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ORIGINAL ARTICLE

T cell–mediated response to SARS-CoV-2 in liver transplant recipients with prior COVID-19

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Whether immunosuppression impairs severe acute respiratory syndrome coronavirus 2-specific T cell–mediated immunity (SARS-CoV-2-CMI) after liver transplantation (LT) remains unknown. We included 31 LT recipients in whom SARS-CoV-2-CMI was assessed by intracellular cytokine staining (ICS) and interferon (IFN)-γ FluoroSpot assay after a median of 103 days from COVID-19 diagnosis. Serum SARS-CoV-2 IgG antibodies were measured by ELISA. A control group of nontransplant immunocompetent patients were matched (1:1 ratio) by age and time from diagnosis. Posttransplant SARS-CoV-2-CMI was detected by ICS in 90.3% (28/31) of recipients, with higher proportions for IFN-γ-producing $CD4^+$ than $CD8^+$ responses (93.5% versus 83.9%). Positive spike-specific and nucleoprotein-specific responses were found by FluoroSpot in 86.7% (26/30) of recipients each, whereas membrane protein-specific response was present in 83.3% (25/30). An inverse correlation was observed between the number of spike-specific IFN-γ-producing SFUs and time from diagnosis

Abbreviations: CI, confidence interval; CMI, cell-mediated immunity; CMV, cytomegalovirus; COVID-19, coronavirus disease 2019; ELISA, enzyme-linked immunosorbent assay; ICS, intracellular cytokine staining; IFN-γ, interferon-γ; IQR, interquartile range; LT, liver transplantation; M, SARS-CoV-2 membrane protein; mAb, monoclonal antibody; N, SARS-CoV-2 nucleoprotein; OD, optical density; PBMC, peripheral blood mononuclear cell; PBS, phosphate-buffered saline; S, SARS-CoV-2 spike glycoprotein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SARS-CoV-2-CMI, SARS-CoV-2-specific T cell–mediated immunity; SD, standard deviation; SFU, spot forming unit; SOT, solid organ transplantation.

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(Spearman's rho: −0.418; *p* value = .024). Two recipients (6.5%) failed to mount either T cell–mediated or IgG responses. There were no significant differences between LT recipients and nontransplant patients in the magnitude of responses by FluoroSpot to any of the antigens. Most LT recipients mount detectable—but declining over time—SARS-CoV-2-CMI after a median of 3 months from COVID-19, with no meaningful differences with immunocompetent patients.

KEYWORDS
clinical research/practice, complication: infectious, immunosuppression/immune modulation, infection and infectious agents, infection and infectious agents - viral, infectious disease, liver transplantation/hepatology, monitoring: immune

1 | INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic has disrupted liver transplantation (LT) programs worldwide. Although the clinical course and outcomes of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection seem to be better for LT recipients compared to other transplant groups,[1] mortality rates among hospitalized patients during the first wave reached 20%.[2,3]

The understanding of the magnitude and durability of natural immunity following SARS-CoV-2 infection among immunocompromised patients is of paramount relevance to determine the individual susceptibility to reinfection. Previous studies have assessed SARS-CoV-2-specific T cell–mediated immunity (SARS-CoV-2-CMI) in kidney transplant (KT) recipients recovered from COVID-19, revealing that interferon (IFN)-γ-producing $CD4^+$ and $CD8^+$ T cells reactive to the spike (S) glycoprotein and other structural proteins may be detected for at least 6 months.[4–7] Specific data for LT recipients, however, are lacking. Variations in the magnitude of SARS-CoV-2-specific responses according to the type of immunosuppression, severity of COVID-19 and timing from transplantation are still largely unknown.

The present study was aimed at analyzing the frequencies of SARS-CoV-2-specific IFN-γ-producing T cell responses and their clinical correlates in a single-center cohort of LT recipients at various times from the diagnosis of COVID-19. To this end, we used two different methods: the intracellular cytokine staining (ICS) and a fluorescent ELISpot for cytokine secretion (IFN-γ FluoroSpot assay).

2 | MATERIALS AND METHODS

2.1 | Study population and setting

All adult LT recipients regularly seen at the University Hospital "12 de Octubre" (Madrid, Spain) with a diagnosis of COVID-19 confirmed by real-time reverse transcription polymerase chain reaction (RT-PCR) or rapid antigen detection test (Panbio™ COVID-19 Ag Rapid Test Device, Abbott Diagnostics) between March 15 and November 13, 2020, were eligible for inclusion. No exclusion criteria regarding clinical severity or timing from transplantation were applied. The assessment of SARS-CoV-2-CMI was performed at a single time point per patient (between December 9 and 21, 2020). The research was carried out in accordance with the ethical standards as laid down in the Declarations of Helsinki and Istanbul. The study protocol was approved by the institutional Research Ethics Committee (ref. 20/314) and all participants provided written informed consent.

The management of LT recipients with post-transplant COVID-19—including the use of antiviral agents, immunomodulatory therapies and supportive care—was in accordance with the national and local guidelines in place at each time, which have evolved throughout the study period according to the available evidence.[8,9] Once recovered, patients were followed-up until January 2021 by phone calls or at the outpatient clinic according to routine practice, in order to assess the development of symptoms compatible with SARS-CoV-2 reinfection.

