DLCO), Diffuse lung capacity for carbon monoxide; (CM), Central memory; (Tfh), Follicular helper T cells; (SEB), Staphylococcal enterotoxin B; (2MW), 2 min walking test; (EM), Effector memory; (TEMRA)., terminal effector memory re-expressing CD45RA.

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there is still a portion that experiences a wide range of persistent symptoms that last months after the acute phase of the illness [1]. The World Health Organization (WHO) estimates that the 10–20 % of those recovering from the SARS-CoV-2 infection manifest at least one symptom after several months. This condition has been dubbed as post-acute sequelae of SARS-CoV-2 infection (PASC) syndrome [2].

PASC syndrome is a multi-systemic disorder characterised by pulmonary, immunological, cardiovascular, neuropsychiatric, gastroenterological, hepatic, renal, endocrine, and dermatological sequelae. While it is not typically life-threatening, it can significantly affect patient's daily activities and social life [3,4]. Pulmonary involvement is a hallmark of PASC syndrome, and includes impaired lung function, that can be assessed through the diffusing capacity of the lung for carbon monoxide (DLCO). DLCO is a key parameter that measures the ability of gases to diffuse from the alveoli into the bloodstream, reflecting the integrity of both the alveolar-capillary membrane and pulmonary blood flow. A reduction in this parameter is frequently shown by PASC patients. A value below 80 % of expected indicates a respiratory impairment, that is more severe in the case of a value below 60 % [5]. Evidence suggests that the underlying mechanisms of pulmonary complications during COVID-19 are closely linked to immune system dysregulation. In particular, immune cells drive alveolar endothelium inflammation leading to long term consequences such as pulmonary fibrosis [6,7,8]. Given the critical role of immune cells, particularly T cells, in mediating these processes, evaluating T cells subsets in PASC patients with reduced DLCO may provide valuable insight into the adaptive immune mechanisms driving pulmonary dysfunction in PASC syndrome.

Despite the prevalence and growing awareness of PASC, the possible pathological mechanisms underlying this syndrome remain poorly defined. The struggle is related to the wide array of symptoms associated with PASC and the complexities of COVID-19 pathogenesis. Nevertheless, different hypotheses have been proposed including viral persistence, immune system activation triggered by SARS-CoV-2 superantigens, and autoimmune responses [9,10]. Overall, a common feature is the involvement of the adaptive immune system [9].

Adaptive immunity comprises circulating antibodies, B and T cells, which protect the organism against re-infections. Among them, Guo *et al.*, have highlighted the crucial role of SARS-CoV-2-specific T-cell responses in protecting against re-infection and providing long-term immunological memory [11]. Additionally, various research efforts have evaluated these T-cell responses, examining their persistence and effectiveness 6 to 10 months after viral clearance. In a study by Hou *et al.,* the authors performed an ELISPOT assay in PASC patients' serum collected one year after the primary infection. They demonstrated that specific T-cell response can last several months after the infection [12]. In their work, Opsteen *et al.* evaluated SARS-CoV-2 specific T-cell response through the activation induced marker (AIM) assay, 6 months post-infection. They found that PASC patients had an increased and sustained SARS-CoV-2-specific $CD4^+$ T-cell response overtime, with respect to those who recover from the infection without any symptoms [13]. These findings, taken together with other data, point to the persistence of viral antigen stimulation, that continuously triggers the adaptive immune system. All these studies share a common limitation: they did not account for correlations between the biological data obtained and the specific clinical parameters evaluated in patients who recovered from COVID-19.

It has been widely established that a persistent and uncontrolled inflammation, together with a dysregulated immune response, can lead to the development of a long-term sequelae following viral infections [14], such as those caused by Ebola, Lassa and Influenza viruses. This uncontrolled inflammation and immune system activation might be implicated in the development and persistence of severe symptoms [15]. In this context, the assessment of the SARS-CoV-2-specific T-cell response and its relationship with clinical data becomes crucial to better characterize the disease and provide a potential diagnostic tool.

There are different strategies to measure humoral immunity and its efficiency (e.g. ELISA and neutralizing antibody assays), but very few allow for the assessment of the entire spectrum of the cellular immunity. Traditional methods for quantifying antigen-specific T-cell responses include proliferation assays, cytokine production detection (ELISPOT), and peptide-MHC multimer staining. However, these methods have limitations such as lack of information on T-cell phenotype, failure to detect low abundant cells like follicular helper T cells (Tfh), and complexity in capturing the complete range of cytokine production. In contrast, the AIM test is an *in vitro* assay, which allows to detect: i) T-cell phenotype, ii) different cytokine production (interferon-γ (IFN-γ), interleukin 2 (IL-2) and tumor necrosis factor (TNF-α)) and iii) the maturation curve [16].

