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Adaptive immune responses to SARS-CoV-2 in recovered severe COVID-19 patients

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ABSTRACT

Background: There is an imperative need to determine the durability of adaptive immunity to SARS-CoV-2. We enumerated SARS-CoV-2-reactive $CD4^+$ and $CD8^+$ T cells targeting S1 and M proteins and measured RBD-specific serum IgG over a period of 2–6 months after symptoms onset in a cohort of subjects who had recovered from severe clinical forms of COVID-19.

Patients and Methods: We recruited 58 patients (38 males and 20 females; median age, 62.5 years), who had been hospitalized with bilateral pneumonia, 60% with one or more comorbidities. IgG antibodies binding to SARS-CoV-2 RBD were measured by ELISA. SARS-CoV-2-reactive $CD69^+$-expressing-IFNγ-producing-$CD4^+$ and $CD8^+$ T cells were enumerated in heparinized whole blood by flow cytometry for ICS.

Results: Detectable SARS-CoV-2-S1/M-reactive $CD69^+$-IFN-γ $CD4^+$ and $CD8^+$ T cells were displayed in 17 (29.3%) and 6 (10.3%) subjects respectively, at a median of 84 days after onset of symptoms (range, 58–191 days). Concurrent comorbidities increased the risk (OR, 3.15; 95% CI, 1.03–9.61; $P = 0.04$) of undetectable T–cell responses in models adjusted for age, sex and hospitalization ward. Twenty-one out of the 35 patients (60%) had detectable RBD-specific serum IgGs at a median of 118 days (range, 60–145 days) after symptoms onset. SARS-CoV-2 RBD-specific IgG serum levels were found to drop significantly over time.

Conclusion: A relatively limited number of subjects who developed severe forms of COVID-19 had detectable SARS-CoV-2-S1/M IFNγ $CD4^+$ and $CD8^+$ T cells at midterm after clinical diagnosis. Our data also indicated that serum levels of RBD-specific IgGs decline over time, becoming undetectable in some patients.

1. Background

Experimental evidence supports a major role of neutralizing antibodies (NtAb) and skewed Th1 functional immune responses in preventing and controlling SARS-CoV-2 infection [1–4]. NtAbs targeting the receptor binding domain (RBD) of the viral spike protein (S) appear to display maximum specificity and potency [5–7]. Broad specificity to structural and non–structural proteins has been reported across SARS-CoV-2-reactive $CD4^+$ and $CD8^+$ T cells, of which S, membrane (M) and nucleocapsid (N) proteins are immunodominant in most individuals [8–18]. Both SARS-CoV-2-specific NtAb and T cells are readily detectable in a large proportion of acute or short-term convalescent COVID-19 patients [8–18]. Data on SARS-CoV infection suggest that memory B and T cells have a potential for long-lasting persistence (over years) [19,20], yet the durability of SARS-CoV-2 adaptive immunity remains to be established. Determining whether SARS-CoV-2 B- and T-cell responses

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persist over time following natural infection or after vaccination seems of paramount relevance in designing effective public health policies to prevent virus transmission and spread.

2. Objectives

We enumerated SARS-CoV-2-reactive $CD4^+$ and $CD8^+$ T cells targeting S and M proteins, and measured IgG antibodies binding to RBD of S protein, along a timeframe of up to 6 months after symptoms onset in a cohort of recovered COVID-19 patients who had been hospitalized due to severe clinical forms of the disease.

3. Study design

3.1. Patients and specimens

A total of 58 non–consecutive patients (38 males and 20 females; median age, 62.5 years; range, 27 – 82 years) were recruited at a median of 85 days (range, 58 – 191 days) after onset of COVID-19 symptoms. SARS-CoV-2 infection was diagnosed by RT-PCR (between February 26 and May 16, 2020) [21]. The only patient inclusion criterion was the availability of serum and/or whole blood specimens for B- and T-cell immunity analyses. Medical history and laboratory data were retrospectively reviewed. Clinical severity of COVID-19 was graded following World Health Organization criteria [22]. Blood was also collected from seven non-SARS-CoV-2-exposed healthy individuals (up to March 2020) who served as controls. The current study was approved by the Research Ethics Committee of Hospital Clínico Universitario INCLIVA (March 2020).