2.2 | SARS-CoV-2-CMI assessment by ICS

Whole blood specimens were collected in sodium heparin tubes and shipped at room temperature to the Hospital Clínico Universitario (Valencia, Spain), where they were processed within 24 h. Enumeration of SARS-CoV-2-specific IFN-γ-producing $CD69^+$ $CD4^+$ and $CD8^+$ T cells was performed by flow cytometry for ICS (BD Fastimmune™ kit, BD Biosciences, San Jose, CA) following a protocol originally designed for the assessment of cytomegalovirus (CMV)-specific cell-mediated immunity.[10,11] In brief, heparinized whole blood (0.5 ml) was simultaneously stimulated for 6 h with two sets of 15-mer overlapping peptides (11-mer overlap) encompassing the SARS-CoV-2 S glycoprotein N-terminal 1- to 643-amino acid sequence (158 individual peptides) (PepMix™ SARS-CoV-2 Spike Glycoprotein, JPT peptide Technologies GmbH, Berlin, Germany) and the entire sequence of the membrane (M) protein (53 peptides) (Epitope Mapping Peptide Set [EMPS] SARS-CoV-2 VME1, JPT) at a concentration of 1 μg/ml per peptide and in the presence of costimulatory monoclonal antibodies (mAbs) targeting CD28 and CD49d (1 μg/ml). Brefeldin A (10 μg/ml) was added for the last 4 h of incubation. Next, blood was lysed (BD FACS lysing solution) and frozen at −80°C. On the day of testing, stimulated blood was thawed at 37°C, washed, permeabilized (BD permeabilizing solution)

and stained with fluorochrome-labeled mAbs (anti-CD3-APC-Cy7, anti-CD69-PE, anti-CD4 or CD8-PerCP-Cy5.5, and anti-IFN-γ-FITC) for 1 h at room temperature. Samples mock-stimulated with phosphate-buffered saline (PBS)/dimethyl sulfoxide solution (without peptides) and costimulatory antibodies or stimulated with phytohemagglutinin (1 mg/ml) as non-specific mitogen (Sigma-Aldrich) were run in parallel in all experiments. Appropriate isotype controls were also used. Next, cells were washed, resuspended in 200 μl of 1% paraformaldehyde in PBS, and analyzed within 2 h on a FACScanto flow cytometer using BD FACSDiva™ software (BD Biosciences). $CD3^+$/$CD4^+$ and $CD3^+$/$CD8^+$ events were gated and analyzed for the T cell activation marker $CD69^+$ and IFN-γ production. SARS-CoV-2-specific IFN-γ-producing $CD69^+$ $CD4^+$ and $CD8^+$ T cells were enumerated by multiplying the corresponding percentages of T cells that produced IFN-γ upon peptide stimulation (after background subtraction) by absolute $CD4^+$ and $CD8^+$ T cell counts, and responses ≥0.1% were considered specific.

2.3 | SARS-CoV-2-CMI assessment by the IFN-γ FluoroSpot assay

Whole blood specimens were processed at the Department of Immunology of the Hospital Universitario "12 de Octubre" within 24 h from sampling. Peripheral blood mononuclear cells (PBMCs) were freshly isolated by density-gradient centrifugation using Ficoll-Paque and seeded at 300 000 cells/well in IFN-γ FluoroSpot™ plates (MabTech, Nacka Strand, Sweden) with cell culture medium containing RPMI, 1% L-glutamine, 1% penicillin/streptomycin, 10% fetal bovine serum and anti-CD28 mAb (1 μg/ml). Test wells were performed in duplicate and supplemented with 15-mer overlapping peptides covering the S1 domain of the S glycoprotein (166 individual peptides) (SARS-CoV-2 S1 scanning pool, MabTech), the nucleoprotein (N protein) (102 peptides) (EMPS SARS-CoV-2 NCAP-1, JPT), and the M protein (53 peptides) (EMPS SARS-CoV-2 VME1, JPT) at a final concentration of 1 μg/ml. Negative control wells lacked peptides, and positive control wells included anti-CD3 mAb (MabTech). The fluorophore conjugates used were anti-BAM-490 and SA-550. Assays were incubated for 16–18 h at 37°C. The readout was performed following the manufacturer's instructions for the Human IFN-γ/IL-2 FluoroSpot^{PLUS} kit and spots were counted using an automated IRIS™ FluoroSpot Reader System (both from MabTech). To quantify antigen-specific responses, spots of the negative control wells were subtracted from the mean spots test wells, and the results were expressed as IFN-γ-producing spot forming units (SFUs) per 10^6 PBMCs. Results were excluded if negative control wells had >80 SFUs/10^6 PBMCs or positive control wells had <400 SFUs/10^6 PBMCs. Reponses were considered positive if the results were at least three times higher than the mean of the negative control wells and above of the following antigen-specific cut-off values (which had been established by using a control group of 30 healthcare workers with no microbiological, serological or clinical evidence of SARS-CoV-2 infection): >25 SFUs/10^6 PBMCs for the S glycoprotein (S1 domain), >14 SFUs/10^6 PBMCs for the N protein, and >21 SFUs/10^6 PBMCs for the M protein.