The primary objective of our work was to evaluate the SARS-CoV-2-specific T-cell response in patients one year after hospital discharge and to correlate these findings with various clinical parameters. The novelty of our work relies in the comprehensive approach, which extends beyond immune profiling. We specifically correlated the dysregulated immune response—encompassing both $CD4^+$ and $CD8^+$ T cells—with distinct clinical symptoms in PASC patients. We performed the AIM assay, which provides a wider depiction of the overall antigen-specific T-cell response and increases the comprehension in both quantitative and qualitative terms of these responses.

2. Materials and methods

2.1. Study participants

We enrolled 92 patients who have been hospitalized due to COVID-19 between March 1st, 2020, and June 29th, 2020. The follow-up occurred with an average of 366 days after recovery and hospital discharge. Different clinical parameters associated with PASC including the presence of residual symptoms, pulmonary function tests and two minutes walking test (2MWT) have been performed as previously described by *Bellan et al.* [4].

2.2. Activation induced marker (AIM) assay

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by standard procedure using Ficoll-paque density gradient centrifugation. After washing, PBMCs were cryopreserved in FBS 10 % DMSO for further analysis.

Cryopreserved PBMCs were thawed in complete RPMI 1640 medium. PBMCs were resuspended in RPMI supplemented with heat-inactivated human serum from male AB plasma (Merck, Darmstadt, Germany). Cells were then stimulated with PepTivator® SARS-CoV-2 Prot_S complete spike peptide pool (1 μg/mL) (Miltenyi Biotec, Germany) in presence of 1 μg/mL anti-CD28 (LifeTechnoloiges, Carlsbad, CA, USA). Cells that were cultured in RPMI medium supplemented only with heat-inactivated human serum in presence of 1 μg/mL anti-CD28 antibody were used as negative control, while cells that were cultured in presence of 1 μg/mL anti-CD28 antibody and 1 μg/mL Staphylococcal enterotoxin B (SEB) (Merck, Darmstadt, Germany) were used as positive control.

For each sample and each condition (stimulated, unstimulated and SEB stimulation), $1x10^6$ cells were plated in 96-well round bottom plates and incubated at 37 °C, 5 % CO2 for 4 h. After 4 h incubation, samples were supplemented with 10 μg/mL of brefeldin A (Becton and Dickinson, Franklin Lakes, NJ, USA) and incubated for the following 12 h.

2.3. Flow cytometry

BD Horizon™ Fixed Viability Stain 575 V (Becton and Dickinson, Franklin Lakes, NJ, USA) was used to assess the cell viability. Surface staining of the cells was performed with titrated concentrations of the following combinations of antibodies: anti-CD4 APC-H7 (clone SK3), anti-CD8 V500C (clone SK1), anti-CD197 PE (clone 2-L1-A), anti-CD45RA BV786 (clone HI100), and anti-CXCR5 in BV421 (clone

RF8B2). After adding the surface markers, samples were incubated for 20 min at 37 °C. After incubation and washing, cells were fixed and permeabilized with Fixation/Permeabilization Kit (Becton and Dickinson, NJ, USA) for intracellular staining.

Intracellular staining was carried out for 30 min at 4 °C with titrated concentrations of the following combinations of antibodies: anti-CD3 PerCP-Cy5.5 (clone SK7) anti-CD137 APC (clone), anti-CD69 PE-Cy7 (clone FN50) anti-IL-2 BV711 (clone 5344.111), anti- IFN-γ FITC (clone B27), anti- TNF-α Alexa Fluor 700 (clone MAb11). All the antibodies were purchased from Becton and Dickinson (Franklin Lakes, NJ, USA).

Acquisition of samples was performed on FACSymphony A5, while analysis was done using FACS Diva software (Version 9.0.0, Becton and Dickinson, Franklin Lakes, NJ, USA).

The percentage of SARS-CoV-2-specific subpopulations was calculated subtracting the negative control for each sample to its spike-specific activation.

The gating strategies were shown in Supplementary Figs. S1–S5.

2.4. Statistical analysis

Mann–Whitney *U* test was used to detect any significant differences in antigen-specific T-cell response between healthy control (HC) and PASC patients. p values r below 0.05 were considered statistically significant. GraphPad Instat software was used (Version 8.3, GraphPad Software, San Diego, CA, USA). The effect size (Cohen's d) was calculated based on the observed means and standard deviations in each group, following the pooled standard deviation approach.

3. Results

3.1. Study cohort

We recruited 92 subjects one year after the recovery from the first wave of COVID-19 infection (March-June 2020). The demographics of participants are listed in Table 1 (gender distribution of PASC in Table 2).