3.2. SARS-CoV-2 RBD IgG immunoassay

IgG antibodies binding to SARS-CoV-2 RBD made in Sf9 cells infected with recombinant baculoviruses (Invitrogen, CA, USA) were measured by an enzyme-linked immunosorbent assay (ELISA) as previously described [23]. Results are reported in Absorbance Units/ml (AU/ml).

3.3. SARS-CoV-2-reactive IFN-γ $CD4^+$ and $CD8^+$ T cells

SARS-CoV-2-reactive $CD69^+$-expressing-IFNγ-producing-$CD4^+$ and $CD8^+$ T cells were enumerated in whole blood by flow cytometry for intracellular cytokine staining (ICS) (BD Fast immune, BD Biosciences, San Jose, CA, USA) as previously described [21]. Two sets of 15-mer overlapping peptides (11 mer overlap) encompassing the SARS-CoV-2 Spike glycoprotein N–terminal 1–643 amino acid sequence (158 peptides) and the entire sequence of SARS-CoV-2 M protein (53 peptides), were used in combination for stimulation (1 μg/ml per peptide) during 6 h, in the presence of CD28 and CD49d costimulatory mAbs and brefeldin A (10 μg/ml), the latter after two hour incubation. Peptide mixes were obtained from JPT Peptide Technologies GmbH (Berlin, Germany). The appropriate positive (phytohemagglutinin) and isotype controls were used. The total number of SARS-CoV-2-reactive $CD4^+$ or $CD8^+$ T cells was calculated by multiplying the percentages of $CD4^+$ or $CD8^+$ T cells producing IFNγ on stimulation (after background subtraction) by the absolute $CD4^+$ or $CD8^+$ T-cell counts. At least 100,000 events/sample were acquired. Responses $\geq$ 0.1% were considered specific [21].

3.4. Laboratory measurements

Clinical laboratory investigation included serum levels IL–6, ferritin and Dimer–D, which were monitored at least twice weekly during hospital stay.

3.5. Statistical methods

Frequency comparisons for categorical variables were carried out using the Fisher exact test. Differences between medians were compared using the Mann–Whitney *U*-test. The Spearman's rank test was used for analysis of correlation between continuous variables. For logistic regression analyses, variables with P values < 0.1 in univariate models were included in multivariate models. Two-sided exact P-values were reported. A P-value < 0.05 was considered statistically significant. The analyses were performed using SPSS version 20.0 (SPSS, Chicago, IL, USA).

4. Results

4.1. Patient clinical features

All 58 patients in this cohort developed severe forms of COVID-19 requiring hospitalization either in the intensive care unit (ICU) (n = 21) or in other hospital wards (n = 37). All patients presented with bilateral pneumonia and 60% had one or more comorbidities, including diabetes mellitus, asthma, hypertension, dyslipidemia, cancer or chronic lung disease. All ICU patients underwent mechanical ventilation. Median hospitalization of patients was 16 days (range, 6–61 days). Patients in ICU or other hospital wards were comparable regarding age, sex and comorbidities (not shown), and all were treated at some point with anti-inflammatory drugs, namely corticosteroids alone ($n = 15$), tocilizumab alone ($n = 6$) and with both drugs ($n = 37$).

