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Altered immune surveillance of B and T cells in patients with persistent residual lung abnormalities 12 months after severe COVID-19

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Abstract

Background Post-COVID-19 respiratory sequelae often involve lung damage, which is called residual lung abnormalities, and potentially lead to chronic respiratory issues. The adaptive immune response, involving T-cells and B-cells, plays a critical role in pathogen control, inflammation, and tissue repair. However, the link between immune dysregulation and the development of residual lung abnormalities remains unclear.

Methods 109 patients discharged with residual lung abnormalities after a critical COVID-19 were followed for 12 months and divided as full recovery patients (FRG, $n=88$) and persistent lung abnormalities (PLAG, $n=21$). Cell profiling analysis was done using flow cytometry at 24 h of not antigen-specific in vitro stimulation. Plasma or supernatant levels of IFN-g, IL-4, IL-10, IgM, and IgG were assessed, and 10 patients (5 FRG, 5 PLAG) were randomly selected for detailed immune cell phenotyping and functional analysis of peripheral blood mononuclear cells using flow cytometry.

Results Compared to the FRG group, PLAG exhibited an increase of unswitched ($p=0.0159$) and decreased double-negative activated B-cells ($p=0.0317$), systemic IL-10 levels were lower, displayed reduced frequency of total B-cells, and impaired spontaneous IgM ($p=0.0357$) and IgG ($p=0.0079$) release in culture. Regarding T-cells, PLAG patients showed a reduction in effector memory CD4 + cells ($p=0.0159$) and an increase in CD4 + TEMRA cells ($p=0.0079$) following in vitro stimulation. Notably, the PLAG group also exhibited higher frequencies of central memory CD4 + Th2 (GATA3+) T-cells in response to activation than the FRG group ($p=0.0079$).

Conclusions Patients with residual lung abnormalities 12 months post-critical COVID-19 exhibit impaired B-cell function, increased unswitched B-cells, and higher frequencies of CD4 + TEMRA T-cells following in vitro activation.

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These immune imbalances may contribute to ongoing lung dysfunction and warrant further investigation as a potential mechanism in residual lung abnormalities. Larger studies are necessary to confirm these findings.

Keywords Post-acute sequelae COVID-19, CD4 + T cells, Th1/Th2 profile, B cells, Antibodies

Introduction

Post-acute sequelae of COVID-19 (PASC) is a current health problem worldwide; estimations indicate that at least 65 million individuals are affected [1]. PASC is defined by multiple adverse outcomes after the resolution of acute infection, which can persist for several months or even years [2]. Indeed, up to 70% of hospitalized patients have exhibited PASC in the first year of recovery [3], with respiratory impairment being one of the primary clinical outcomes [4]. However, although the implications of immune activation and inflammation are clinically relevant, they are not yet fully understood. One unresolved question is why PASC symptoms persist for so long and what role the immune system plays in this process.

Reports suggest that the persistence of immunological alterations, such as redistribution of mucosal T-cells, immunoglobulin (Ig) A, and dysregulated CD8 + T-cells, could be associated with post-acute COVID-19 lung sequelae [5, 6]. Another report indicated that at twelve months post-COVID-19, patients show significant differences in diffusing capacity for carbon monoxide (DL_{CO}), with changes occurring between 6- and 12 months post-discharge. Additionally, T-cells from patients with lung sequelae and long COVID exhibit higher responsiveness to SARS-CoV-2 peptides but display exhaustion features [7].

Some studies suggest that post-acute COVID-19 lung sequelae could be associated with the repeated recognition of SARS-CoV-2 proteins by host adaptive immune cells due to a viral reservoir. This may induce the down-regulation of host interferon signaling and cross-reactive autoimmunity through pre-existing or de-novo autoantibodies [8, 9]. B and T lymphocytes do not act independently; for instance, the cytokine microenvironment generated by T-cells affects the differentiation of B cells and, consequently, antibody production [10].

To clarify the contribution of the adaptive immune response in the pathogenesis of post-acute COVID-19 lung sequelae, particularly in those with persistent lung abnormalities (residual lung abnormalities), we followed patients discharged with interstitial damage post-critical COVID-19. At 12 months, some patients continued to experience respiratory symptoms, altered pulmonary function, and interstitial lung lesions as seen on computed tomography, while others had fully recovered. Therefore, we investigated the proinflammatory (Th1) or anti-inflammatory (Th2) profiles and their impact on the frequency and function of B-cells.

Materials and methods

The reader is referred to the supplementary material for details of the materials and methods.

Subjects

Our Institute was a COVID-19 center of attention to severe or critical illness during the pandemic. The survivors' patients were followed in our post-COVID clinic implemented by the Institute and evaluated by a multidisciplinary group of specialists. 150 patients who had critical COVID-19 (all patients required invasive mechanical ventilation) were discharged from the hospital with persistent interstitial lung changes identified by high-resolution computed tomography (HRCT). Of these, 32 patients did not agree to participate in the study protocol, leaving 118 enrolled patients. These patients were followed up every four months with lung functional tests (simple spirometry, single-breath DL_{CO}, and the six-minute walk test [6MWT]). At twelve months of follow-up, in addition to functional tests, another HRCT was performed, obtaining a CT Score (Fig. 1a).

Then, after a year, nine patients were diagnosed with interstitial damage unrelated to COVID-19, and they were excluded from the study. Eighty-eight patients were classified as recovered because they showed ≥ 80% of predicted lung functional values (including FVC and DL_{CO}), no respiratory symptoms, and CT without abnormalities [hereafter called fully recovery group (FRG)]. Twenty-one patients persisted with symptoms, mainly dyspnea on exercise and abnormal lung function related to residual lung abnormalities (< 80% in FVC and DL_{CO}, excluding obstructive patterns such as decreased FEV1). This group showed after one-year, decreased DLco and walked meters in 6MWT, and CT still showing interstitial lung abnormalities, parenchymal bands, and abnormal CT score [hereafter called persistent lung abnormalities group (PLAG)] (Fig. 1b). Ten COVID-19 patients were randomly selected to evaluate the phenotype and function of immune cells, five from PLAG and five from FRG (Fig. 1c); to note a specific panel was used for T-cells phenotype and another for B-cells (details in Table S1 in the Additional file). Each group's sample size was selected using a sample size calculated from our two study groups (PLAG vs. FRG) and considered the DL_{CO} at twelve months post-COVID-19 as the primary endpoint (see sample size in the Additional file). Previously, DL_{CO} has been associated with immune alterations in post-COVID patients [7]. Using this data as a reference, DL_{CO} was chosen as the primary endpoint for the power analysis based