Briefly, out of the 92 participants, 71 % were males and 29 % females, with an average age of 60 years. In this cohort, average half of the subjects displayed a reduced DLCO (47.8 %) and more than 60 %, residual symptoms (which include fever, cough, dyspnea, asthenia, diarrhea, dysgeusia, anosmia, arthralgia and myalgia) with 70 % alteration of the 2MWT. In contrast, the HC group included in this study, although they had also been hospitalized during the first wave of COVID-19, showed no signs of PASC 12 months post-infection. Specifically, they did not exhibit any residual symptoms, DLCO impairment, or abnormalities in the 2MWT. Overall, the cohort consisted of 70 % (n = 64) PASC patients and 30 % (n = 28) HC (Table 3).

3.2. Association of demographic factors with PASC

To explore the relationship between demographic characteristics (i.e., gender and age) and PASC, we conducted chi-square tests with a twofold approach. Although, due to sample size constraints, this finding should be taken carefully, this result indicates that male patients are more likely to experience PASC compared to female patients. Second, we assessed the relationship between age, categorized as ≤ 60 years and > 60 years, and PASC. We observed that PASC is significantly associated (p = 0.0246), to patients older than 60 years.

Table 1
General features of the study participants.

Demographic characteristics	
Age, years	60 [50–70]
Sex M/F	65 (70.6)/27(29.3)
Clinical parameters at 12 months	
DLCO, <80 %/>80 %	44 (47.8)/(48(52.1)
Residual symptoms, N/Y	61 (66.3)/31 (33.6)
Alterated 2MWT, N/Y	70 (76)/22 (23.9)
PASC, N/Y	28 (30.4)/64 (69.5)

Qualitative variables are shown as frequencies (%).

Table 2
Distribution of PASC and HC patients by gender. A chi-square test revealed a statistically significant association between gender and PASC (p = 0.0120).

	PASC	HC	Total	p value
Male	40	25	65	**0.0120**
Female	24	3	28	
Total	64	28	92	

Table 3
Distribution of PASC and HC patients by age. A chi-square test revealed a statistically significant association between gender and PASC (p = 0.0246).

	PASC	HC	Total	p value
≤60 years	29	20	49	**0.0246**
>60 years	35	8	43	
Total	64	28	92	

3.3. Increased $CD4^+$ memory T-cell response to SARS-CoV-2 peptides in PASC patients

To investigate the extent of a SARS-CoV-2 T-cell memory response one year after the primary infection, we performed an *in vitro* T-cell activation method (the AIM test) capable to phenotype T cells, assess their functionality (cytokine expression) and maturation stage, thorough quantitative and qualitative examination of T cell-memory response against spike peptides. We classified our subjects into responders and non-responders based on the presence or absence of T-cell response against the SARS-CoV-2 peptides. As expected from a 1 year far activation to spike peptides, we found non-responders i.e subject lacking a memory response, in both the helper and the cytotoxic compartment. Interestingly, the portion of responders were higher in the $CD4^+$ AIM^+ compartment (71 %) compared to the $CD8^+$ AIM^+ one (43 %) (Fig. 1). We subsequently analysed the data of each T-cell compartment by stratifying PASC and HC. Surprisingly, we found that the majority of *responders* of the $CD4^+$ AIM^+ compartment was mainly represented by PASC patients, 78 % vs 53 % of HC (p = 0.025) (Table 4.). Instead, in the $CD8^+$ AIM^+ compartment there were no differences between the two groups (Fig. 2).

3.4. The magnitude of multifunctional $CD4^+$ CM T cells is higher in PASC patients compared to HC

The AIM test allows to deeply characterize the phenotype of the T cells that respond to the SARS-CoV-2 peptides, evaluating both the magnitude of the SARS-CoV-2-specific T cells and their multifunctionality, i.e. cells secreting one or more pro-inflammatory cytokines simultaneously.

We found significant differences between HC and PASC patients, regarding mainly the $CD4^+$ AIM^+ compartment. Indeed, the subset of central memory (CM) T cells (identified as $CD45RA^-$, $CD197^+$) that secrete cytokines were higher in PASC patients. In contrast, evaluation of the magnitude and the multifunctionality of the $CD8^+$ AIM^+ compartment did not reveal any difference between HC and PASC patients (Fig. 3).