2.4 | SARS-CoV-2 serology

Serum SARS-CoV-2 IgG antibodies targeting the S1 protein were detected with the Euroimmun Anti-SARS-CoV-2 enzyme-linked immunosorbent assay (ELISA) (Euroimmun AG, Lübeck, Germany) according to manufacturer's instructions. Optical density (OD) values were measured at 450 nm using the PR 3100 microplate reader (Bio-Rad Life Science, Marnes-La-Coquette, France). Results were evaluated semi-quantitatively by calculating the ratio of the OD value of the sample over the OD value of the calibrator (relative OD), with the following cut-off values: ratio <0.8: negative; ratio ≥0.8 to <1.1: borderline; and ratio ≥1.1: positive.

2.5 | Nontransplant controls

In order to analyze the impact of post-transplant immunosuppression on the development and magnitude of SARS-CoV-2-CMI measured by the IFN-γ FluoroSpot assay, we selected a group of immunocompetent patients diagnosed with COVID-19 that were hospitalized at our institution in March 2020. Nontransplant controls were matched in a 1:1 ratio with LT recipients according to age and time interval from the diagnosis of COVID-19 to the assessment of SARS-CoV-2-CMI. None of the controls had other causes of immunosuppression (i.e., active malignancy, HIV infection or chronic corticosteroid therapy).

2.6 | Statistical analysis

Quantitative data were shown as the mean ± standard deviation (SD) or the median with interquartile range (IQR), and qualitative variables were expressed as absolute and relative frequencies. Categorical variables were compared using the χ^2 test. The Mann-Whitney U test was applied for continuous variables. Correlations between continuous variables were evaluated using the Pearson's correlation coefficient or the Spearman's rho. The agreement between both methods used for assessing SARS-CoV-2-CMI (ICS and FluroSpot) was evaluated by the Kappa index. Statistical analysis was performed with SPSS version 20.0 (IBM Corp.).

3 | RESULTS

3.1 | Study population

Overall, 35 LT recipients regularly followed-up at our institution with a RT-PCR-confirmed diagnosis of SARS-CoV-2 infection between March 15 and November 13 were approached. Four

of them refused to participate, and 31 patients (88.6%) were finally included (Table 1). The median interval from the diagnosis of COVID-19 to the assessment of SARS-CoV-2-CMI of 103 days (IQR: 72.8–261.5). Mean tacrolimus and mycophenolic acid trough levels at that point were 4.9 ± 2.4 and 2.7 ± 1.5 ng/ml, respectively. Nineteen patients (61.3%) have a repeated SARS-CoV-2 RT-PCR after a median of 20 days (IQR: 14–66) from the initial positive testing. Eight of them (42.1%) had a persistently positive RT-PCR test for a median of 19 days (IQR: 11–26.5). A 46-year-old female patient that had undergone transplantation 6.3 years earlier and was on tacrolimus monotherapy developed a mildly symptomatic new episode of SARS-CoV-2 infection that was documented by RT-PCR after 5.5 months from the initial diagnosis of COVID-19.

3.2 | Post-transplant SARS-CoV-2-CMI assessed by ICS

Any SARS-CoV-2-CMI (≥0.1%) was detectable by ICS in 90.3% (28/31) of patients. In detail, SARS-CoV-2-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cell responses were observed in 90.3% (28/31) and 83.9% (26/31), respectively. Absolute counts were 2.8 ± 2.0 $CD4^+$ T cells/μl and 1.9 ± 2.1 $CD8^+$ T cells/μl. One recipient—a 57-year-old female patient that had undergone LT 16 years earlier and was on tacrolimus—lacked any detectable T cell response after 290 days from the diagnosis of COVID-19.

When SARS-CoV-2-specific T cell counts were analyzed according to the interval from the diagnosis of COVID-19, there was a non-significant decrease over time for both $CD4^+$ (3.3 ± 2.3 versus 2.1 ± 1.6 cells/μl from 30–90 days to >180 days; *p* value = .153) and $CD8^+$ T cell subsets (2.7 ± 2.7 versus 1.6 ± 1.8 cells/μl, respectively; *p* value = .223) (Figure 1).

3.3 | Post-transplant SARS-CoV-2-CMI assessed by the IFN-γ FluoroSpot assay

Any SARS-CoV-2-CMI was detectable by the IFN-γ FluoroSpot assay in 90.0% (27/20) of patients (one sample was not evaluable). In detail, positive S glycoprotein-specific (>25 SFUs/10^6 PBMCs) and N protein-specific (>14 SFUs/10^6 PBMCs) responses were present in 86.7% (26/30) each, whereas the rate of M protein-specific response (>21 SFUs/10^6 PBMCs) was 83.3% (25/30).