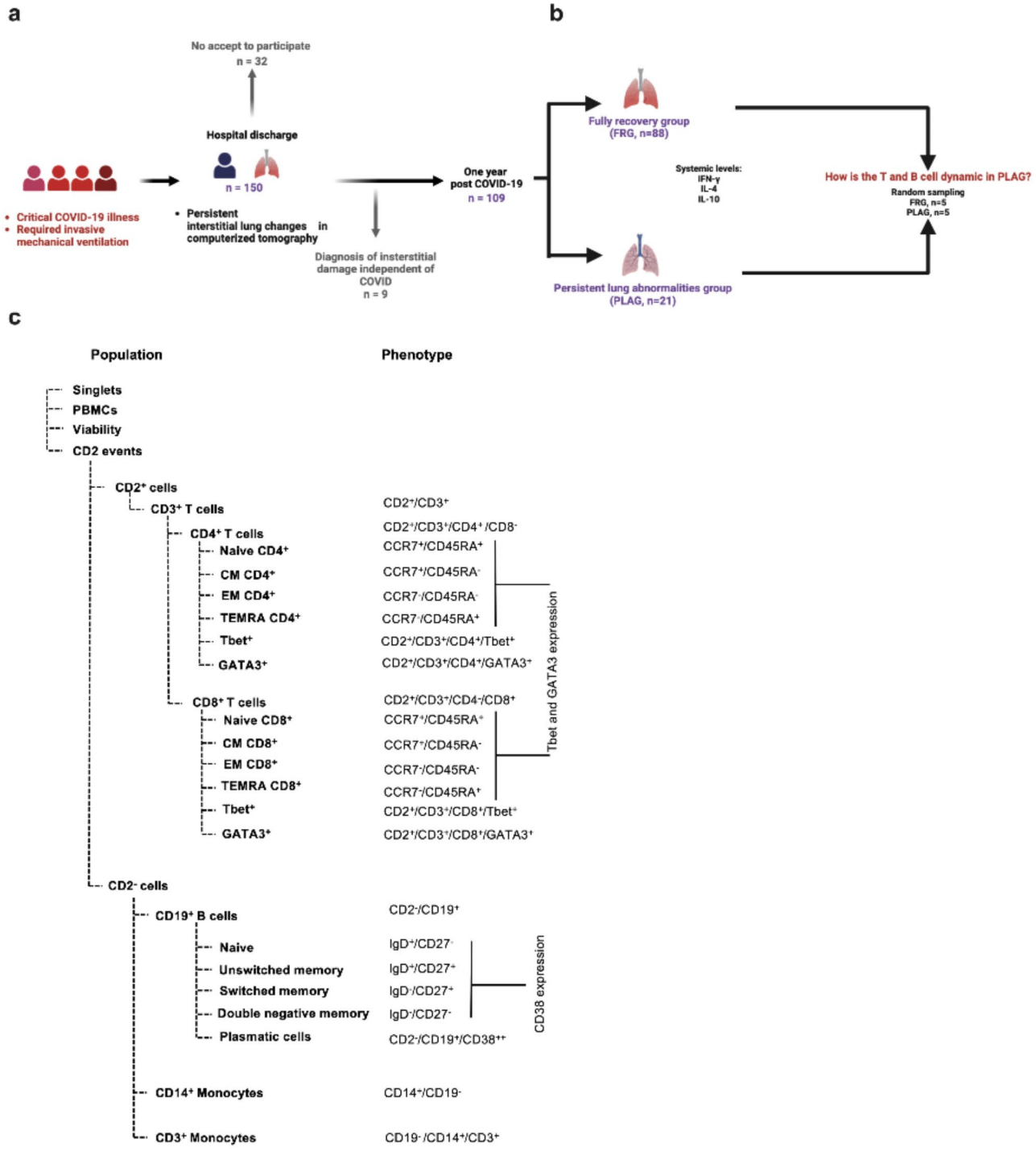

Fig. 1 Patient follow-up and cell phenotype. (**a)** Follow-up of 150 COVID-19 patients discharged with interstitial lung changes after critical illness. (**b)** Of these, 118 patients agreed to participate. Nine were excluded due to COVID-independent interstitial lung damage diagnoses. The remaining 109 underwent clinical evaluations, functional lung tests, and high-resolution computed tomography (HRCT), leading to their classification into a Full Recovery Group (FRG, $n=88$) and a Persistent Lung Abnormalities Group (PLAG, $n=21$). Systemic cytokine and antibody levels were measured, and a randomized selection was performed for flow cytometry analysis. (**c)** The hierarchy and phenotypes of each cell population were evaluated. Cells and plasma for evaluations were obtained from patients 12 months post-discharge

on the significant differences observed between the FRG and PLAG groups at baseline and follow-up.

This protocol was approved by the ethical committee of the Instituto Nacional de Enfermedades Respiratorias Ismael Cosio Villegas (INER, Protocol number C53-20 and B04-22). All individuals signed a consent letter to participate in this study. All procedures were performed in agreement with the 1964 Helsinki Declaration and the ethical standards of the Institutional Ethics Committees.

Peripheral blood mononuclear cells (PBMCs) and culture

PBMCs were isolated by standard Lymphoprep™ (Accurate Chemical-Scientific, Westbury, NY, USA) centrifugation gradient and cryopreserved until use. The PBMCs culture was maintained for 24 h, at 37 °C in a humidified atmosphere containing 5% CO2 and stimulated with monoclonal antibody (mAb) anti-CD3 and CD28 (1ug/mL, BD Pharmingen) (more details in Table S1 in the additional file). PBMCs and plasma were obtained from patients 12 months post-discharge.

Flow cytometry staining and analysis

PBMCs were recovered and prepared for flow cytometry at the end of the culture.

Details of the analysis strategy are observed in Fig. S1 in the additional file, and the subsets hierarchy is shown in Fig. 1c. Details of antibodies are provided in Table S1 in the additional file.