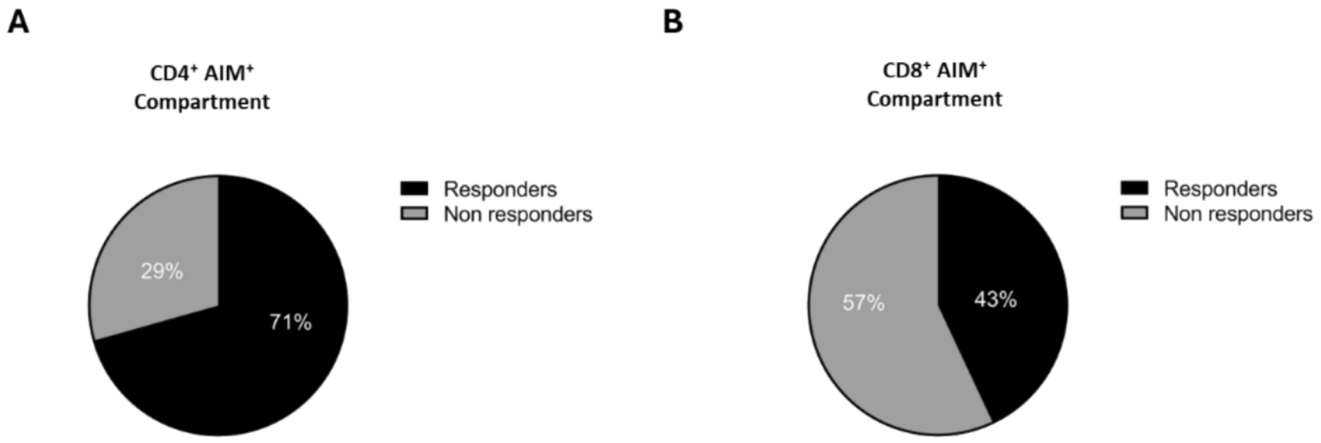

Fig. 1. Portion of *responders* and *non-responders* among the enrolled subjects. (**A**) In the $CD4^+$ AIM+ compartment, the percentage of *responders* (71 %) was higher compared to the *non-responders* group (29 %). (**B**) In the $CD8^+$ AIM+ compartment, the percentage of *responders* (43 %) among all the participants was lower respectively to the *non-responders* (57 %).

Table 4
Differences of the percentage of *responders* between HC and PASC in the $CD4^+$ AIM+ and $CD8^+$ AIM+ compartment. *p < 0.05.

	CD4+ AIM+ responders	CD8+ AIM+ responders
HC	15/28 (54 %)	10/28 (36 %)
PASC	50/64 (78 %)	30/64 (47 %)
p value	0.025*	0.3667

3.5. High magnitude of SARS-CoV-2 memory T cells is linked to PASC patients with reduced pulmonary function

PASC patients were divided into two distinct groups: those with a DLCO < 80 % and those with a DLCO ≥ 80 %, using this cut off to identify patients with a certain degree of respiratory impairment. Recent studies reveal that a large proportion of PASC patients exhibit a reduction of the DLCO < 80 %, indicating a persistent damage in the alveolar-capillary space and possible fibrosis [17].

Considering both the magnitude and the multifunctionality, the total $CD4^+$ AIM^+ cytokine + population, as well as all the maturation curve e phases ($CD4^+$ naïve, CM, EM $CD4^+$ and TEMRA) AIM^+ cytokines, were higher in patients with a DLCO < 80 % (Fig. 4**A–G**). Additionally, also the $CD4^+$ AIM^+ Tfh were higher in patients with a reduced DLCO (Fig. 4**H**).

3.6. High magnitude of SARS-CoV-2 T cells of the $CD4^+$ AIM^+ compartment is linked to residual symptoms in PASC patients

PASC patients were stratified based on the presence or absence of residual symptoms and the magnitude and the multifunctionality of the