We also analyzed T cell responses in a continuous manner according to the time elapsed from COVID-19. The magnitude of S glycoprotein-specific (514 ± 387 versus 174 ± 267 SFUs/10^6 PBMCs; *p* value = .004) and M protein-specific responses (263 ± 226 versus 104 ± 87 SFUs/10^6 PBMCs; *p* value = .038) decreased from the first (30–90 days) to the last time interval analyzed (>180 days), respectively (Figure 2). An inverse correlation was accordingly found between the number of S glycoprotein-specific IFN-γ-producing SFUs per 10^6 PBMCs and the interval from COVID-19 diagnosis

TABLE 1 Demographics and clinical characteristics of the study population (*n* = 31)

Variable	
Age at diagnosis, years, mean ± SD	61.7 ± 10.6
Male gender, *n* (%)	24 (77.4)
Ethnicity, *n* (%)	
Caucasian	28 (90.3)
Latino	3 (9.7)
Major chronic comorbidities, *n* (%)	
Hypertension	13 (41.9)
Diabetes mellitus	11 (35.5)
Obesity	3 (9.7)
Heart disease	2 (6.5)
Underlying cause of end-stage liver disease, *n* (%)	
HCV infection	15 (48.4)
Alcoholic cirrhosis	7 (22.6)
HBV infection	3 (9.7)
HBV/HDV coinfection	2 (6.5)
Drug-induced acute liver failure	2 (6.5)
Other[a]	2 (6.5)
Diagnosis of hepatocellular carcinoma, *n* (%)	13 (41.9)
Time from transplantation to diagnosis of COVID−19, months, median (IQR)	76.5 (15.2–185.0)
Immunosuppression at diagnosis of COVID−19, *n* (%)	
Prednisone	8 (25.8)
Daily dose, mg, median (IQR)	5 (5–15)
Tacrolimus	19 (61.3)
Mofetil mycophenolate	19 (61.3)
mTOR inhibitor	4 (12.9)
Azathioprine	1 (3.2)
Type of immunosuppressive regimen	
Mofetil mycophenolate monotherapy	7 (22.6)
Tacrolimus, mofetil mycophenolate and prednisone	6 (19.4)
Tacrolimus and mofetil mycophenolate	6 (19.4)
Tacrolimus monotherapy	6 (19.4)
mTOR inhibitor monotherapy	4 (12.9)
Other[b]	2 (6.5)
Clinical severity of COVID−19, *n* (%)	
Outpatient management	19 (61.3)
Hospitalization, no supplemental oxygen requirements	5 (16.1)
Hospitalization, low-flow oxygen (FiO_2 <40%)	6 (19.3)
Hospitalization, high-flow oxygen (FiO_2 ≥40%)	1 (3.2)
Mode of diagnosis of SARS-CoV−2 infection, *n* (%)	

(Continues)

TABLE 1 (Continued)

Variable	
RT-PCR	29 (93.5)
Rapid antigen detection test	2 (6.5)
Radiological diagnosis of COVID–19 pneumonia, *n* (%)	11 (35.5)
Laboratory values at presentation, mean ± SD	
Lymphocyte count, cells ×10^9	0.9 ± 0.5
Lactate dehydrogenase, IU/L	248.0 ± 68.9
C-reactive protein, mg/dl	4.9 ± 6.3
Treatment, *n* (%)	
Hydroxychloroquine	8 (25.8)
Azithromycin	1 (3.2)
Lopinavir/ritonavir	1 (3.2)
Remdesivir	2 (6.5)
Low-to-intermediate-dose corticosteroids	4 (12.9)
Methylprednisolone boluses	3 (9.7)
Tocilizumab	1 (3.2)
Follow-up from the diagnosis of COVID–19, days, median (IQR)	183 (110.5–305.3)

Abbreviations: COVID-19, coronavirus disease 2019; HBV, hepatitis B virus; HCV, hepatitis C virus; HDV, hepatitis D virus; IQR, interquartile range; mTOR, mammalian target of rapamycin; RT-PCR, reverse transcription polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SD, standard deviation.

[a]Autoimmune hepatitis and mushroom poisoning.

[b]Everolimus and prednisone, and azathioprine, everolimus, and prednisone.

(Spearman's rho: −0.418; *p* value = .024), reflecting a decrease in the magnitude of response over time.

3.4 | Agreement between ICS and IFN-γ FluoroSpot assay

We restricted this analysis to T cell responses against the S glycoprotein and the N protein by the FluoroSpot assay, since these were the viral antigens used for stimulation in the ICS method. We found a moderate agreement between positive (≥0.1%) SARS-CoV-2-specific IFN-γ-producing $CD4^+$ T cell responses by ICS and S-specific T cell responses (>25 SFUs/10^6 PBMCs) by FluoroSpot (kappa: 0.526; *p* value = .001). The agreement with the M-specific (>21 SFUs/10^6 PBMCs) T cell response was only slight (kappa: 0.211; *p* value = .190). No agreement was found between $CD8^+$ T cell responses and the results of the FluoroSpot assay to either S (kappa: 0.040; *p* value = .827) or M antigens (kappa: 0.010; *p* value = .273).

