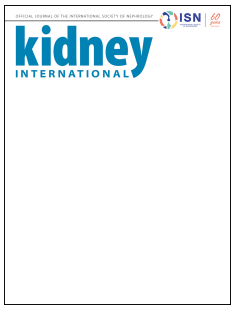


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A comprehensive assessment of long-term SARS-CoV-2-specific adaptive immune memory in convalescent COVID-19 Solid Organ Transplant recipients

Alexandre Favà, Laura Donadeu, Thomas Jouve, José Gonzalez-Costello, Laura Lladó, Carolina Santana, Néstor Toapanta, Manuel Lopez, Vincent Pernin, Carme Facundo, Nuria Serra Cabañas, Olivier Thaunat, Marta Crespo, Laura Llinàs, Ignacio Revuelta, Nuria Sabé, Alexander Rombauts, Laura Calatayud, Carmen Ardanuy, Juliana Esperalba, Candela Fernandez, Juan J. Lozano, Rosemarie Preyer, Kevin Strecker, Carlos Couceiro, Elena García-Romero, Alba Cachero, Maria Meneghini, Alba Torija, Moglie Le Quintrec, Edoardo Melilli, Josep Maria Cruzado, Carolina Polo, Francesc Moreso, Elena Crespo, Oriol Bestard

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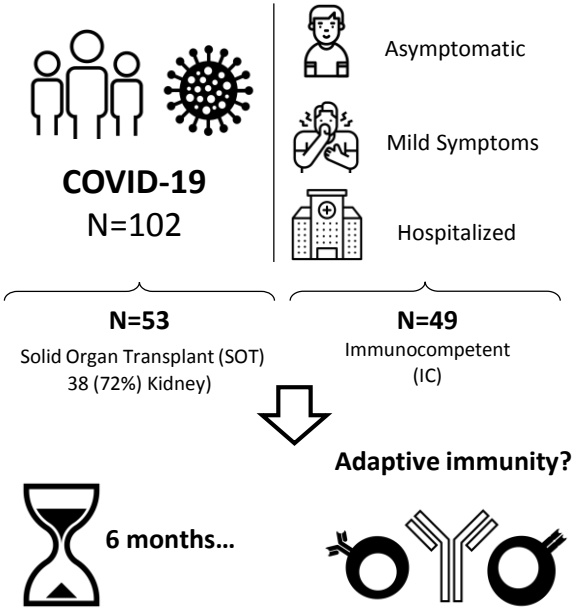
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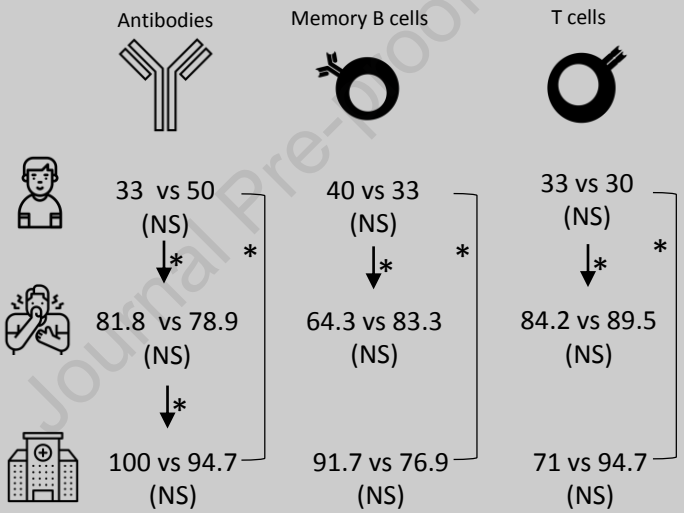
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HYPOTHESIS AND AIMS



Detectable immunity SOT vs IC (%) at 6 months



*p<0.05; SOT: Solid Organ Transplant; IC: Immunocompetent; NS: non-significant

YEARS AFTER TRANSPLANT:

- ***Nucleoprotein IgG:***
  OR 1.2; p=0.02
- ***Spike IFNγ T-cells:***
  OR 1.4; p=0.01
- ***Spike IFNγ-IL2 T-cells:***
  OR 1.2; p=0.03

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CONCLUSION : Solid organ transplant recipients are capable of mantaining detectable immunity 6 months after COVID-19, which is mainly determined by infection severity. Long-lasting adaptive immunity might be challenged among those recently transplanted.