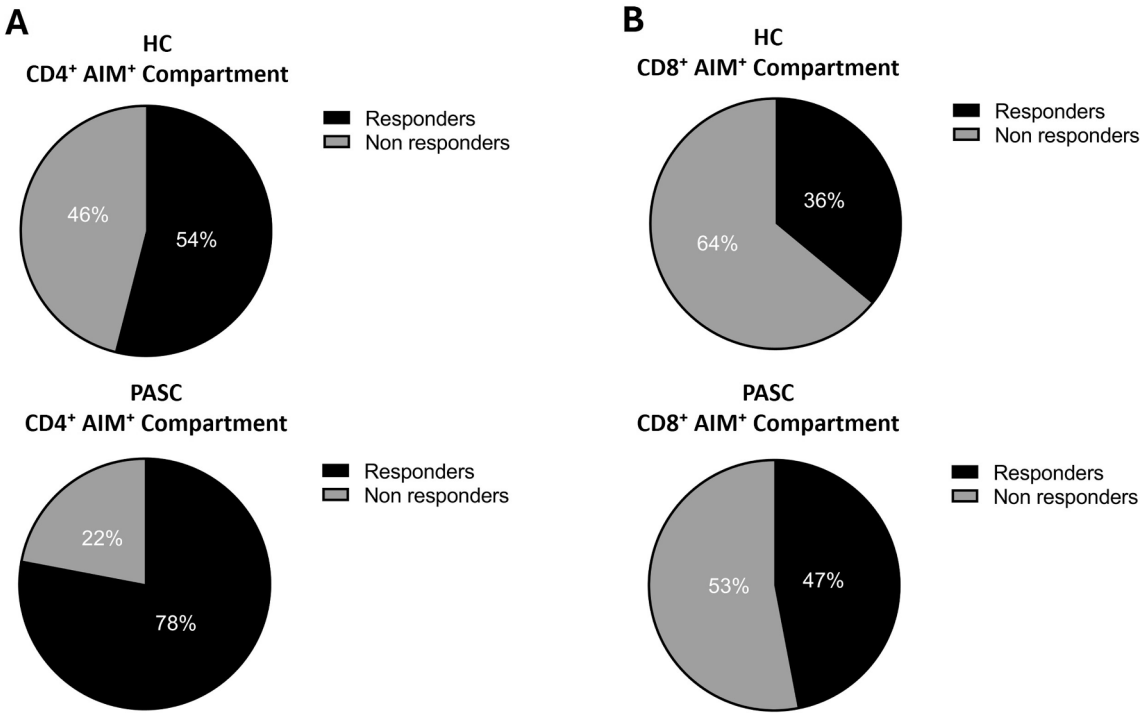

Fig. 2. Portion of *responders* and *non-responders* between HC and PASC patients, in the $CD4^+$ AIM^+ and $CD8^+$ AIM^+ compartment. (A) The portion of *responders* was higher compared to *non-responders* in both HC and PASC patients for the $CD4^+$ AIM^+ compartment. (**B**) For the $CD8^+$ AIM^+ compartment, the portion of *responders* was higher in comparison to *non-responders* in HC, instead for PASC patients the percentage of *responders* was lower compared to *non-responders*.

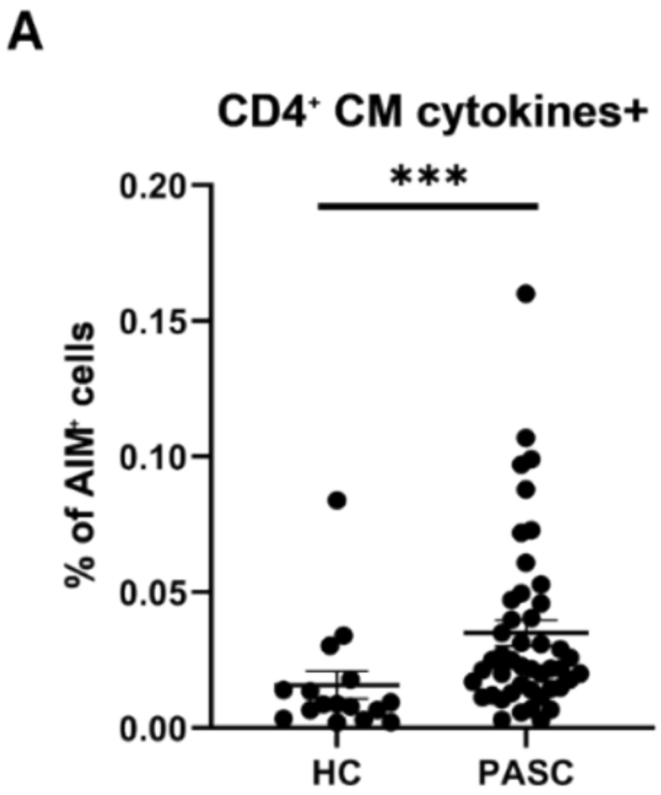

Fig. 3. Evaluation of the magnitude of the CD4$^+$ AIM+ compartment between HC and PASC patients. (**A**) Dot plot shows the percentage of CD4$^+$ CM T cells that secrete pro-inflammatory cytokines. Mann-Whitney *U* test was used *** $p < 0.001$.

T-cell subsets was evaluated.

Notably, in the CD4$^+$ AIM$^+$ compartment, we found an increase of the CD4$^+$ cytokines, CD4$^+$ EM cytokines, CD4$^+$ Tfh cytokines in those patients with residual symptoms (Fig. 5**A, B and C**).