4.2. SARS-CoV-2-reactive IFN-γ $CD4^+$ and $CD8^+$ T cells in recovered COVID-19 patients

SARS-CoV-2-S1/M-reactive $CD69^+$-IFN-γ $CD4^+$ and $CD8^+$ T cells were enumerated at a median of 84 days after symptoms onset (range, 58–191 days) (Supplementary Figure 1). Of the 58 patients, 17 (29.3%) and 6 (10.3%) had detectable SARS-CoV-2 $CD4^+$ and $CD8^+$ T-cell responses, respectively. Only two patients displayed both SARS-CoV-2-reactive T-cell subsets. SARS-CoV-2 $CD4^+$ and $CD8^+$ T-cell counts ranged from 0.98 to 43.75 cells/μL, and from 0.48 to 2.98 cells/μL, respectively (median, 4.83 and 1.13 cells/μL, respectively).

Fig. 1A shows SARS-CoV-2 T-cell reactivity according to the sampling timeframe after symptoms onset (arbitrarily categorized as 2–3 months vs. >3 months). Overall, we found no difference between the percentage of patients with or without detectable $CD4^+$ ($P = 0.40$) or $CD8^+$ ($P = 0.12$) T cells across comparison groups; nevertheless, none of the patients sampled beyond day 130 after COVID-19 presentation (n = 3) exhibited either SARS-CoV-2 $CD4^+$ or $CD8^+$ T-cell responses. Of note, patients with or without detectable SARS-CoV-2-reactive T cells were monitored within a comparable timeframe (median, 91 days; range, 60 – 118 days vs. median 83 days, range, 58 – 191 days; $P = 0.18$).

4.3. SARS-CoV-2 RBD-specific IgGs in recovered COVID-19 patients

Serum specimens for quantitation of SARS-CoV-2 RBD-specific IgGs were available from 35 patients, and collected at a median of 118 days (range, 60 – 145 days) from onset of symptoms. Twenty-one of the 35 patients (60%) had detectable RBD-specific IgGs (median, 1.8 AU/mL; range, 0.99–4.14 AU/mL). RBD-specific IgG reactivity according to time of sampling is shown in Fig. 1B. A comparable number of patients had detectable responses at both time points ($P = 0.84$).

Out of the 21 patients exhibiting RBD-specific IgG reactivity, 10 (47.6%) had measurable SARS-CoV-2 T-cell responses ($CD4^+$ T cells in 6 patients and $CD8^+$ T cells in the remainder). Of the 14 patients lacking RBD-specific IgGs, 5 had detectable SARS-CoV-2 $CD4^+$ T cells and none had SARS-CoV-2 $CD8^+$ T cells.

Eighteen of the 35 patients had paired serum samples collected at the time of hospitalization (median, 22 days after symptoms onset; range,