Table 1 Demographics, pulmonary function, and imaging measurements between subjects fully recovered and those with residual lung abnormalities

Variable	FRG $n=88$	PLAG $n=21$	p-value
Age, years (±SD)	54 ± 11	62 ± 9	0.002
Sex, male (%)	60 (68)	9 (43)	0.04
Values at hospital discharge time.			
FVC % predicted (±SD)	93 ± 19	89 ± 26	0.5
DL_{CO} % predicted (±SD)	82 ± 18	68 ± 18	0.001
Meters in 6MWT, (±SD)	438 ± 129	358 ± 150	0.02
% SpO2 at rest, (±SD)	94 ± 2	92 ± 3	0.01
% SpO2 post-exercise, (±SD)	90 ± 3	86 ± 5	< 0.001
Values at 1-year hospital discharge.			
FVC % predicted (±SD)	91 ± 14	86 ± 15	0.3
DL_{CO} % predicted (±SD)	85 ± 15	64 ± 10	< 0.001
Meters in 6MWT (±SD)	449 ± 126	330 ± 140	0.04
% SpO2 at rest (±SD)	93 ± 2	92 ± 3	0.3
% SpO2 post-exercise (±SD)	88 ± 4	83 ± 6	0.005
Basal CT Score (±SD)	20 ± 4	21 ± 4	0.2
CT Score 1 year (±SD)	4 ± 4	8 ± 5	< 0.001

FRG: fully-recovery group; PLAG: persistent lung abnormalities group; SD: standard deviation, FVC: forced vital capacity, DL_{CO}: single breath carbon monoxide diffusing capacity, 6MWT: 6-minute walk test, SpO2: peripheral oxygen saturation. CT: computed tomography

Evaluation of soluble molecules

Plasma and supernatants were collected and stored at -70 °C until use. Interferon-gamma (IFN-g), Interleukin (IL-) 4, IL-10, IgM, and IgG were measured by direct sandwich ELISA assay (antibody-uncoated plate) (more details of antibodies in Table S1 in the additional file).

Statistical analysis

Data were analyzed using GraphPad Software (v 9.0.1). Data are shown as median values and interquartile ranges (IQR, 25–75). According to the number of samples and normality test, statistical analysis was performed using a Kruskal–Wallis's test with Dunnett's post-test for multiple comparisons and a Mann–Whitney U test to compare two groups.

A normality test with the Kolmogorov-Smirnov test was used to analyze clinical data. The descriptive data were presented as frequency, percentages, and mean +/- standard deviation. A comparison between groups was performed using the Fisher exact test for qualitative variables and U-Mann-Whitney for quantitative variables.

In all tests, the standard of significance was set at $p < 0.05$.

Results

Description of the enrolled patients in this study cohort

All 109 patients in this study had critical COVID-19 illness, requiring invasive mechanical ventilation. The patients were categorized into FRG or PLAG groups based on clinical parameters at 12 months post-COVID. The clinical and demographic characteristics of these patients (Total $n=109$) are detailed in Table S2 in the Additional File. A comparison of the groups revealed that the PLAG group was significantly older ($p=0.002$), while the FRG group had a higher proportion of male patients ($p=0.04$). Other parameters, such as comorbidities, duration of invasive mechanical ventilation, symptom onset duration, and hospitalization time, showed no significant differences (Table S2 in the Additional File). Patients randomly selected for the phenotypical and functional evaluation of cells exhibited characteristics similar to the total group, indicating that the sample is representative (Table S3 in the Additional File).

Table 1 shows a comparison between groups of their demographic, pulmonary function results, and CT scores at 12 months of discharge. As is expected, compared to FRG, PLAG had abnormal pulmonary functional tests that persisted after a year; differences in DL_{CO}, meters walking, and SpO2 post-exercise were reported in the PLAG group.

Moreover, Fig. 2 shows a comparative HRCT from a representative patient from both groups. At discharge, patients displayed lung alterations (Fig. 2a and c);