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A comprehensive assessment of long-term SARS-CoV-2-specific adaptive immune memory in convalescent COVID-19 Solid Organ Transplant recipients

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KEYWORDS: COVID-19 infection, Solid Organ Transplantation, adaptive immunity

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ABSTRACT

Long-term adaptive immune memory has been reported among immunocompetent individuals up to eight months following SARS-CoV-2 infection. However, limited data is available in convalescent patients with a solid organ transplant. To investigate this, we performed a thorough evaluation of adaptive immune memory at different compartments (serological, memory B cells and cytokine [IFN-γ, IL-2, IFN-γ/IL12 and IL-21] producing T cells) specific to SARS-CoV-2 by ELISA and FluoroSpot-based assays in 102 convalescent patients (53 with a solid organ transplants (38 kidney, 5 liver, 5 lung and 5 heart transplant) and 49 immunocompetent controls) with different clinical COVID-19 severity (severe, mild and asymptomatic) beyond six months after infection. While similar detectable memory responses at different immune compartments were detected between those with a solid organ transplant and immunocompetent individuals, these responses were predominantly driven by distinct COVID-19 clinical severities (97.6%, 80.5% and 42.1%, all significantly different, were seropositive; 84% vs 75% vs 35.7%, all significantly different, showed IgG-producing memory B cells and 82.5%, 86.9% and 31.6%, displayed IFN-γ producing T cells; in severe, mild and asymptomatic convalescent patients, respectively). Notably, patients with a solid organ transplant with longer time after transplantation did more likely show detectable long-lasting immune memory, regardless of COVID-19 severity. Thus, our study shows that patients with a solid organ transplant are capable of maintaining long-lasting peripheral immune memory after COVID-19 infection; mainly determined by the degree of infection severity.

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INTRODUCTION

The Coronavirus Disease-2019 (COVID-19) caused by the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) has challenged global health in an unprecedented manner, resulting in a widespread morbidity and mortality. Even though most people develop mild symptoms or remain asymptomatic after SARS-CoV-2 infection[1,2] some patients develop a severe respiratory syndrome which associates with an excessive systemic inflammatory process, ultimately leading to respiratory failure and death.[3,4] Notably, some specific group of patients seem to be at significantly higher risk of fatal outcomes such as recipients of solid organ transplants (SOT), most likely due to their chronic immunosuppressive therapy that broadly targets adaptive T-cell immunity. [5,6]

Recent important studies have shown that during acute COVID-19 and early convalescence, infected patients develop robust adaptive immune responses by means of high SARS-CoV-2-specific IgG antibody titers and T-cell frequencies, both CD4 and CD8 T cells, in peripheral blood. [7] Remarkably, the strength of these adaptive immune responses seems to also vary according to distinct COVID-19 disease severity [8,9,10,11,12] thus, suggesting a key role of SARS-CoV-2-specific immunity controlling and limiting primary viral.[13,14,15,16] On the other hand, a long-lasting protective immunity, both serological and cellular, has also been reported among convalescent COVID-19 patients from the general population between 5 and 8 months after infection.[17]

In the setting of SOT, however, scarce information has been reported regarding the degree, durability and biological interplay between different adaptive immune mechanisms in response to SARS-CoV-2. In this regard, our group recently showed that SOT patients developing a moderate/severe COVID-19 infection are able to

generate, albeit with a notable initial delay, similarly strong SARS-CoV-2-specific serological and T-cell immune responses during early convalescence as compared to immunocompetent patients with the similar severe infection.[18,19] Nevertheless, unlike SARS-CoV-2 convalescent immunity, weak adaptive immune responses have been reported in SOT recipients after two doses messenger RNA-based vaccination. [20,21] Importantly, understanding whether memory immune responses specific to SARS-CoV-2 last for long convalescent periods and how the serological and B and T cellular compartments behave over time is key to establish guided preventing strategies among this high-risk patient population.

Herein, we performed a thorough evaluation of both serological and functional T and B-cell immune memory against main immunogenic SARS-CoV-2 antigens using functional cell-based immune assays, in a cross-sectional cohort of 102 convalescent SOT (n=53) and immunocompetent (IC) healthy individuals (n=49) with distinct disease severity, beyond 6 months after COVID-19 infection.

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MATERIAL AND METHODS

Patients of the Study and clinical definitions

One-hundred and two COVID-19 convalescent patients from different European transplant centers were evaluated in this study (Bellvitge University Hospital [N=67]; Vall d'Hebron University Hospital [N=13]; Montpellier University Hospital [N=4]; Fundació Puig-Vert [N=3]; Lyon University Hospital [N=2]; Hospital del Mar [N=2], Hospital Clinic [N=1]) and a general medical assistance center (N=10). 53 SOT recipients (38 kidney, 5 liver, 5 lung and 5 heart transplants) and 49 immunocompetent (IC) healthy individuals, in whom peripheral blood mononuclear cells (PBMCs) and serum samples could be obtained, with a median follow-up after COVID-19 infection beyond 6 months (199 days, IQR 170-215), were included in this study. Additionally, 35 subjects (21 SOT and 14 IC) having developed a severe COVID-19 were compared to their 1-month post-infection immune memory status. None of the participants had been vaccinated, before or during the study follow up.

All patients had been tested positive for SARS-CoV-2 infection by a rt-PCR analysis on nasopharyngeal swab samples and diagnosed for COVID-19 between March/October 2020. Samples from 16 pre-pandemic uninfected individuals were used as negative controls for T cell assays, as described elsewhere[18]. Additional 10 historic biological samples were employed as a controls for the B cell functional assays.