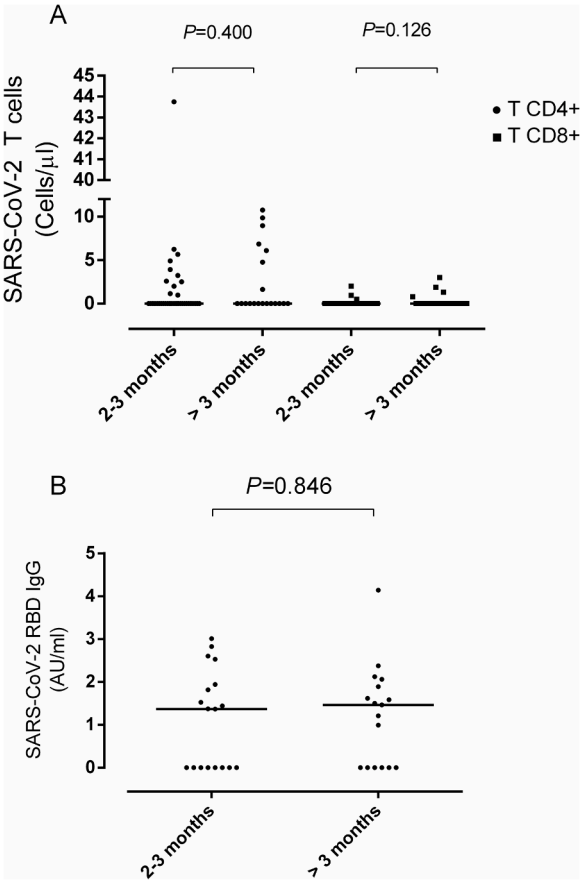

Fig. 1. SARS-CoV-2 T- and B-cell responses in individuals recovered from severe COVID-19. Peripheral blood SARS-CoV-2-S1/M-reactive $CD69^+$-expressing IFN-γ-producing $CD4^+$ and $CD8^+$ T cells (A) and SARS-CoV-2-RBD-specific IgG levels (B) according to the time of sampling following symptoms onset. Bars indicate median levels. *P* values are shown.

8–34 days) and after recovery (median, 120 days after symptoms onset; range 93–145 days). SARS-CoV-2 RBD-specific IgGs were detectable in 17 patients at the first time point and 14 at the latter. As shown in Fig. 2, SARS-CoV-2 RBD-specific IgG serum levels were found to drop significantly over time (from a median of 4.97 AU/ml to a median of 1.51 AU/ml; $P < 0.001$).

Finally, we found no correlation between SARS-CoV-2 RBD-specific IgG levels and SARS-CoV-2 $CD4^+$ and $CD8^+$ T-cell counts ($P = 0.12$ and $P = 0.14$, respectively).

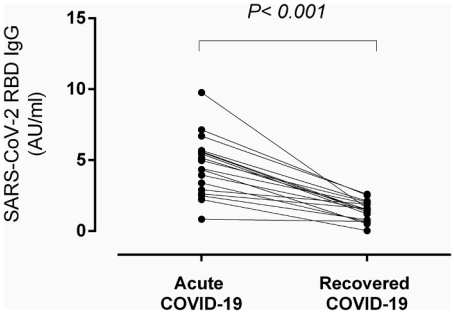

Fig. 2. Kinetics of SARS-CoV-2-RBD-specific IgG levels in individuals recovered from severe COVID-19. Serum levels of such an antibody specificity were measured at the time of hospitalization (Acute COVID-19; median, 22 days after symptoms onset; range, 8–34 days) and after recovery (recovered COVID-19; median, 120 days after symptoms onset; range 93–145 days). AU refers to Absorbance Units. *P* value is shown.

4.4. Factors associated with detectable SARS-CoV-2-reactive T cells or RBD-specific IgGs in recovered COVID-19 patients

The detection rate of SARS-CoV-2-reactive T cells (either $CD4^+$, $CD8^+$ or both) was comparable across patients admitted to ICU or other medical wards ($P = 0.82$) (Table 1). Likewise, both median SARS-CoV-2 $CD4^+$ and $CD8^+$T-cell counts were similar between groups ($P = 0.31$ and $P = 0.1$ for $CD4^+$ and $CD8^+$ T cells, respectively). Neither age nor sex was found to influence either the likelihood (Table 1) or magnitude ($P > 0.21$ for all comparisons) of detectable SARS-CoV-2 T-cell responses. In contrast, patients displaying one or more comorbidities were significantly ($P = 0.04$) less likely to exhibit detectable T-cell responses (Table 1), although median T-cell counts were not significantly dissimilar across comparison groups ($P = 0.1$ and $P = 0.19$ for $CD4^+$ and $CD8^+$ T cells, respectively). In fact, comorbidities increased the risk (OR, 3.15; 95% CI, 1.03–9.61; $P = 0.04$) of undetectable T-cell responses in logistic regression models adjusted for age, sex and hospitalization ward (ICU vs. others).