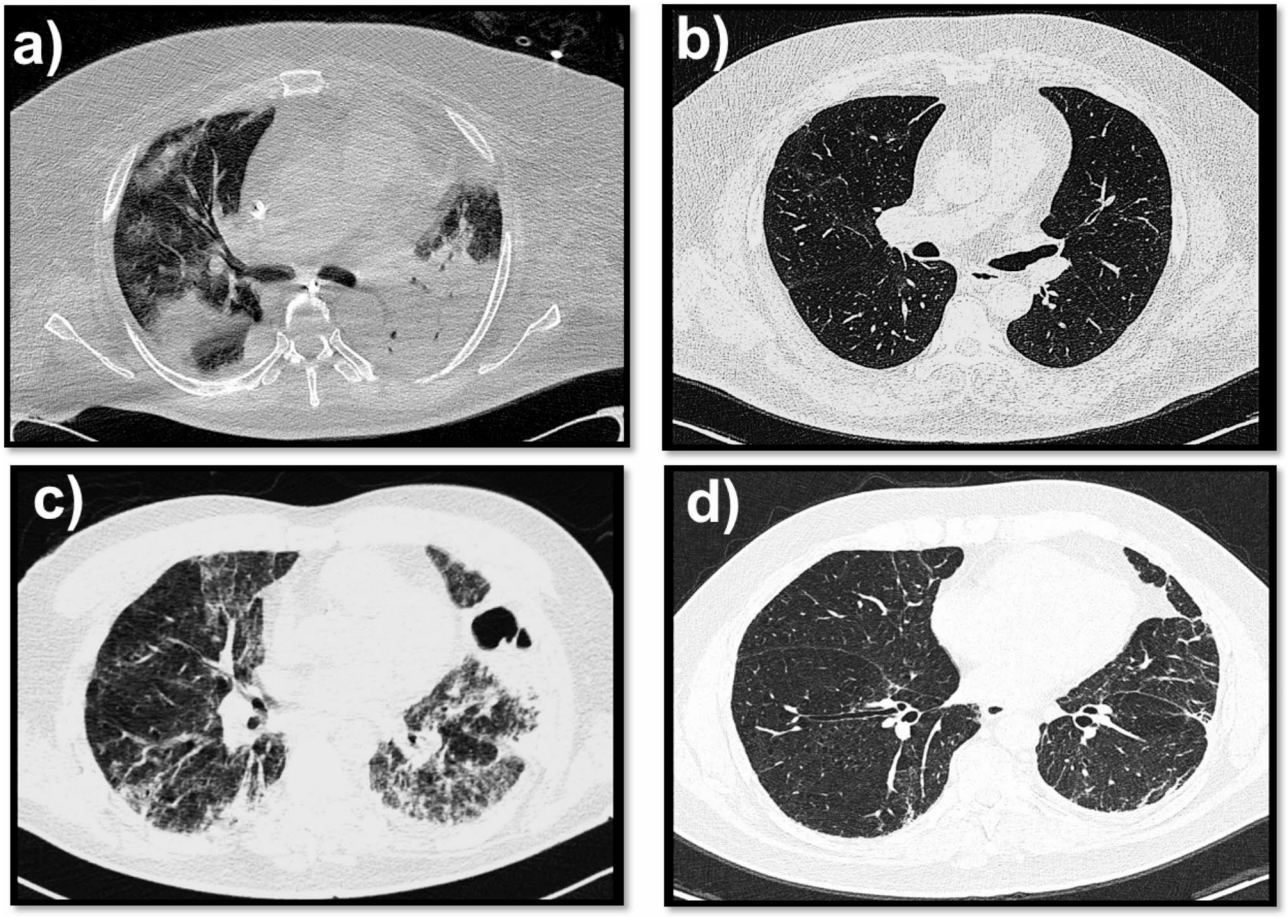

Fig. 2 Computed tomography of a patient fully recovered group (FRG) and a persistent lung abnormalities group (PLAG). (**a)** Male, 40 years old, showing ground-glass attenuation and parenchymal consolidation. (**b)** The same patient showed in (**a**) but at 1 year of follow-up, asymptomatic and normal pulmonary function. (**c)** Male, 58 years old, showing diffuse ground glass and some parenchymal consolidation. (**d)** the same patient showed in (**c**) at 1 year with dyspnea on exercise, decreased DL_{CO}, persistence of reticular lesions, and lost volume in the left lung

however, after 12 months, one group of patients showed a complete recovery (Fig. 2b), and another did not (Fig. 2d).

Patients from PLAG display low systemic IL-10 and IgM levels

Systemic cytokines levels are an indirect indicator of the Th1/Th2 profile. IFN-γ was evaluated as a classical pro-inflammatory Th1 cytokine, and IL-4 and IL-10 were assessed as classical anti-inflammatory Th2 cytokines. Data show that plasma levels of IFN-γ and IL-4 were similar between patients from FRG and PLAG (Fig. 3a and b); however, PLAG showed significantly lower IL-10 levels ($p = 0.0001$) (Fig. 3c).

Among other functions, IL-10 promotes human B-cell differentiation into IgM- or IgG-secreting plasmablasts [11]. Therefore, we analyzed plasma levels of total IgM and IgG in a random sampling of patients, and we found no differences in IgG or IgM levels (Fig. S2 in the Additional file).

Patients from PLAG have decreased total B-cells and lower levels of released immunoglobulins

Five representative patients from the FRG and PLAG groups were randomly selected to evaluate the phenotype and function of immune cells. As shown in Table S3, the selected patients have similar demographic data to the complete cohort, ensuring that the cell evaluation is representative of the FRG and PLAG profiles.

The B-cell gate (CD19+) was identified by flow cytometry. We observed that, compared to FRG, PLAG cells had a decrease in the percentages of B-cells ($p = 0.0317$) (Fig. 3d). Moreover, these B-cells showed lower concentrations of IgM ($p = 0.0357$) (Fig. 3e) and IgG ($p = 0.0079$) after 24 h culture (Fig. 3f).

However, the frequency of B-cell subsets, identified based on IgD and CD27 expression into the CD19 + gate (Fig. S3a in the Additional file), showed a similar distribution of double negative (DN), naïve, unswitched, and switched B-cells between both groups (Fig. S3b and c in the Additional file).