3.5 | SARS-CoV-2 ELISA IgG serology

SARS-CoV-2 IgG positivity was observed in 90.3% (27/31) of the patients. Two LT recipients (6.5%) were negative for IgG, whereas

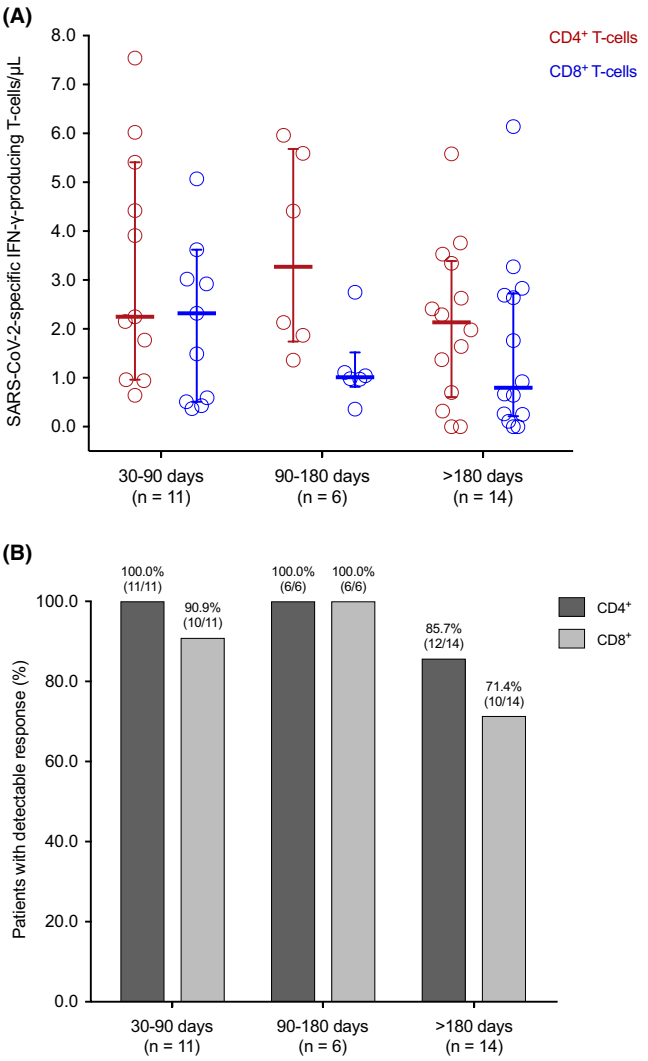

FIGURE 1 SARS-CoV-2-specific IFN-γ-producing T cell $CD4^+$ (red) and $CD8^+$ (blue) T cell counts (A) and proportion of patients with detectable (≥0.1%) responses (B) by the ICS method according to the time interval from the diagnosis of COVID-19 to the assessment. Horizontal bars and whiskers represent median values and interquartile ranges, respectively. IFN-γ, interferon-γ; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2

one further patient (3.2%) had a borderline result in the ELISA. The two recipients with negative IgG titers also lacked SARS-CoV-2-specific T cell responses by the IFN-γ FluoroSpot assay. One of them—a 54-year old male patient that had undergone transplantation 2.5 months before COVID-19 diagnosis and was on triple therapy with tacrolimus, mofetil mycophenolate and prednisone—had no detectable SARS-CoV-2-specific IFN-γ-producing $CD4^+$ T cells by ICS either. There was a significant correlation between the semi-quantitative results of the IgG ELISA (relative OD) and the number of M protein-specific (r: 0.380; *p* value = .038) (Figure 3) but not S glycoprotein-specific (r: 0.064; *p* value = .749) IFN-γ-producing SFUs per 10^6 PBMCs. No correlation was observed with the SARS-CoV-2-specific $CD4^+$ T cell count measured by ICS (r: 0.110; *p* value = .555) either.