The likelihood of detecting SARS-CoV-2-RBD-specific IgGs was not influenced by age ($P = 0.67$), or presence of comorbidities ($P = 0.65$), but was higher in males ($P = 0.004$) and in ICU patients ($P = 0.019$) (Table 1). Both factors were found to increase the likelihood of detecting an antibody response in multivariate models adjusted for age and comorbidities (OR, 11.71; 95% CI, 1.86–73.7; $P = 0.009$ and OR, 6.57; 95% CI, 1.04–41.4; $P = 0.04$, respectively). Nevertheless, neither of these parameters had a significant impact on serum SARS-CoV-2 IgG levels (not shown).

The net state of inflammation shortly after viral infections may shape the quality and strength of ongoing adaptive immunity responses [24]. In this context, we next compared serum peak levels of several inflammatory biomarkers, including IL-6, ferritin and Dimer-D, measured within the first 15 days after hospitalization among recovered COVID-19 patients with or without detectable SARS-CoV-2 T-cell responses. No significant differences ($P > 0.45$ for all comparisons) were found between comparison groups for any of these parameters. A similar observation was made for SARS-RBD-specific IgGs ($P > 0.2$ for all comparisons). Furthermore, either weak or no correlation between serum peak levels of IL-6, ferritin or Dimer-D and SARS-CoV-2 $CD4^+$ and $CD8^+$ T-cell counts or SARS-CoV-2 RBD-specific IgG levels were observed (Table 2).

5. Discussion

To our knowledge, previous studies on the features of SARS-CoV-2-specific adaptive immunity in convalescent COVID-19 patients [1–4, 8–18,23] mostly involved recently recovered patients (up to 3 months from onset of symptoms), of whom a large percentage (45–95%) consistently exhibited both T- and B-cell responses. The dynamics of such immune responses beyond this time point remains largely

Table 1
Detectable SARS-CoV-2-reactive T cells and RBD-specific IgGs in COVID-19 patients according to demographics and clinical factors.

Parameter	SARS-CoV-2 $CD4^+$ or $CD8^+$ T cell response			SARS-CoV-2 RBD-specific IgGs		
	Yes	No	*P* value	Yes	No	*P* value
Sex: Male/female	13/8	25/12	0.66	18/3	5/9	0.04
Age: ≤62.5 / >62.5 years[a]	13/8	16/21	0.07	9/12	7/7	0.67
ICU admission: Yes/No	8/13	13/24	0.82	13/8	3/11	0.019
Comorbidities: Yes/No	9/12	26/11	0.04	15/6	9/5	0.65

[a] Median age of patients.
RBD, receptor binding domain of S protein.

Table 2
Correlation between serum levels of inflammatory biomarkers and SARS-CoV-2 $CD4^+$ or $CD8^+$T cells and SARS-CoV-RBD-specific IgGs.

Parameters	Spearman Rho value	P value
IL-6/ SARS-CoV-2 $CD4^+$ T cells	0.07	0.68
IL-6/ SARS-CoV-2 $CD8^+$ T cells	0.11	0.51
IL-6/ SARS-CoV-2 RBD-IgGs	−0.01	0.96
D-D/ SARS-CoV-2 $CD4^+$ T cells	−0.10	0.46
D-D/ SARS-CoV-2 $CD8^+$ T cells	0.20	0.12
D-D/ SARS-CoV-2 RBD-IgGs	0.4	0.01
Ferritin/ SARS-CoV-2 $CD4^+$ T cells	−0.04	0.76
Ferritin/ SARS-CoV-2 $CD8^+$ T cells	0.16	0.24
Ferritin/ SARS-CoV-2 RBD-IgGs	0.34	0.06

D-D, Dimer-D; IL-6, interleukin-6; RBD, receptor binding domain.

unexplored. Here, we assessed SARS-CoV-2 T- and B-cell immune responses in patients who had recovered from severe COVID-19 at medium term (up to 6 months) after disease presentation. We used a whole blood flow cytometry ICS method for enumeration of activated and functional (IFN-γ-producing) SARS-CoV-2-reactive $CD4^+$ and $CD8^+$ T cells, targeting the S1 region of the S protein and the M protein, which are readily detectable ($CD4^+$ T cells more frequently), in most short-term recovered COVID-19 patients regardless of disease severity [10–18]. Our ICS assay appears capable of quantifying coronavirus cross-reactive T cells, as two out of seven non-exposed individuals exhibited detectable responses (not shown).