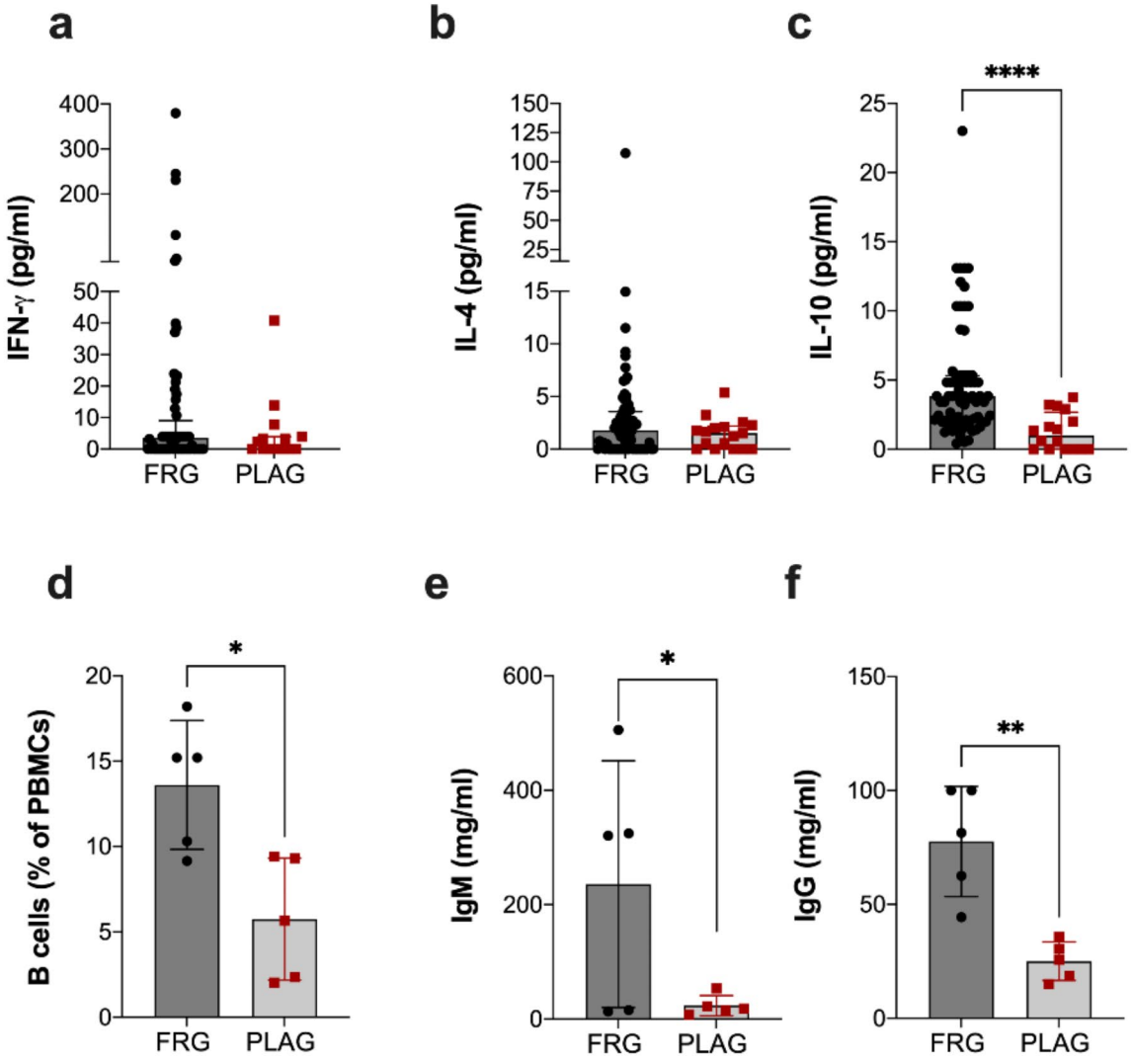

Fig. 3 Patients with persistent lung abnormalities have low systemic IL-10 with a lower frequency of B cells and are less able to spontaneously produce immunoglobulins. Cytokines (**a**) IFN-γ, (**b**) IL-4, and (**c**) IL-10 levels were evaluated in plasma patients from FRG (black dots, $n=88$) and PLAG (red square, $n=21$). Mononuclear cells of FRG (black dots) and PLAG (red square) patients were randomly selected (5 per group), and the phenotype and function were evaluated. (**d**) the frequency of B-cells (CD19+) was evaluated by flow cytometry. Mononuclear cells were cultured, and (**e**) IgM and (**f**) IgG levels were evaluated in the supernatant after 24 h of unstimulated culture. Data are represented as median and IQR values; each symbol represents an individual patient. The Mann–Whitney U test performed statistical comparisons, * $p<0.05$, ** $p<0.01$, **** $p<0.0001$

Patients from PLAG show an imbalance of activated double negative and unswitched B-cells

As shown, patients from PLAG had decreased total B cells and immunoglobulins production, but subsets were not altered. The CD38 expression on B-cells is considered an activation marker [12], and we used it to determine if activated B-cell subsets are different between both groups. First, we evaluated the frequency of B-cell (CD19+), then into the CD19 + gate, the CD38 expression was evaluated to identify activated B-cells, and we observed that the frequency of activated B-cells is similar between FRG and PLAG (Fig. 4a).

Following, based on the IgD and CD27 expression, B-cell subsets were evaluated into activated B-cells (Fig. 4b). Data showed that compared to FRG, cells from PLAG had decreased the frequency of DN ($p=0.0317$) (Fig. 4c), whereas the unswitched was increased ($p=0.0159$) (Fig. 4e); naïve and switched subsets were not modified (Fig. 4d and f, respectively).

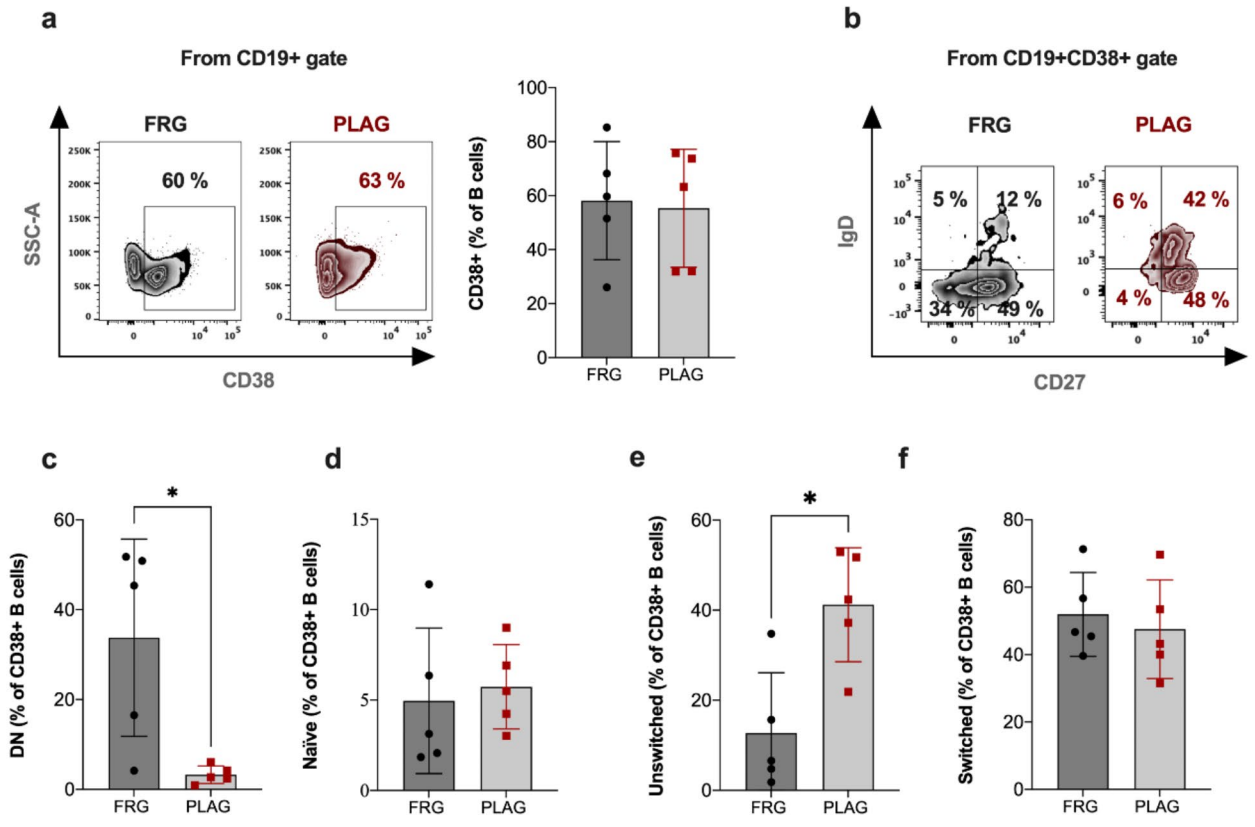

Fig. 4 Patients with persistent lung abnormalities have decreased activated B cells with phenotype double negative and increased activated B cells with phenotype unswitched. Peripheral mononuclear cells from patients with full recovery (FRG; black dots, $n=5$) and persistent lung abnormalities (PLAG, red square, $n=5$) were prepared for flow cytometry after 24 h of unstimulated culture. (**a**) First, the CD19 + gate was done; then, the CD38 expression on CD19 + cells was identified to obtain the frequency of activated total B-cells. (**b**) Into CD19 + CD38 + cells gate, IgD and CD27 expression were evaluated to identify activated B-cell subsets. The frequency of activated (**c**) double negative (DN), (**d**) naïve, (**e**) unswitched, and (**f**) switched B-cells is shown in graphics. Data are represented as median and IQR values; each symbol represents an individual patient. The Mann–Whitney U test performed statistical comparisons, * $p<0.05$