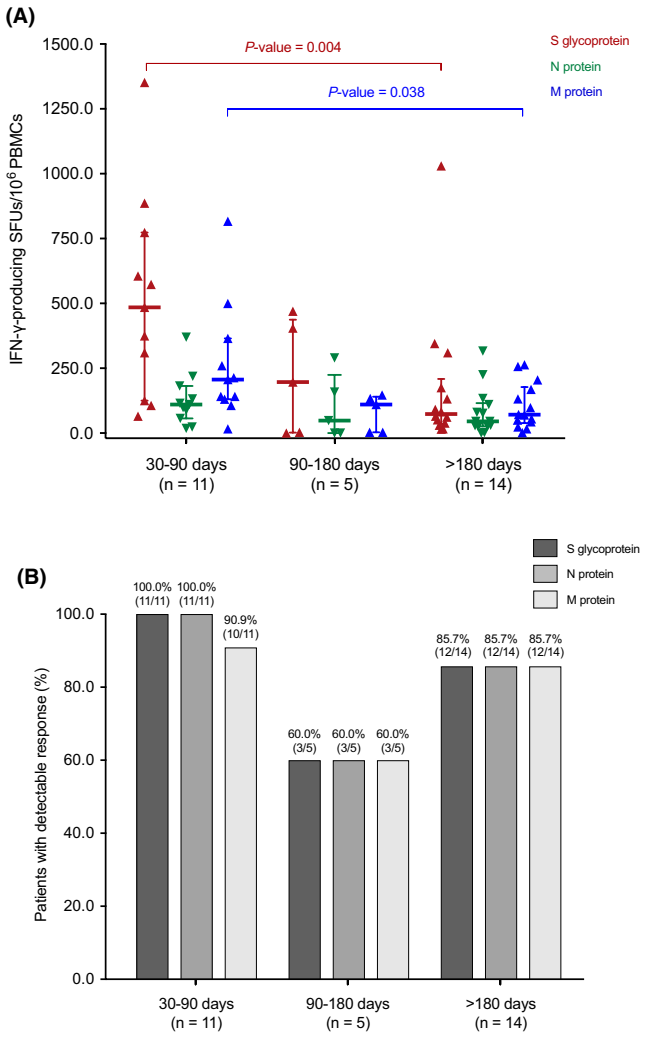

FIGURE 2 SARS-CoV-2-specific IFN-γ-producing T cell responses reactive to the S glycoprotein (red), the N protein (green), and the M protein (blue) (A) and proportion of patients with positive (>25 S-reactive, >14 N-reactive and >21 M-reactive SFUs/10^6 PBMCs) responses (B) by the IFN-γ FluoroSpot assay according to the interval from the diagnosis of COVID-19 to the assessment. Horizontal bars and whiskers represent median values and interquartile ranges, respectively. Comparisons were performed with the Mann-Whitney U test. IFN-γ, interferon-γ; PBMC, peripheral blood mononuclear cell; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SFU, spot forming unit

3.6 | Clinical correlates of SARS-CoV-2-CMI

The potential correlations between the magnitude of SARS-CoV-2-specific T cell responses and clinical variables, including the severity of COVID-19 and the type of immunosuppression, were also investigated. No significant differences in SARS-CoV-2-specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cell counts enumerated by ICS were observed according to the presence of diabetes mellitus, the requirement of hospitalization or oxygen therapy, or the use of tacrolimus, an antiproliferative agent (i.e., mofetil mycophenolate or azathioprine) or prednisone at the time of SARS-CoV-2-CMI assessment (Figure S1). No apparent impact on SARS-CoV-2-specific T cell responses by FluoroSpot was noted for the presence of diabetes or clinical severity either (Figure S2). On the other hand, M protein-specific responses were significantly lower among patients receiving tacrolimus (median: 101.3 versus 157.5 SFUs/10^6 PBMCs; *p* value = .052) or prednisone (median: 16.7 versus 141.7 SFUs/10^6 PBMCs; *p* value = .009) (Figure S3). Patients receiving prednisone were less likely to exhibit S glycoprotein-specific (57.1% [4/7] versus 95.7% [22/23]; *p* value = .031), N protein-specific (57.1% [4/7] versus 95.7% [22/23]; *p* value = .031), and M protein-specific (42.9% [3/7] versus 95.7% [22/23]; *p* value = .006) T cell responses. A similar association was also observed between the use of tacrolimus and M protein-specific response (72.2% [13/18] versus 100.0% [12/12]; *p* value = .066). Finally, no significant correlations were observed between SARS-CoV-2-CMI and other clinical and laboratory variables, including time from transplantation and patient age (Table S1) or immunosuppressive drug levels (Table S2).

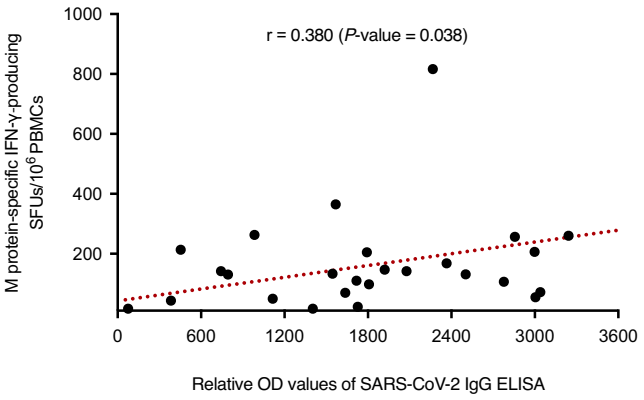

FIGURE 3 Correlation (Spearman's rho) between the semiquantitative results of SARS-CoV-2 IgG ELISA and the number of M protein-specific IFN-γ-producing SFUs per 10^6 PBMCs by the IFN-γ FluoroSpot assay. IFN-γ, interferon-γ; PBMC, peripheral blood mononuclear cell; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SFU, spot forming unit

3.7 | Comparison with nontransplant controls

Finally, we compared SARS-CoV-2-specific T cell responses by the IFN-γ FluoroSpot assay in LT recipients and a nontransplant control group of 30 immunocompetent patients matched by age and time from COVID-19 (Table S3). As expected, the interval from the initial diagnosis of SARS-CoV-2 to the SARS-CoV-2-CMI measurement was similar both groups (158.0 ± 94.9 versus 162.5 ± 29.9 days, respectively; *p* value = .809).

We observed no significant differences between LT recipients and nontransplant patients in the proportion of detectable T cell responses to the S glycoprotein (86.7% [26/30] versus 86.7% [26/30], respectively; *p* value = 1.000), the N protein (86.7% [26/30] versus 83.3% [25/30]; *p* value = 1.000), or the M protein (83.3% [25/30]

versus 90.0% [27/30]; *p* value = .706). When the results of the FluoroSpot assay were analyzed as continuous variable, the number of SFUs per 10^6 PBMCs for any of these antigens was also comparable (Figure 4; Table 2).