Likewise, RBD-specific IgG levels strongly correlate with NtAb titers as measured using live or S-pseudotyped SARS-CoV-2, so they can be used as a proxy for inferring the neutralizing activity of sera [6,7,23].

Several major findings arose from our study. First, SARS-CoV-2-S1/M-reactive-IFN-γ $CD4^+$ and $CD8^+$ T cells were detected in a limited number of recovered patients from severe COVID-19 (around 30% and 10% for $CD4^+$ and $CD8^+$ T cells, respectively). Furthermore, we were unable to document SARS-CoV-2 T-cell reactivity beyond day 130 after COVID-19 diagnosis. This latter observation must be interpreted with caution given the low number of patients tested within that timeframe. In a previous study recruiting severe COVID-19 patients [21], we reported that around 40% of patients were capable of mounting SARS-CoV-2-S1/M-reactive IFN-γ-producing $CD8^+$ T cell responses shortly after infection; in that study, SARS-CoV-2-reactive $CD4^+$ T cell responses were not assessed. Nevertheless, other studies including hospitalized COVID-19 patients [11,12] found detectable functional $CD4^+$ T-cell responses to both S1 and M proteins in a large fraction of patients at short-term convalescence. Taken collectively, the above and our observations herein suggest that functional SARS-CoV-2-S1/M-reactive T-cell frequencies in peripheral blood may wane over time in patients with severe COVID-19. In this sense, suboptimal expansion of functional T cells, may be a hallmark in patients developing the severest forms of COVID-19 [1,25]. The impact of COVID-19 severity on the durability of SARS-CoV-2 T-cell responses over time remains to be elucidated. Patients with mild COVID-19 appear to develop more robust and durable (up to 6 month since the onset of symptoms) responses than individuals experiencing SARS-CoV-2 asymptomatic infection [26–28]; yet, SARS-CoV-2-(both S and M)-specific $CD4^+$ and $CD8^+$ T cells appeared to decline over time in a cohort skewed toward mild forms of COVID-19, although they remained detectable in many subjects at more than 6 months after COVID-19 diagnosis [29]. Nevertheless, memory $CD4^+$ T cell frequencies trended lower in hospitalized COVID-19 cases compared to non-hospitalized cases by around 8 months after COVID diagnosis [29]. In contrast, long-term (≥200 days after the onset of symptoms) SARS-CoV-2-specific T cell memory, as evaluated by IFN-γ ELISpot and activation induced markers, and the proportion of polyfunctional cells and T_{SCM} cells among SARS-CoV-2-specific memory T cells, was found to be comparable between mild patients and moderate/severe/critical patients, although only 23 patients in this series developed severe/-critical disease [30]. Dissimilarities across the above studies and ours in terms of the methods used for enumeration of SARS-CoV-2-reactive T cells, and notably regarding the clinical characteristics of patient in the cohorts may account for the discrepancy.

Second, in the current series, 60% of individuals displayed detectable SARS-CoV-2-RBD-specific IgGs at 2–5 months following COVID-19 diagnosis. In this context, it has been consistently reported that RBD IgG seroconversion occurs almost universally in moderate to severe COVID-19 patients within 3–4 weeks after onset of symptoms [6,7,22]. The above data thus suggest that the likelihood of detecting such antibody specificity in sera diminishes over time. Moreover, by analyzing paired serum specimens collected within the first month after symptoms onset and by 3–5 months after COVID-19 diagnosis in recovered individuals, we showed that the detection rate decreased over time, as was the case of SARS-CoV-2-RBD-specific serum IgG levels.