As shown in the flow-chart of the study (**Figure 1**), both SOT recipients and IC patients included in the study were classified according to 3 distinct COVID-19 clinical presentations; 41 had been hospitalized for a severe COVID-19 (SEV) requiring oxygen supply (22 SOT and 19 IC), 42 presented with mild symptoms (MILD) and were not hospitalized (22 SOT, 20 IC) and 19 were asymptomatic (ASYMP) and found

positives for SARS-CoV-2 by a rt-PCR on nasopharyngeal swab in routine screening or contact tracking tests (9 SOT and 10 IC). Main clinical, demographic and immunological patient characteristics were recorded.

The study was approved by the Ethical Review Boards (PR115/20) at each center and patients were recruited in the study after providing a signed informed consent.

Collection and management of Serum and PBMCs samples

Detailed description is depicted in supplementary methods.

Assessment of SARS-CoV-2-specific Humoral Immunity

SARS-CoV-2-specific Serological Memory

Serum IgG antibodies were assessed against two main SARS-CoV-2 antigens: the Nucleoprotein and Spike glycoprotein in 101/102 (99%) study patients using two distinct ELISA platforms. Detailed information of the methodology and interpretation is provided as supplementary material.

SARS-CoV-2-specific IgG-producing memory B cells

Circulating SARS-CoV-2-specific IgG-producing memory B-cell (mBC) frequencies was assessed against the receptor binding domain of SARS-CoV-2 Spike (RBD-S) protein in 71/102 (69.6%) individuals of the study using a colorimetric B cell ELISPOT assay. A thorough description of the method and interpretation (**Supplementary Figure S1**) of this assay is depicted in supplementary material.

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SARS-CoV-2-reactive cytokine-producing Memory T cells

Circulating SARS-CoV-2-reactive cytokine-producing memory T-cell frequencies could be assessed in 97/101 (95.1%) patients of the study using a multicolor FluoroSpot Immune assay (AID® Gmbh, Strassberg, Germany), in which 4 distinct cytokine-producing T-cell frequencies were simultaneously assessed: effector (IFN-γ), proliferative (IL-2), central (IFN-γ/IL-2) Th1 and IL-21 Th2 memory responses. These responses were evaluated against the 4 main structural SARS-CoV-2 proteins: Spike Glycoprotein (S), Membrane Protein (M), Nucleoprotein (N) and Envelope Small Membrane Protein (E) (JPT®, Berlin, Germany). A strong positive correlation of T-cell immune responses between all viral antigens was observed but for antigen E, which were barely detectable (**Supplementary table 1**). Thus, all the analyses were focused against antigens S, M and N. Global SARS-CoV-2 reactive T cell immune responses were calculated by means of the median T cell frequencies against the three main immunogenic antigens (S, M and N) in each patient. Furthermore, since a hierarchical T-cell immune response was observed and was predominantly driven by IFN-Y-producing T cells against antigen S (**Supplementary Figure S2**), the qualitative assessment of T-cell immune memory was based on this response. A detailed description of the methodology and interpretation (**Supplementary Figure S3**) is provided as supplementary material.

Statistics

Continuous variables were expressed as mean ± SD or median and IQR and categorical variables as number of total (n) and percentage (%). A comparison between groups was performed using Pearson' $\chi 2$ test for categorical data. Continuous measurements were compared among groups using Kruskal-Wallis and

Mann-Whitney U test for non-normally distributed data, while ANOVA and t tests were used when data were normally distributed. P-values <0.05 were considered statistically significant. Univariate logistic regression models were used to investigate the influence of clinical covariates (age, gender, symptomatic/asymptomatic infection and years after transplant) by means of Odds Ratio (OR) with 95% confidence interval for humoral and cellular responses. Those covariates that were associated with a P-value <0.1 were introduced into a multivariate binary logistic regression model. SARS-CoV-2- reactive cellular and humoral responses were centered and scaled and heatmap was built by means of the pheatmap R package 16 using Euclidean distance and complete method as agglomeration method. R package version 1.0.12 was used https://CRAN.R-project.org/package=pheatmap. All other analyses were performed using SPSS version 26 software, and graphs were generated using GraphPad Prism version 8.0 software (Graphpad Software, San Diego, California).

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RESULTS

Patients of the study

As shown in the study flow-chart (**Figure 1**) and **Table 1**, 102 COVID-19 convalescent patients after a median time of 199 days (IQR 170-215) post-infection were investigated (53 SOT and 49 IC). SOT had a median time after transplantation of 5 years (IQR 1-12) and most of them were receiving a calcineurin-inhibitor (CNI) based immunosuppression (83%). All patients were classified and matched according to the clinical severity of COVID-19 infection: 41 SEV (22 SOT, 19 IC), 42 MILD; (22 SOT, 20 IC) and 19 ASYM (9 SOT, 10 IC).