4 | DISCUSSION

In the present cross-sectional study, the functionality and magnitude of SARS-CoV-2-CMI have been analyzed by means of two methods in a cohort of 31 LT recipients at different time intervals from the diagnosis of COVID-19. The clinical severity of SARS-CoV-2 infection was also variable, with about two thirds of the recipients being managed as outpatients. The main finding is that the majority of LT recipients recovered from COVID-19 had detectable SARS-CoV-2-CMI (90.3% by ICS and 90.0% by ELISpot-based IFN-γ FluoroSpot assay) after a median of 103 days, and exceeding 6 months in some patients. In addition, anti-SARS-CoV-2 S1 protein IgG was detected in 90.3% of patients. No differences were evident in the proportion of positive SARS-CoV-2-specific T cell responses or in the number of IFN-γ-producing SFUs targeting each of the structural proteins investigated between LT recipients and nontransplant patients matched by age and time from diagnosis. These findings collectively indicate that immunosuppression has no meaningful impact on the long-term ability to mount specific T cell–mediated immunity elicited by natural SARS-CoV-2 infection after LT.

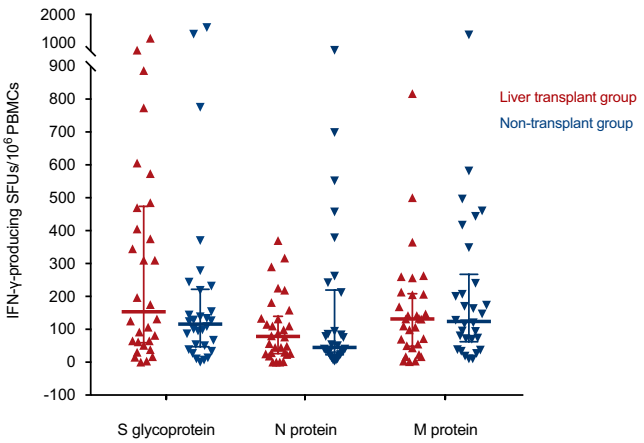

FIGURE 4 SARS-CoV-2-specific IFN-γ-producing T cell responses measured by the IFN-γ FluoroSpot assay in 30 LT recipients (red) and 30 nontransplant patients (blue) matched (1:1 ratio) by age and time interval from the diagnosis of COVID-19. Horizontal bars and whiskers represent median values and interquartile ranges, respectively. IFN-γ, interferon-γ; PBMC, peripheral blood mononuclear cell; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SFU, spot forming unit

Only a few studies have analyzed the kinetics of SARS-CoV-2-CMI in solid organ transplant (SOT) recipients following COVID-19,[4–7,12,13] and none of them were focused on the LT population. By means of an ELISpot assay, Candon et al. characterized SARS-CoV-2-specific T cells in a small group of KT recipients at a shorter interval from symptom onset than in our cohort (median of 38.5 days). All recipients with confirmed SARS-CoV-2 infection displayed $CD4^+$ and $CD8^+$ T cells reactive to at least 6 of 9 viral peptides, with frequencies similar to the nontransplant patients on hemodialysis.[4] In a larger cohort, we previously reported that SARS-CoV-2-CMI was present by ICS in 57.1% and 57.9% of KT recipients at months 4 and 6 after the diagnosis, respectively.[5] In other study, SARS-CoV-2-specific $CD4^+$ polyfunctional T cell responses were detected in 9 out of 10 patients—most of them KT recipients—during the acute phase of COVID-19.[6] Hartzell et al. reported that stable KT recipients on tacrolimus-based immunosuppression hospitalized due to COVID-19 exhibited changes in peripheral blood immune cell phenotypes similar to those observed in the general population.[12]

All-cause mortality rates among SOT recipients hospitalized due to COVID-19 during the first pandemic wave have been reported to exceed that of the general population.[1,3] Although outcomes for LT recipients seem to be relatively better as compared to other transplant groups, the pooled estimate for in-hospital mortality in a recent meta-analysis reached 20%.[14] However, it has been recently proposed that the more severe course observed for post-transplant COVID-19 could be attributed to the higher burden of comorbidities and more common presence of risk factors for poor outcome (such as older age or acute kidney injury) rather than to the transplant status itself.[15–18] The absence of significant differences in between LT recipients and matched nontransplant patients in the magnitude of SARS-CoV-2-CMI would support this notion. Thieme et al. also reported that SARS-CoV-2-specific

TABLE 2 SARS-CoV-2-specific T cell responses assessed by the IFN-γ FluoroSpot assay in LT recipients and nontransplant patients matched by age and time interval from the diagnosis of COVID-19

SARS-CoV–2-specific IFN-γ-producing SFUs per 10^6 PBMCs, median (IQR)	LT group (*n* = 30)	Nontransplant group (*n* = 30)	*p* value
S glycoprotein	153 (59–473)	115 (47–221)	.297
N protein	78 (26–139)	45 (22–219)	.836
M protein	131 (48–208)	123 (62–267)	.579

Abbreviations: IFN-γ, interferon-γ; IQR, interquartile range; LT, liver transplantation; M, membrane protein; N, nucleoprotein; PBMC, peripheral blood mononuclear cell; S, spike glycoprotein; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SD, standard deviation; SFU, spot forming unit.

Comparisons were performed with the Mann-Whitney *U* test.