4. Discussion

PASC syndrome encompasses a range of non-specific health problems whose onset, persistence or worsening follow an acute SARS-CoV-2 infection. Its treatment and pathophysiology represent a major challenges [18], and current evidence indicates the possible role of T cells and cellular immunity in disease pathogenesis [19]. Our results highlighted that PASC syndrome was more frequent in males compared to females. These findings contrast with the existing, although limited data in the literature, which suggest that females are predominantly affected by PASC and are more prone to develop the syndrome. Stewart et al and others reported that hormones and menopause may contribute to the increase of pro-inflammatory cytokines such as IL-6, IL-1 and TNF-α, resulting in the establishment of inflammatory status and persistence of diseases [20]. We cannot exclude the possibility that this discrepancy is due to the gender imbalance in our cohort. Nevertheless, on the opposite, and in line with literature we found that individuals older than 60 years were more prone to develop PASC syndrome. Since older patients are more susceptible to comorbid conditions, which can impact their overall health and weaken the immune system's ability to effectively respond to subsequent illnesses [21].

In our study, we inquire about the potential correlation between PASC symptoms and immune perturbation by adopting the AIM test, an *in vitro* T-cell activation method that allows for the investigation of long-term protective immunity following spike peptide stimulation [22]. Being capable to phenotype T cells, assessing their functionality (cytokine expression) and maturation stage, this technique offers a thorough quantitative and qualitative examination of cell-mediated memory

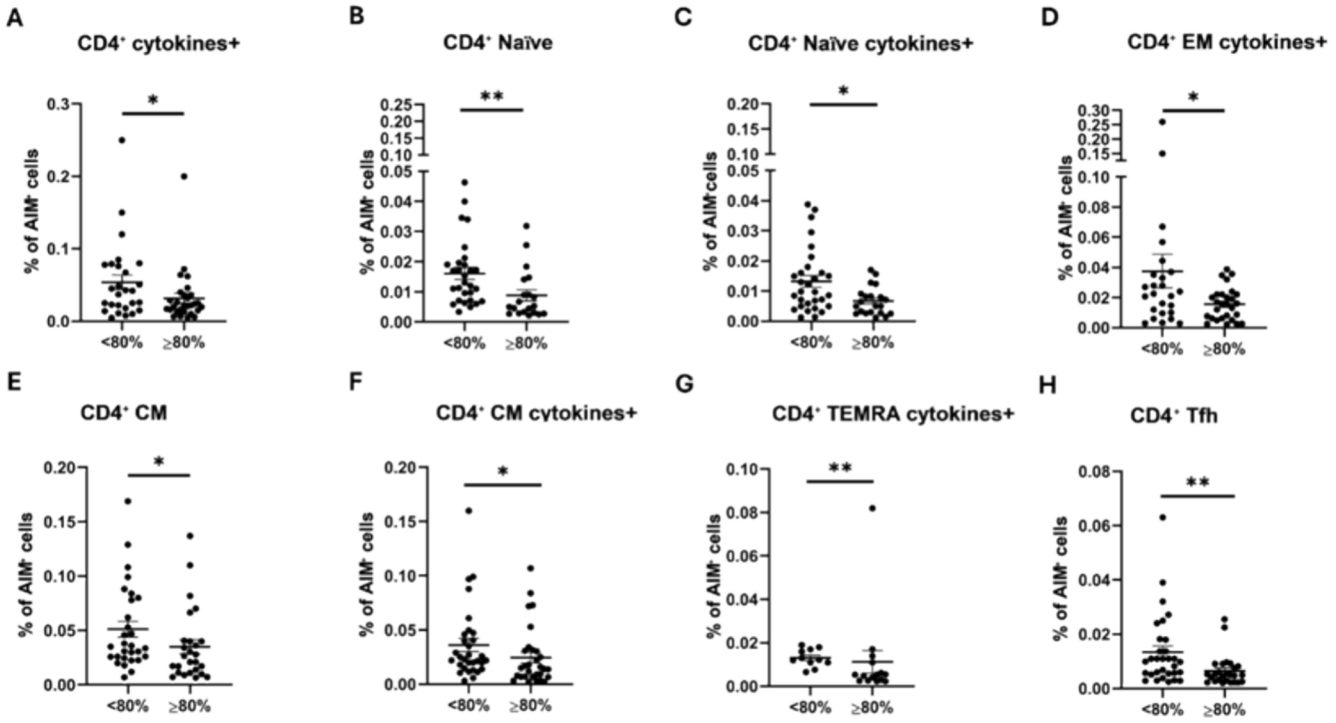

Fig. 4. Evaluation of the magnitude of the CD4$^+$ AIM+ compartment between patients with a DLCO < 80 % and ≥ 80 %. (**A**) Dot plot shows the percentage of CD4$^+$ T cells that secrete the pro-inflammatory cytokines. **(B)** Dot plot shows the percentage of CD4$^+$ naïve T cells. (**C**) Dot plot shows the percentage of CD4$^+$ naïve T cells that secrete pro-inflammatory cytokine. (**D**) Dot plot shows the percentage of CD4$^+$ EM that secrete pro-inflammatory cytokines. (**E**) Dot plot shows the percentage of CD4$^+$ CM T cells (**F**) Dot plot shows the percentage of CD4$^+$ CM AIM$^+$ T cells. (**G**) Dot plot shows the percentage of CD4$^+$ TEMRA T cells that secrete pro-inflammatory cytokines. (**H**) Dot plot shows the percentage of CD4$^+$ Tfh cells. Mann Whitney *U* test was used *$p < 0.05$; **$p < 0.01$.