In response to polyclonal stimulus, T-cells from FRG, but not PLAG, show a decreased frequency of CD4 + Th2 T-cells

Due to the relevance of T-cells to induce the cytokines microenvironment, we first assessed the frequency of T-cells (CD2 + CD3+) (Fig. 5a) and the CD4 + and CD8 + subsets by flow cytometry (Fig. 5b). We observed that T-cell frequency is similar in patients from FRG and PLAG; similarly, the distribution of CD4 + and CD8 + T-cells is comparable between study groups (Fig. 5c).

Then, we evaluated by flow cytometry the Th1 (Tbet+) and Th2 (GATA3+) profiles in total CD4 + T-cells (Fig. 5d and f, respectively); cells were treated with a polyclonal stimulus to confirm if the profile is different between patients from FRG and PLAG before and after an activation.

At baseline, the frequency of Th1 CD4 + cells was not different, and this behavior is maintained even after an activation process; however, PLAG induces a discreet increase of Th1 CD4 + cells, and it is not statistically different from FRG (median 40% vs. 17%, respectively) (Fig. 5e).

Regarding the frequency of Th2 CD4 + cells, at baseline, FRG showed a higher but not significant level of Th2 CD4 + cells compared with PLAG (median 37% vs. 30%, respectively). However, under an activation process, FRG showed a down-regulation of Th2 CD4 + cells ($p=0.0159$), which was not observed in PLAG; therefore, under activation, the frequency of Th2 CD4 + in PLAG is significantly higher compared with FRG ($p=0.0079$) (Fig. 5g).

Patients from PLAG have altered frequency of CD4 + T memory subsets and Th1/Th2 profiles in response to an activation process

According to the CCR7 and CD45RA expression, T-cells are divided into effector memory (EM), central memory (CM), naïve, and CD4 effector memory T-cells re-expressing CD45RA (TEMRA) subpopulations. We examined the distribution of these CD4 + T memory