M- and S-reactive polyfunctional $CD8^+$ T cell counts and neutralizing antibody-titers did not differ between SOT recipients and nontransplant controls.[6] In a mixed cohort of SOT recipients, Favà et al. demonstrated the development of robust serological and functional responses comparable to nontransplant patients during early convalescence.[7] Studies performed in other types of immunocompromised hosts have reached similar conclusions.[19] Overall, these findings emphasize the need of taking into account not only the seroconversion rate but also the development of T cell–mediated immunity in studies assessing the immunogenicity of SARS-CoV-2 vaccines in the SOT population.[20]

While there is a long-standing technical and clinical experience with the quantification of CMV-specific T cell responses in the SOT setting,[21] the optimal methodological approach for the assessment of post-transplant SARS-CoV-2-CMI remains to be established. Thus, we have applied two different techniques that offer complementary results. The ICS method is able to phenotype T cell responses ($CD4^+$ versus $CD8^+$), whereas the IFN-γ FluoroSpot assay informs on the viral antigen-specificity of such responses. In accordance with our previous study performed in KT recipients,[5] the results of the FluoroSpot assay correlate with $CD4^+$ but not $CD8^+$ T cell responses enumerated by ICS. In addition, the categorical agreement—albeit moderate—was higher for the immunodominant S glycoprotein. It should be noted, however, that both methods are not entirely comparable in terms of sample analyzed (whole blood versus PBMCs) and duration of antigen stimulation (6 versus 16–18 h). On the other hand, there were differences in the S peptides used for in vitro stimulation. Specific T cells enumerated by ICS were reactive to both subdomains (S1 and S2) of the viral glycoprotein, whereas the FluoroSpot assay only detected responses against the S1 subdomain. It could be hypothesized that the ICS method would be able to detect a larger T cell repertoire elicited by both natural infection and vaccination.

In line with studies performed in the general population,[22] we observed a significant decline over time in the S glycoprotein-specific T cell response assessed by the IFN-γ FluoroSpot assay (which was not mirrored by the results obtained for other antigens or with the ICS method). In addition, the high proportion of detectable SARS-CoV-2-CMI by ICS after a median of 3.4 months contrasts with that observed by our group with the same method among KT recipients, which only reached 57.1% and 19.0% for SARS-CoV-2-specific $CD4^+$ and $CD8^+$ T cell responses, respectively, at month 4.[5] Differences in the type and amount of immunosuppression may account for such discrepancy. Indeed, most of the KT recipients in our previous study were receiving a triple regimen based on a calcineurin inhibitor, an antiproliferative agent and prednisone,[5] whereas 54.8% of LT recipients were on monotherapy (mainly mofetil mycophenolate). Consistent with this, patients receiving prednisone or tacrolimus or prednisone at the time of immune assessment exhibited lower SARS-CoV-2-specific T cell responses by the FluoroSpot assay. The deleterious impact of tacrolimus-containing regimens on the capacity of mounting SARS-CoV-2-CMI was also observed in the setting of KT.[5] Interestingly, we observed no apparent effect of the clinical severity of COVID-19 (i.e. requirement of hospitalization or oxygen therapy) on the magnitude of T cell responses. Some [23–25] but not all authors [22,26] have found a positive correlation between clinical severity at early phases of infection and SARS-CoV-2-specific T cell counts in nontransplant patients, although studies differ in the timing of evaluation. Increasing evidence shows that subjects with asymptomatic SARS-CoV-2 infection are able to mount specific T cell responses comparable to those observed in patients recovering from COVID-19.[22] It is to be assumed that this concept also extends to LT recipients, since most of the patients in our cohort had only mild clinical manifestations and were managed as outpatients.

Although this is the largest study to date investigating SARS-CoV-2-CMI after SOT (and the only one focused on LT recipients and applying different methods), some limitations should be acknowledged. The number of patients included was relatively low, particularly for the more severe courses of disease. Therefore, no multivariate analysis was performed and the clinical associations observed (i.e., type of immunosuppression) should be taken with caution. Despite matching by age and time from diagnosis, LT recipients and nontransplant controls differed in some clinical characteristics, since all the patients in the latter group required hospital admission (as compared to only 38.7% in the LT group). Thus, the lack of apparent differences in the magnitude of SARS-CoV-2-CMI could be at least partially explained by the characteristics of the control cohort, since immunocompetent patients unable to mount robust T cell–mediated responses early after infection are more likely to progress to severe symptoms (P. Almendro-Vázquez, Instituto de Investigación Sanitaria Hospital "12 de Octubre", personal communication, 2021). Nevertheless, a sensitivity analysis restricted to hospitalized LT recipients and their corresponding controls (12 pairs) did not reveal differences between both groups in the size of T cell responses (data not shown). Finally, the timing for the evaluation of SARS-CoV-2-CMI was variable, and the cross-sectional design precluded the analysis of post-transplant kinetics over time.

In conclusion, SARS-CoV-2 infection—even if mild and not requiring hospitalization or oxygen therapy—elicited specific T cell–mediated immunity detectable in nine out of ten LT recipients after a median of 103 days from the episode of COVID-19. The magnitude of post-transplant SARS-CoV-2 T cell responses did not significantly differ from that observed in nontransplant patients hospitalized due to COVID-19. Further studies are needed to characterize the functionality and durability of natural or vaccine-induced SARS-CoV-2-CMI among LT recipients.

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DISCLOSURE

The authors of thismanuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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