In general, IC patients were slightly younger (50.1±15.8 vs 57.9±12.5 mean age, p=0.017) and their convalescence period was longer (206 [185-232] vs 184 [159-207] median days, p=0.005) than SOT (**Figure 1**) and these differences were mainly driven by the MILD IC group, which was composed of healthcare workers (**Table 1**). Among the remaining groups, IC and SOT were matched for age and time after infection. There were no differences regarding the type of immunosuppression, SOT or time after transplantation between the 3 distinct clinical groups.

None of the included patients was diagnosed of transplant rejection during the acute SARS-CoV-2 infection or the follow-up, but one kidney transplant individual who presented a sub-clinical antibody mediated rejection in a 12-month protocol biopsy.

Disease severity but not patient condition drives long-lasting immune memory.

As illustrated in an unsupervised heatmap in **Figure 2,** SARS-CoV-2-specific immune memory responses, both at the serological and functional B and T-cell compartments were predominantly explained by the clinical severity of COVID-19 infection rather than by the patient condition, either SOT or IC. As shown, all differences were

fundamentally driven by the severe clinical groups but not for IL21-producing T cells, which did not significantly differ across different clinical severities (**Supplementary table 2**).

Long-term SARS-CoV-2-specific Humoral memory Serological Memory

Beyond 6 months after infection, 81/101 (80.2%) and 78/101 (77.2%) of patients showed detectable SARS-CoV-2 IgG antibody levels against antigens spike (S) and Nucleoprotein (N), respectively. As illustrated in **Figures 3a-b**, there were no differences regarding both seroconversion rates (81.13% vs 79.17%; p=0.805) and IgG titters (108 [28.85-396.5] vs 85.8 [16.5-398.5] UA/ml; p=0.58) against antigen S between SOT and IC, respectively. Conversely, while similar seroconversion against antigen N was observed between SOT and IC, N-specific IgG titers were lower among SOT patients (6.7 [0.67-33] vs 34.3 [4.43-75.63] UA/ml; p=0.027).

A clear seroconversion gradation according to the 3 distinct clinical severities was observed, regardless of patient condition, either SOT or IC (40/41 [97.56%] vs. 33/41 [80.48%] vs. 8/19 [42.1%]; p<0.001) and (38/41 [92.68%] vs. 31/41 [75.6%] vs. 9/19 [47.36%]; p<0.001) for SEV, MILD and ASYMP against antigens S and N, respectively (**Figures 3c-d; Supplementary Table 3**). The same observation was found for IgG titers (435 [189 – 775.5] vs 39.4 [15.85 - 113] vs 4.94 [0 - 68]; p<0.001; and 35.7 [8.63 – 81.25] vs 9.39 [0.89 - 50.6] vs 0.08 [0.08 - 13.7]; p<0.001) in SEV, MILD and ASYMP patients against antigens S and N, respectively (**Figures 3e-f)**.

Nonetheless, despite higher IgG titers against antigen S was observed among MILD-SOT than MILD-IC patients (76.7 [30.4-209.8] vs 20.9 [15.5-45.2] UA/ml; p=0.034), most likely due to the later time of analysis of MILD-IC subjects (**Table 1**), SEV-IC

patients displayed numerically higher IgG titers against antigen N than SEV-SOT patients (61.8 [36.2-92.1] vs 15.7 [4-33.4] UA/ml; p<0.001).

B-cell Memory

49/71 (69%) patients displayed detectable circulating (RBD-S)-specific IgG-producing memory B cells (mBC), with similar proportion (22/31 vs 28/40 p=0.929) and frequencies (0.0134 [0-0.0557] vs 0.0116 [0-0.054]; p= 0.883) between SOT and IC patients, respectively (**Figure 4a-b**). Likewise to serology, detection of mBC was highly influenced by the 3 different clinical presentations (84% vs 75% vs 35.7%; p=0.004, in SEV, MILD and ASYMP, respectively), regardless of patient condition (**Figures 4c-d; Supplementary Table 4**). Even though no statistical differences were observed regarding IgG-producing mBC frequencies between groups, SEV patients showed numerically higher frequencies, being this difference especially evident between SEV-IC vs ASYMP-IC patients (0.059 [0.013–0.189] vs 0 [0-0.031]; p=0.024) (**Figure 4d).**

Long-term SARS-CoV-2-specific T-cell Memory

Overall, there were no differences regarding the proportion of patients with detectable SARS-CoV-2-reactive T cells or their frequencies for any of the evaluated cytokine-producing T cells between SOT and IC patients against the different viral antigens (**Supplementary Figures S4 and S5; Supplementary Table 5**).

A hierarchical T-cell immune response was observed that was mainly dominated by antigen S (**Supplementary Figure S2; Supplementary Table 6**). Similarly to humoral immunity, the proportion of T-cell responders significantly decreased along with the different clinical presentations (**Figure 5**), and these differences were independent of

the patient condition (**Supplementary Figure S6)**. As described in **Figure 6,** a clear decrease in all SARS-CoV-2-reactive cytokine-producing T-cell frequencies but not for IL21-producing T cells was observed in line with the less severe clinical presentation. Of note, a less pronounced SARS-CoV-2-specific T-cell gradient was observed among SOT as compared to IC patients, especially between severe and mild convalescent COVID-19 patients.