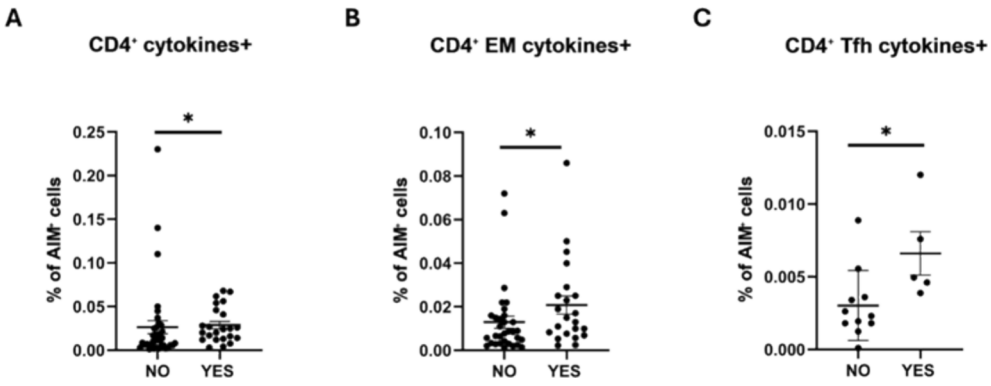

Fig. 5. Evaluation of the magnitude of the CD4$^+$ AIM$^+$ compartment between patients with and without residual symptoms. (**A**) Dot plot shows the percentage of CD4$^+$ T cells that secrete pro-inflammatory cytokines. (**B**) Dot plot shows the percentage of CD4$^+$ EM T that secrete pro-inflammatory cytokines. (**C**) Dot plot shows the percentage of CD4$^+$ Tfh cells that secrete pro-inflammatory cytokines. Mann Whitney *U* test was used *p $<$ 0.05.