Third, we found no correlation between SARS-CoV-2-reactive IFN-γ $CD4^+$ T cells and RBD-specific antibody levels, suggesting that SARS-CoV-2 T- and B-cell responses may follow divergent kinetics. In contrast other studies [10,31] found a strong correlation between NtAb antibody titers and the numbers of virus-specific T cells targeting S or N proteins in short-term convalescent individuals. Our data are congruent with the idea that $CD4^+$ Th1-cell help is not strictly required to sustain SARS-CoV-2-RBD-specific IgG responses over the medium term, at least in the population group analyzed herein; rather, T follicular helper (Tfh) cells, which unfortunately were not enumerated here, have been shown to play a major role in helping B cells to generate highly matured antibodies [see 32 for review].

Fourth, the presence of one or more comorbidities, but not age, sex or COVID-19 severity (ICU vs. non–ICU), was identified as a factor presumably hampering the persistence of peripheral blood SARS-CoV-2-S1/M-reactive T cells in recovered COVID-19 patients. As for RBD IgGs, both sex (male) and COVID-19 severity appeared to increase the probability of detectable responses at medium term after disease presentation, although neither of these had an impact on RBD IgG levels.

Fifth, despite uncontrolled inflammation driven by innate immune response shortly following viral infection may negatively affect the strength and durability of arising T-cell responses [24], we found serum peak levels of inflammatory biomarkers measured early after COVID-19 presentation not to differ across recovered COVID-19 patients with or without detectable SARS-CoV-2-reactive T cells or SARS-COV-2-RBG IgGs. Moreover, little or no correlation was observed between the magnitude of systemic inflammation and SARS-CoV-2 immune parameters.

The current study has several limitations, predominantly, the reduced cohort size which precluded performing statistically meaningful sub analyses such as those potentially addressing the impact of the use of corticosteroids and tocilizumab on the development of SARS-CoV-2 T-cell responses. First, T-cell immunity analyses were conducted at a single time point. We cannot rule out that detectability of the two SARS-CoV-2-reactive T cells by the immunoassay used herein may fluctuate over time. Moreover, no whole blood specimens drawn at the time of patient hospitalization were available for T-cell immunity assessment. Second, T cells and antibodies targeting other antigen specificities, which may afford protection against SARS-CoV-2, were not evaluated. Third, T-cell functionalities other than IFN-γ production were not explored. Fourth, we cannot be certain whether our whole blood T-cell immunoassay might be less sensitive than others using isolated PBMCs. Fifth, taking serum peak levels of IL-6, Dimer-D and ferritin during the first 15 days after symptoms onset is an admittedly arbitrary time point for analyses which may not have captured the true net state of systemic inflammation generated shortly after SARS-CoV-2 infection. Sixth, the possibility that long-term memory SARS-CoV-2 reactive T or B cells could be present in extravascular compartments was not explored.

In summary, we have shown that a relatively low number of subjects who developed severe forms of COVID-19 had detectable SARS-CoV-2-S1/M IFNγ $CD4^+$ and $CD8^+$ T cells at medium term after clinical diagnosis (up to 6 months), particularly those with concurrent

comorbidities. Our data also indicated that serum levels of RBD-specific IgGs decline over time, becoming undetectable in some patients. Elucidating whether individuals lacking these specific adaptive immune responses are susceptible to reinfection is beyond the scope of the current study and needs to be addressed in future research.

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Ethical approval

The current study was approved by the Research Ethics Committee of Hospital Clínico Universitario INCLIVA (March 2020).

Author statement

B.O., E.A., I.T., P.A., M.J.R. and R.G.-R.: Methodology and data validation. B.O, E.A., I.T.: Formal analysis. J.R.-D., J.B., D.N.: Conceptualization, supervision. M.L.B., J.R. and J.S.-C.: Clinical Staff in charge of patients. D.N.: writing the original draft. All authors reviewed and approved the original draft.

Declarations of Competing Interest

None.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcv.2021.104943.

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