Relationship between serological and cellular SARS-CoV-2-specific Immunity

A significant positive correlation between serum IgG titers and frequencies of IgG and cytokine-producing memory B and T cells, respectively, against protein S was observed, which was more robustly observed within the IC group (**Figure 7)**. Conversely, no correlation was found between serologic and cellular responses against protein N and between frequencies of IgG-producing mBC and cytokine-producing T cells.

Next, we compared all memory immune compartments in each individual according to the different clinical presentations in all patients in whom the humoral (either mBC or antibodies) and cellular immune responses could be investigated (97/102 [95.1%] patients; 40 SEV, 38 MILD and 19 ASYMP). As shown in **Figure 8**, while all SEV patients showed some detectable SARS-CoV-2-specific immune memory, 5.3% (2/38) of MILD and up to 42.1% (8/19) of ASYMP patients did not show detectable anti-viral immunity in any of the three immune compartments ($p<0.001$). No differences were found between SOT and IC (data not shown).

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Longitudinal analysis of SARS-CoV-2 immune memory in Severe convalescent COVID-19 patients.

In a subgroup of 35 severe convalescent patients (21 SOT and 14 IC), SARS-CoV-2 immune memory could be compared to a previous initial time point after COVID-19 infection (49 days; IQR 43-53). As illustrated in **Figure 9a,** while no differences were observed regarding percentages of seropositivity against antigens S and N as well as in IgG titers against antigen N between the two time points in the two groups, anti-S IgG titers significantly dropped in both groups (800[285-800] vs 277.5 [186.5-800]; p=0.029 for SOT; 800 [524-800] vs 571 [263-713]; p=0.002 for IC).

Notably, a significant decline was observed in all cytokine-producing SARS-CoV-2-reactive T-cell frequencies but not in IL-21, in both groups (**Figure 9b),** with a higher proportion of SOT becoming non T-cell responders than IC (**Figure 9c**).

Main clinical determinants influencing long-term immune memory

We then investigated whether main demographic characteristics such as age, gender or time after transplant influenced long-term immune memory at the distinct compartments, adjusting for the type of COVID-19 clinical severity, given its preponderance leading to distinct long-lasting SARS-CoV-2-specific immune responses.

Contrary to infection severity, age and gender did not impact on long term immunity of IC individuals; (data not shown).

Among SOT, however, in addition to COVID-19 disease severity, time (years) since transplantation was also revealed as an independent factor modulating the maintenance of long-term peripheral immune memory (**Supplementary Table 7**), specifically for anti-N IgG antibodies (OR 1.2, 95%CI 1.02-1.40, p=0.02), IFN-γ and

IFN-γ/IL2 reactive T cells (OR 1.4 95%CI 1.08-1.83, p=0.013 and OR 1.14 95%CI 1.01-1.28, p=0.028; respectively).

Discussion

In this study, we investigated the persistence and magnitude of adaptive immune memory specific to SARS-CoV-2 beyond 6 months after infection in a large cohort of convalescent SOT recipients and IC individuals having experienced three distinct clinical presentations, severe, mild or asymptomatic COVID-19. Herein, we show that SOT patients are capable of maintaining a long-lasting functional immune response specific to SARS-CoV-2 similarly to IC individuals both at the serological and T and B-cell memory immune compartments. Most importantly, we found that the persistence and magnitude of this response is mainly influenced by the degree of COVID-19 clinical severity, thus a high proportion of asymptomatic and some mild convalescent patients did not display any detectable adaptive immune memory in any biological compartment. To note, even though no major differences were generally observed between SOT and IC, SOT individuals displayed weaker humoral responses to SARS-CoV-2 antigen N, a weaker correlation between serologic and cellular responses than convalescent IC patients with the same clinical disease severity and a more pronounced decline of SARS-CoV-2-reactive cytokine-producing T-cell frequencies over time. Furthermore, our data highlights a more impaired long-term immune preservation among most recently transplanted SOT individuals.