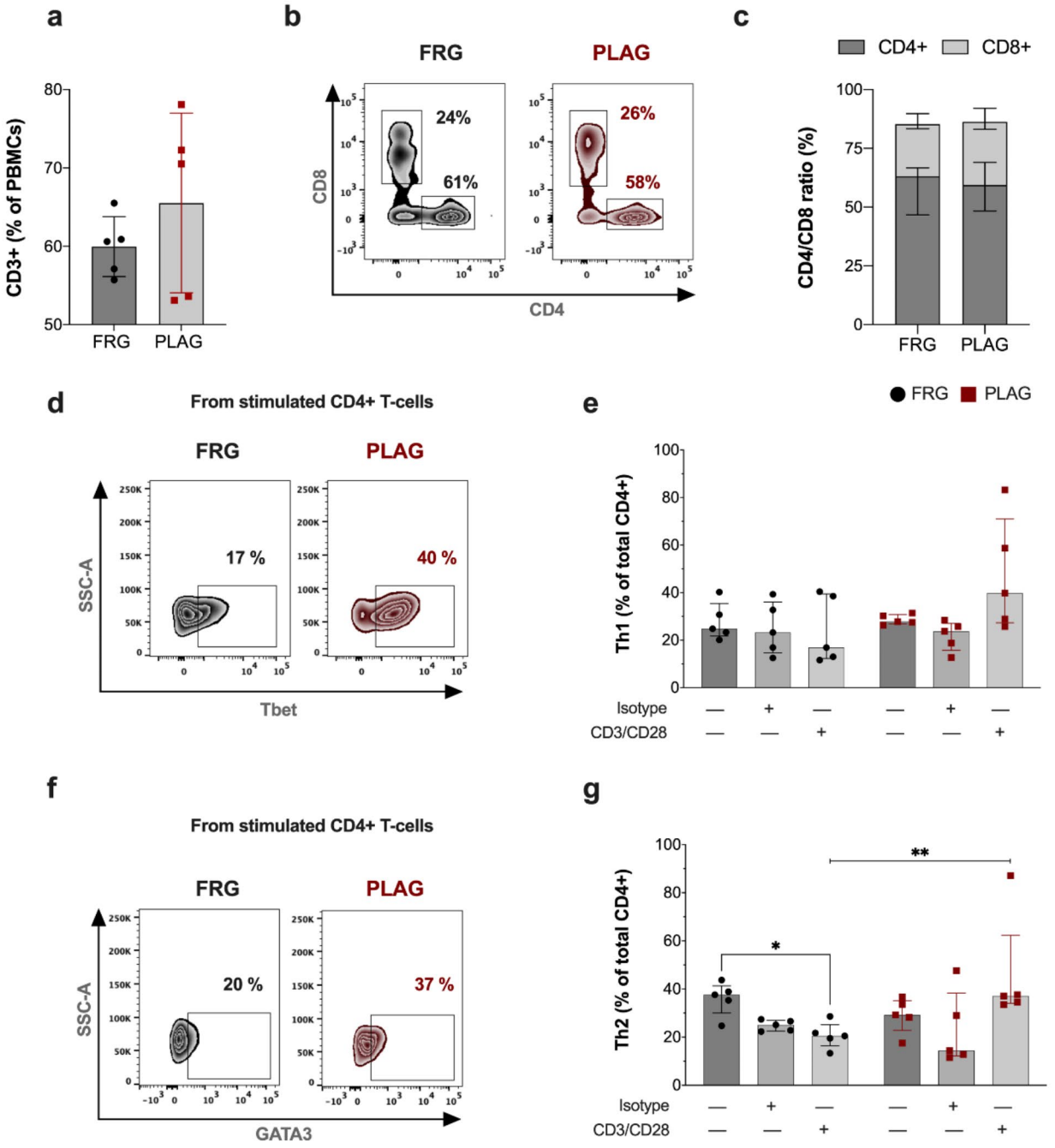

Fig. 5 Full recovery patients, but not those with persistent lung abnormalities, can down-regulate CD4 + GATA3 + T-cells as a response to polyclonal stimulus. Peripheral mononuclear cells are from patients FRG (black dots, $n = 5$) or PLAG (red square, $n = 5$), stimulated for 24 h with anti-CD3/CD28 (1 µg/mL). An unstimulated condition and isotype control were included as control stimulation. (**a**) The frequency of CD3 + cells (T lymphocytes) at baseline and (**b**) co-receptor CD4 and CD8 expression was evaluated, and (**c**) is shown in the graphic. (**d**) The Tbet + expression and (**e**) frequency were assessed in CD3 + CD4 + T-cells cultured with a polyclonal stimulus. (**f**) The GATA3 + expression and (**g**) frequency were assessed in CD3 + CD4 + T-cells cultured with a polyclonal stimulus. Data are represented as median and IQR values; each symbol represents an individual patient. The Kruskal-Wallis test performed statistical comparisons, ** $p < 0.01$, * $p < 0.05$

subsets by flow cytometry (Fig. S4a in the Additional file); data showed that FRG and PLAG have similar frequencies of CD4 + T memory subsets at baseline, but in response to an activation process, cells from PLAG patients display a decrease in the frequency of $CD4+_{EM}$ ($p = 0.0159$) and an increase of $CD4+_{TEMRA}$ ($p = 0.0079$) compared with its baseline frequency (Fig. S4b-d in the Additional file). CD8 + T-cell subsets showed no

differences at baseline or under the activation process (data not shown).

Moreover, we observed that under an activation process, cells from PLAG show an increased frequency of subsets CD4 + naïve, CM and TEMRA positive to Tbet+ (Th1) compared with its baseline condition ($p = 0.0079$, $p = 0.0159$, and $p = 0.0079$, respectively); and this condition was also different even compared to activated CD4 + T-cells from FRG (naïve: $p = 0.0159$; TEMRA: $p = 0.0079$). CD4 + EM cell subsets did not show differences at baseline or under the activation process (S5a-b in the Additional file).

On the other hand, the Th2 (GATA3+) profile showed that cells from the FRG have a decreased frequency of Th2 in the subsets CD4 + CM ($p = 0.0079$) and EM ($p = 0.0159$) in response to the activation process, compared to its baseline. Whereas CM under an activation process is higher in PLAG than FRG ($p = 0.0079$), and naïve and TEMRA cell subsets did not show differences at baseline or under the activation process (S5c-d in the Additional file).

Monocytes remain unaltered in patients with persistent lung abnormalities

Monocytes are the main precursor of macrophages, and reports suggest that a small subpopulation of monocytes expressing CD3 is associated with inflammation status [13]. To discard the possibility of this cell subset being monocyte/T-cell complexes, which have been reported [14]. First, a gate of CD2- cells was done (to discharge T cells fraction); posteriorly, the CD14 + single cells were identified (Fig. S1 in the Additional file). Our data indicated that the frequency of total monocytes (CD2-CD14 + cells) and the monocytes CD3 + subset (CD2-CD14-CD3+) is similar between FRG and PLAG (Fig. S6 in the Additional file).

Discussion

Studies of patients discharged with interstitial lung abnormalities are scarce, and how the immune system is affected in patients who maintain lung PACS is still being determined. In this study, we followed a group of convalescent COVID-19 patients discharged with lung abnormalities after severe acute disease. At 12 months post-COVID-19, respiratory symptoms, pulmonary function, and computed tomography scans allowed us to classify them into two groups: those fully recovered and those with persistent lung abnormalities. We then evaluated the function and distribution of the main immune cells.

Age has been established as a known contributor to the development of fibrotic lung lesions and lower total lung capacity and DL_{CO} in COVID-19 convalescent patients [15]. From an immune perspective, high serum levels of IL-17, IL-2, IL-1β, IL-6, and TNF, and low levels of IL-4 and IL-10 appear to constitute a cytokine profile of long COVID-19 [16, 17].

Several lines of evidence have also indicated that high IL-10 levels are associated with the anti-inflammatory and tissue-protective properties of the lungs during COVID-19 [18]. The results of this study are consistent with this finding and suggest that alterations in pulmonary function tests, and residual lung abnormalities in COVID-19 convalescents could be related to low IL-10 levels, affecting the cells of the immune system.

Our data suggest a low B-cell frequency in patients with persistent lung abnormalities. During severe SARS-CoV-2 infection, a reduction in the unswitched memory B cell population and an expansion of double-negative (DN) B cells have been found [19, 20]. In our study, using the gating strategy for CD27 and IgD on B-cells, we observed that DN B-cells significantly decreased while the frequency of unswitched memory B-cells increased.

We hypothesize that the affection of DN B-cells may play a crucial role in the pathogenesis of pulmonary sequels. However, given that these cells can acquire different functions based on the various subtypes of "conventional" DN B-cells [21], future studies should address the relative expression of Tbet, CD21, and CD11c and explore the four subsets of DN B cells.

While DN2 and DN3 subset expansion has been associated with a poor prognosis of COVID-19 and negatively correlated with several ventilatory parameters, such as respiratory rate and oxygen saturation levels [19, 20], its distribution in patients with PASC and persistent lung abnormalities has not been determined. Several lines of evidence have indicated that DN2 and DN3 B cells could maintain autoimmune responses to COVID-19 [22].

When we explored the expression of CD38 on B cells, which is known to be strongly upregulated by activated B cells [23], we found that patients from PLAG had a decreased frequency of double-negative B cells, suggesting characteristics of premature exhaustion. This early exhaustion may contribute to the defective responses to produce IgM or IgG, as observed in this work. This finding could be associated with the high level of inhibitory receptors, as members of the FcRL family reported in the context of several infectious diseases [24].

During severe SARS-CoV-2 infection, a reduction in unswitched memory B cells has been reported [19, 25]. However, another report regarding these unswitched memory B cells in the convalescent stage mentioned a frequency recovery, with no differences compared to healthy controls [26]. In keeping with these reports, we did not find any difference in the frequency of unswitched memory B cells, indicating this population has minimal fluctuation in recovery and non-recovery patients.