against spike peptide in post-acute SARS-CoV-2 infection, which may help in dissecting the intricate immunological background of its sequelae. Based on the immunological T-cell response against the SARS-CoV-2 peptides, we were able to discriminate, among enrolled subjects those who showed an activation, either in CD4$^+$ or CD8$^+$ compartment. Our data highlighted a defective CD8$^+$ response compared to the CD4$^+$ subset, one after year the primary infection. Given their ability to eliminate virus-infected cells and produce effector cytokines, CD8$^+$ T cells are crucial in the regulation of viral infection; in the contraction phase that follows antigen clearance, a small proportion of effector CD8$^+$ T cells differentiate into memory CD8$^+$ T cells [23]. However, previous studies indicated that, in cases of viral persistence or cancer, the formation of memory CD8$^+$ T cells is compromised. This may explain the huge impairment of CD8$^+$ activation observed in our cohort [24,25]. Moreover, it has been evaluated that, among COVID-19 survivors, the proliferative capacity of memory CD8$^+$ T cells was not as robust as that of CD4+ memory T cells, even if SARS-CoV-2-specific cells remained functionally responsive, demonstrating enhanced effector abilities including activation, proliferation, and secretion of immune mediators [26]. Longitudinal studies have revealed sustained dysregulation of immune responses in subjects with PASC [27]; accordingly, the stratification of our cohort in PASC and non-PASC subjects, revealed an increased proportion of subjects with a persistent memory response in the CD4$^+$ subset in the symptomatic group, while no significant differences emerged in the CD8$^+$ compartment, even though the number of *responders* among symptomatic subjects outnumbered those of asymptomatic ones. This result may provide a deeper insight in the immune regulation in recovered patients, since CD8+ response seems to be generally more hampered, and it does not discriminate subjects according to post-infection repercussions. On the other hand, a more efficient response against spike peptides, in terms of magnitude, emerged to be restricted to CD4$^+$ compartment in PASC subjects. Significant differences came out in CM CD4$^+$ AIM$^+$ cells, producing at least one of the evaluated cytokines. Our data are in line with a previous study that found a higher proportion of CD4$^+$ CM cells in PASC compared to recovered individuals, potentially supporting a persistent reservoir [19]. Indeed, CD4$^+$ CM cells are essential for the establishment of immunological memory and to provide long-lasting protection against pathogens, by activating and orchestrating the functions of other immune cells through the secretion of cytokines (IL-2, TNF-α, IFN-γ) [27]. Our results support previous evidence, suggesting a persistent activation of CD4$^+$ T cells after severe SARS-CoV-2 infection [28]. To investigate more specifically the association between immune activation and post-infection manifestations, we stratified our cohort according to DLCO, a common clinical predictor of residual lung function, and residual symptoms (fever, arthromyalgia, asthenia, cough, dyspnea, diarrhea, dysgeusia and anosmia). We found an increased magnitude of the immune activation in subjects with impaired lung function (DLCO $<$ 80 %) and persistent symptomatology. The association between reduced DLCO resulting from severe COVID-19 and increased immune activation is not entirely clear-cut and may involve multiple determinants, since DLCO reduction after severe lung infection can be ascribed to various factors, including inflammation, fibrosis, microvascular thrombosis, and damage to the alveolar-capillary membrane [29]. Both acute and post-acute COVID-19 cases show a deregulated immune response, which include cytokine storms and excessive inflammation [27], which contribute to lung damage and impaired gas exchange [30]. Similarly, other post-infection symptoms may result from underlying increased inflammation, that may also be explained by antigenemia and/or viral persistence in various anatomical locations [13]. To our knowledge, the deep phenotyping of spike-specific activated T cells and the magnitude of activation in PASC subjects with impaired DLCO or residual symptoms has never been characterized before. Our results confirm the clear hyperactivation of CD4$^+$ T-cells at different maturation stages and with diverse multifunctionality features, already suggested by previous studies [22,31]. The concomitant activation of subsets with different maturation stage, may be counterintuitive, but not unexpected, given the complexity of immune system recovery following severe COVID-19 infections. The persistent activation of naïve T cells may be bystander-driven, and this hypothesis is supported by the elevated levels of T-cell-related cytokines found in the plasma of severe patients even after recovery [29]. CD4$^+$ Tfh AIM$^+$ cells resulted to be significantly increased in subjects with reduced lung function, while multifunctional AIM$^+$ Tfh cells were significantly increased in patients showing residual symptoms. These cells play a crucial role in adaptive immune regulation. A previous study reported that after SARS-CoV-2 infection and/or vaccination, Tfh cells are recalled by antigen re-exposure [32]. We can so speculate that enduring reservoirs of circulating Tfh cells and memory CD4$^+$ T cells, developed during primary infection, may be effectively mobilized upon encountering the antigen again, potentially playing a pivotal role in inducing a persistent immune response. Whether the immune activation state is a cause or consequence of post-infection symptoms remains an unclear matter. Multiple studies suggest an underlying autoimmune pathology dealing with this constant immune hyperactivation [33,34], but the topic is still debated. The AIM assay stands out for its sensitivity and ability to characterize T-cell subpopulations and their responses and permitted a deep characterization of the complex immunological mechanisms associated to post-acute infection sequelae. Additionally, examining different T-cell subpopulations can help to assess whether the prevalence of a particular subset correlates with a higher risk of other specific infections, such as herpes viruses, hepatitis B, or cytomegalovirus reactivation. Our work represents a step forward into understanding the complexity of the immunological picture related to this and multi-faced condition.

However, it presents some limitations, specifically, i) AIM test is an in-vitro test and as such it does not fully replicate the complexity of the in-vivo microenvironment. Indeed, in a physiological setting, interactions between immune cells, cytokines, and tissues create a dynamic environment that can influence T cell activation and response. ii) the limit size of our cohort especially the discrepancy among HC and PASC patients. Therefore, further investigations are needed to confirm and expand upon our findings.

Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the University of Eastern Piedmont (protocol 117/20; NO-MORE COVID study).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

CRediT authorship contribution statement

Chiara Venegoni: Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Davide Raineri:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Camilla Barbero Mazzucca:** Writing – original draft, Methodology, Formal analysis, Data curation. **Ali Ghazanfar:** Data curation. **Giuseppe Cappellano:** Writing – review & editing. **Alessio Baricich:** Writing – review & editing. **Filippo Patrucco:** Writing – review & editing. **Patrizia Zeppegno:** Writing – review & editing. **Carla Gramaglia:** Writing – review & editing. **Piero Emilio Balbo:** Writing – review & editing. **Vincenzo Cantaluppi:** Writing – review & editing. **Giuseppe Patti:** Writing – review & editing. **Mara Giordano:** Writing – review & editing. **Marcello Manfredi:** Writing – review & editing. **Roberta Rolla:** Writing – review & editing. **Pier Paolo Sainaghi:** Writing – review & editing. **Mario Pirisi:** Writing – review & editing. **Mattia Bellan:** Writing – review & editing, Funding acquisition. **Annalisa Chiocchetti:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2025.114103.

Data availability

The authors declare that the data supporting the findings of this study are included within the article or Supplementary Materials and are available from the corresponding author upon reasonable request.

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