Recent studies have shown that for seasonal coronaviruses, protective immunity seems to be predominantly short-lived.[22] However, detectable long-term immune memory against SARS-CoV-2 within three main compartments (serological and B and

T cell memory), has been described in convalescent IC individuals beyond 6 months after COVID-19. [8,17]

In our study, while we confirm that COVID-19 provides detectable peripheral immunity at the three main immune compartments (serological and functional B and T-cell immune responses) beyond 6 months after infection in convalescent IC individuals, we show for the first time that SOT patients are similarly capable of maintaining a long-lasting immune memory response at the serological and functional memory B and T-cell level. In fact, the robust immune responses detected at the different immune compartments in many SOT with the longest follow up (up to 355 days after infection), strongly suggest that memory immune responses in this patient population may last even further despite receiving chronic immunosuppression. Rather, the most relevant feature determining the persistence and magnitude of protective immunity was the degree of COVID-19 clinical severity. Notably, a clear gradient of immune responses from the more severe to the mild and asymptomatic groups was clearly delineated in our patients. [8,9,10,11,12] In fact, while more than 80% of severe convalescent COVID-19 subjects were seropositive and displayed robust SARS-CoV-2-specific IgG and cytokine-producing memory B and T cells, respectively, only in 40% were detectable among the asymptomatic group. A potential explanation for these findings may rely in recent observations showing that severe hospitalized cases, both IC and SOT, display higher viral loads, viremia and longer viral shedding as compared to milder COVID-19 cases,[23,24,25,26] which may lead to higher antigen exposure ultimately triggering stronger and long-lasting immune responses.

In line with previous studies,[8,27] we also found a high correlation between the different immune compartments specific to SARS-CoV-2. However, these differences were predominantly driven by the IC group, suggesting a more impaired functional immune

response of SOT related to chronic immunosuppression. Indeed, the gradient of strength and detection of immune responses at the T-cell level between the distinct clinical COVID-19 presentations within SOT was not pronounced as compared to IC patients, especially between severe and mild patients. Moreover, in a longitudinal analysis of immune response progression within severe convalescent COVID-19 patients, SOT displayed a clearer decline of functional T-cell immune memory than IC patients, again illustrating a certain deleterious effect of chronic immunosuppression on anti-viral immunity over time. In addition, despite similar seropositivity rates for both antigens S and N between IC and SOT, the later displayed significantly lower anti-N IgG titters than IC patients. Unlike in the general population,[17] it has recently been described that SOT patients seem to show lower anti-N IgG titers,[28] especially those with higher immunosuppressive burden,[29] suggesting higher susceptibility of anti-N seroconversion to chronic immunosuppression.

Finally, when we investigated major determinants influencing the presence of SARS-CoV-2-specific immune memory within SOT patients, besides COVID-19 clinical severity, we found that more recently transplanted patients exhibited an independent higher risk of not maintaining detectable serological and T-cell immunity than those with a longer functioning graft. These data highlight the negative effect of the initial immunosuppressive burden challenging adaptive anti-viral immune responses.

Our study has some limitations. First, we have to consider an inherent selection bias in our cohort, since all the included individuals had successfully recovered from SARS-CoV-2 infection, which is not the general COVID-19 outcome among this at-risk patient population. On the other hand, our immune evaluation was restricted to the original SARS-CoV-2 strain, due to the infection time-period (March to October 2020) so we are not able to fully ensure whether these data would replicate with the more virulent

viral strains. Finally, since this study was done prior to the successful vaccination campaigns [30] so we cannot completely extrapolate these findings to breakthrough infections in patients after unsuccessful vaccination.

Also, the mild infection group of the study, which was fundamentally based on healthcare workers was a bit younger and were analyzed at a later time. However, in general these differences did not impact on the immune responses compared to the same mild SOT group. While the number of asymptomatic patients was lower than the others two groups, the consistency of the results observed within this group counterbalance this constraint. Finally, we could not describe the predominant T or B cell subsets, responsible of these SARS-CoV-2-reactive T and B cells. However, our FluoroSpot assay allowed us to functionally assess the frequencies of different IgG- and cytokine-producing B and T cells specific to SARS-CoV-2 at the single cell level.

In conclusion, our findings show that robust humoral and cellular immune memory persists among IC and SOT convalescent COVID-19 patients for more than 6 months after infection, and these responses are highly dependent on the clinical degree of COVID-19 severity, which might ultimately illustrate distinct level of viral antigen exposure. However, long-lasting adaptive immunity seems to be challenged to some extent by chronic immunosuppression, especially among those more recently transplanted. Our data may have some relevant implications regarding the long-lasting immune response achieved after vaccination, highlighting the need of an accurate and broader assessment of SARS-CoV-2 immune response to establish guided preventive strategies.

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FIGURES

Figure 1. Flow-chart of the study. *p<0.05 (Chi-square Test and T-Test).

Figure 2. Heatmaps generated by hierarchical clustering of SARS-CoV-2-specific immune responses for SOT and IC patients, according to the COVID-19 disease severity (Moderate/Severe, Mild or asymptomatic). Immune responses used for clustering were differentially expressed (fold change >2, false discovery rate p < .05). Gray fields indicate missing values.

Figure 3. IgG antibody responses to SARS-CoV-2 Spike and Nucleocapsid proteins. (a) Proportion of SOT and IC individuals with detectable IgG antibodies. (b) IgG antibody titters against antigens Spike and Nucleoprotein among SOT and IC; *p<0.05. (c-d) Seropositive proportion of patients for Spike(c) and Nucleoprotein (d), according to infection severity at the onset. In columns, immunosuppression status for every cluster of severity. (e-f) IgG-Spike (e) and IgG-Nucleoprotein (f) titters according to severity and immunosuppression group; *p<0.05. Detailed data on antibody titters available in Supplementary Table 3.