However, how activation modulates the unswitched memory B-cell population in convalescent patients is unclear.

Our results indicate that patients with persistent lung abnormalities show a decrease in the unswitched memory population after stimulation. Besides, they maintained an activated status before and after stimulation, contributing to a sustained function that could result in persistent immune activation, as reported in other studies [27], and potentially leading to cellular exhaustion, as seen in other immune cells [28]. Nonetheless, the role of unswitched memory B-cells in contributing to or protecting against PASC remains to be determined. Interestingly, a higher frequency of non-stimulated unswitched memory B-cells correlated with reduced symptom duration after mild SARS-CoV-2 infection [29], indicating a protective response by this subset, although its frequency after activation has not been explored. Therefore, an imbalance in DN and activated-unswitched could contribute to the persistence of residual lung abnormalities.

Beyond B-cells, inflammation and T-cells, particularly the TEMRA subset, have been associated with developing post-COVID sequelae [30]. Previous data suggested that long-COVID patients show an increased frequency of CD8 + CD28- CD27- T-cells, indicating an exhausted profile in response to SARS-CoV-2 peptides [7]. In this context, we found that CD4 + T-cells from patients with residual lung abnormalities, after activation, led to an increase in the TEMRA subset. However, when proinflammatory CD4 + T-cell subsets were identified, naïve, and TEMRA cells increased.

Our study did not clarify the exhaustion or senescence status of these T-cells. However, our data suggest an imbalance of T-cell subsets that likely impacts B-cell function. The distribution of CD4 + T-cell subsets has been associated with differential clinical outcomes. For instance, higher levels of $CD4+_{TEMRA}$ cells could explain the tissue damage observed in PACS patients. Interestingly, $CD4+_{TEMRA}$ cells can be stimulated without requiring TCR cross-linking by a cognate antigen, implying the low TCR avidity reported in T-cells of PASC patients [30, 31].

To identify the Th1/Th2 profile induced after T-cell activation, we induced a polyclonal system. Inflammation has been a key focus in studying COVID-19-related immune responses. Recently, we reported that post-COVID-19 lung alterations could be linked to changes in CD4 + and CD8 + T-cells distribution, with immunosuppression-related mechanisms, such as PD-L2-mediated pathways, impacting T-cell activation [32]. Evidence shows that the PD/PD-L axis, associated with immunosuppression, negatively impacts T-cell function [33, 34]. Our findings and other reports [35] provide evidence that a prolonged Th2 activation may play a role in inducing or maintaining post-COVID-19 lung damage.

This study did not explore the regulatory T cells (Treg), characterized by the expression of the CD4 coreceptor and the forkhead box P3 (FOXP3) and exhibit regulatory function. Evidence suggests that a Treg subset expressing the T-cell immunoglobulin and ITIM domain (TIGIT) exerts robust suppressive activity on cellular immunity and predisposes septic individuals to opportunistic infections [36]. Reports indicate that TIGIT + Treg cells are central in suppressing Th1 and Th17 responses [37]. In the context of COVID-19, high levels of TIGIT + Treg cells have been associated with lung dysfunction and hospital-acquired bacteremia [38]. Given the strong immunosuppressive function of this subset, future studies are necessary to assess the presence of TIGIT + Treg cells in patients with persistent lung abnormalities and whether they contribute to suppressing a Th1 microenvironment, potentially exacerbating post-COVID-19 lung damage.

This study is not without limitations. First, the flow cytometry data should be validated with a larger sample size to ensure more robust conclusions. Second, we did not include an age-matched healthy group that had never contracted COVID-19, which would help clarify age-normal immune status. Finally, future studies should evaluate the specific immune response to SARS-CoV-2 peptides to determine whether the observed changes are part of a general immune response or specific to antigen exposure.

Conclusions

Our study reveals significant changes in the phenotype of B and CD4 + T-cells in convalescent COVID-19 patients with PACS and persistent lung abnormalities. These findings suggest that an imbalance between double-negative and unswitched B-cells, along with the expansion of a proinflammatory, terminally differentiated $CD4+_{TEMRA}$ population after stimulation, may contribute to residual lung abnormalities. These observations reflect the immune responsiveness of these cells when activated, suggesting that these cell subsets should be further explored as potential targets for future interventions or biomarkers; however, larger studies are necessary to confirm these findings.

Abbreviations

IPF	Idiopathic Pulmonary Fibrosis
FRG	Full Recovery Patients
PLAG	Persistent Lung Abnormalities
PASC	Post-Acute Sequelae of COVID-19
Ig	Immunoglobulin
HRCT	High-Resolution Computed Tomography
DLco	Single-Breath Diffusing Capacity of CO (Carbon Monoxide)
6MWT	Six-Minute Walk Test
CT	Computed Tomography score
FVC	Forced Vital Capacity
PBMCs	Peripheral Blood Mononuclear Cells

IL	Interleukin
Th	T helper cells
IFN-γ	Interferon gamma
CD	Cluster of Differentiation
Tbet	T-box transcription factor TBX21
GATA3	GATA Binding Protein 3
CCR7	C-C Motif Chemokine Receptor 7
EM	Effector Memory
CM	Central Memory
TEMRA	Effector Memory T-cells Re-Expressing CD45RA

Supplementary Information

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Supplementary Material 1

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Author contributions
Conceptualization: IBR, MS, and LCG; methodology: JFG, FTQ, CPB, and LARL; validation: IBR, MS, and LCG; formal analysis: JFG, RFV, GPR, and LCG; investigation: JFG, and LCG; patients' recruitment and clinical data curation: IBR, FTQ, ACM, and MS; writing-original draft preparation: JFG, MS and LCG; writing-review and editing: JFG, IBR, FTQ, CPB, LARL, ACM, RFV, GPR, MS, and LCG.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

Conflict of interest
The authors declare that they have no competing interests.

Ethical approval
This protocol was approved by the ethical committee of the Instituto Nacional de Enfermedades Respiratorias Ismael Cosio Villegas (INER, Protocol number C53-20 and B04-22). All individuals signed a consent letter to participate in this study. All procedures were performed in agreement with the 1964 Helsinki Declaration and the ethical standards of the Institutional Ethics Committees.

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