Figure 4. Frequencies of RBD-specific IgG producing mBC. (a) Proportion of SOT and IC individuals with detectable RBD-IgG producing memory B cells (mBC). (b) Frequencies of RBD-IgG-producing mBC between SOT and IC. (c) Proportion of individuals with detectable RBD-IgG-producing mBC according to infection severity at the onset. In columns, immunosuppression status for each severity group. (d) Frequencies of RBD-IgG-producing mBC according to severity and

immunosuppression group; *p<0.05. Detailed data on ratio of RBD-IgG-producing mBC is provided in Supplementary Table 4.

Figure 5. Proportion of patients with detectable SARS-CoV-2-reactive cytokine-producing T cell responses according to infection severity. IFN-γ, IL-2, IFN-γ/IL-2 and IL-21 were assessed. *p<0.05

Figure 6. Global T cell responses specific to SARS-CoV-2 (median T cell frequencies against the three SARS-CoV-2 immunogenic antigens: S, M and N). Significant intra and intergroup differences (SOT SEV, SOT MILD, SOT ASYMP, IC SEV, IC MILD and IC ASYMP) are shown; *p<0.05.

Figure 7. Correlations between serologic and cellular immune compartments against (Spike)SARS-CoV-2 antigen. IgG titters against antigen S and circulating (RBD-Spike)-specific mBc frequencies exhibited a significant positive correlation (r=0.355, p=0.003), which was fundamentally driven by IC subjects (r=0.548, p<0.001). A similar pattern was observed between Spike-specific IgG titers and the different (Spike)SARS-CoV-2-reactive cytokine-producing T-cell frequencies but for IL21 (data not shown) was mainly observed within IC individuals (IFNγ: r=0.358, p=0.013; IL2: r=0.404, p=0.005 and IFNγ/IL2: r=0.458, p=0.001). SFU, spot forming unit; PBMCs, peripheral blood mononuclear cells.

Figure 8. Dot plots showing the proportion of subjects with detectable responses at the different immune compartments according to disease severity. Humoral memory (H) + T-cell memory (T) = detectable (RBD-Spike)-specific mBC

and/or anti-Spike IgG and Spike-specific IFN-γ producing T cells. Humoral memory = detectable (RBD-Spike)-specific mBC or anti-Spike IgG**.** T-cell memory = Detectable Spike-specific IFN-γ producing T cells. None (N): No detectable humoral or cellular immunity. SEVERE group: 80% (H+T), 17.5% (H), 2.5% (T), 0% (N); MILD group: 78.9% (H+T), 7.9% (H), 7.9% (T), 5.3% (N); ASYMP group: 26.3% (H+T), 26.3% (H), 5.3% (T), 42.1% (N); $p<0.001$.

Figure 9. Kinetics of SARS-CoV-2 IgG antibodies and T-cell responses in severe COVID-19 individuals between months 1 and 6 after infection. 35 convalescent patients (21 SOT, 14 IC) were longitudinally assessed at two time points: T1= 49 (IQR 44-53) days and T2= 201 (IQR 185-208) days after infection. (a) Quantitative and qualitative antibody (Spike and Nucleoprotein) responses; IgG titters (UA/ml). (b) T-cell frequencies for IFN-γ, IL2, IFN-γ, IL2 and IL21; T cell frequencies (SFU/$2{\cdot}10^5$ PBMC). (c) Proportion of patients with detectable T cell responses for IFN-γ, IL2, IFN-γ, IL2 and IL21.*$p<0.05$. SFU=spot forming unit; PBMCs= peripheral blood mononuclear cells.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the Kidney International.

SUPPLEMENTARY MATERIAL

Supplementary methods.

Supplementary Figure S1. Representative images of RBD-specific and Polyclonal IgG detection from mBC, prior differentiation to antibody secreting cells (ASC). No RBD-specific IgG detection was found among healthy donors.

Supplementary Figure S2. Hierarchical cytokine profile of T-cell responses against main structural SARS-CoV-2 proteins Spike (S), Membrane (M) and Nucleoprotein (N).

Supplementary Figure S3. Representative images of a convalescent SOT from severe COVID-19 and an un-exposed individual, including specific INF-γ responses against the Spike overlapping peptide pool; Pokeweed mitogen (PWM), as internal positive control; isolated medium, as internal negative control; and the readouts after subtraction.

Supplementary Figure S4. Percentage of SOT and IC patients with detectable (Spike)SARS-CoV-2-specific for different cytokine-producing T cells

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Supplementary Figure S5: Global T-cell responses specific to SARS-CoV-2 (median [IQR] T-cell frequencies against the three main SARS-CoV-2 immunogenic antigens: Spike(S), Membrane (M) and Nucleoprotein (N)) for SOT and IC patients and for each cytokine assessed (IFN-γ, IL-2, IFN-γ/IL-2 and IL-21).

Supplementary Figure S6. Proportion of patients with detectable cytokine producing T-cell responses against SARS-CoV-2, according to the immunosuppression status and infection severity.

Supplementary Table S1. Correlations between SARS-CoV-2-specific T-cell and B-cell responses.

Supplementary Table S2. Statistical differences and false discovery rate (fdr) for all the immune responses clustered in Figure 2 Heatmap.

Supplementary Table S3. IgG titters against Spike and Nucleoprotein (median UA/ml [IQR]), according to immunosuppression status and infection severity.

Supplementary Table S4. Ratio of IgG producing memory B cells (median [IQR]) against RBD, according to immunosuppression status and infection severity. RBD = receptor binding domain.

Supplementary Table S5. Specific T cell responses (median spots [IQR]) against SARS-CoV-2 (median of SMN antigens). according to immunosuppression status and infection severity.

Supplementary Table S6. Hierarchical cytokine profile of T cell responses against main structural SARS-CoV-2 proteins Spike (S), Membrane (M) and Nucleoprotein (N). Frequencies of IFN-γ, IL-2, IFN-γ/IL-2 and IL-21-producing T cells were assessed among the six groups of study.

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Supplementary Table S7. Univariate and multivariate analyses based on binary logistic regression model for major determinants influencing persistence of SARS-CoV-2 antibody and cellular responses among SOT after 6 months (OR [95% CI]).

Supplementary References.

Supplementary information is available at *Kidney International's* website.

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Table 1. Demographic and clinical characteristics of patients infected by SARS-CoV-2.

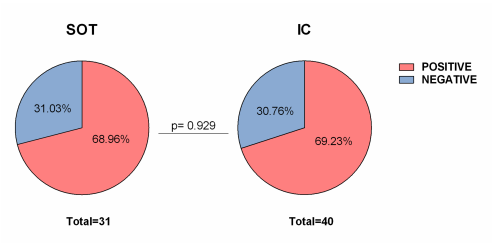
COVID-19 patients (n=102)	**Severe** (n=41)		**Mild** (n=42)		**Asymptomatic** (n=19)		P value
	SOT (n=22)	**IC** (n=19)	**SOT** (n=22)	**IC** (n=20)	**SOT** (n=9)	**IC** (n=10)	
Age (Years, mean±SD)	56.7±13.7	60.4±9.2	60.6±9.6	35.2±10.6[a]	54.3±15.3	60.5±8.9	<0.001
Sex (Female; n [%])	4(18.2)	7(36.8)	8(40)	11(55)	4(44.4)	6(60)	0.145
Time after infection (days; median [IQR])	196 (181-213)	201 (185-206)	177 (132-203)	231[a] (213-252)	161 (121-168)	163 (139-185)	<0.001
Transplant Organ (n [%])							
• Kidney	16 (72.7)	NA	16 (80)	NA	6 (66.7)	NA	
• Liver	2 (10)	NA	3 (15)	NA	0	NA	0.161
• Heart	0 (0)	NA	3 (15)	NA	2 (22.2)	NA	
• Lung	4 (18.2)	NA	0	NA	1 (11.1)	NA	
Type Immunosuppression							
- Calcineurin-inhibitors	16 (72.7)	NA	20 (100)	NA	8 (88.9)	NA	0.241
- Mycophenolate Mofetil	21 (94.5)	NA	19 (95)	NA	6 (66.7)	NA	0.099
- mTor-inhibitors	4 (18.2)	NA	2 (10)	NA	2 (22.2)	NA	0.566
- Steroids	18 (81.8)	NA	17 (85)	NA	9 (100)	NA	0.304
Time after Transplant (Years; median [IQR])	8.05±7.45	NA	5.82±6.79	NA	7.56±7.09	NA	0.571

Abbreviations: NA: Not applicable; SOT: Solid Organ Transplant; IC: Immunocompetent.

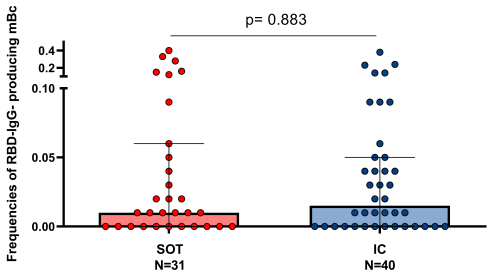
[a] Statistical differences were only observed between mild SOT and IC patients.

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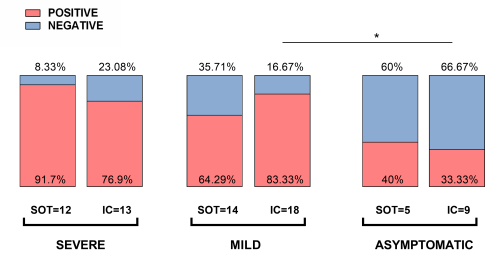
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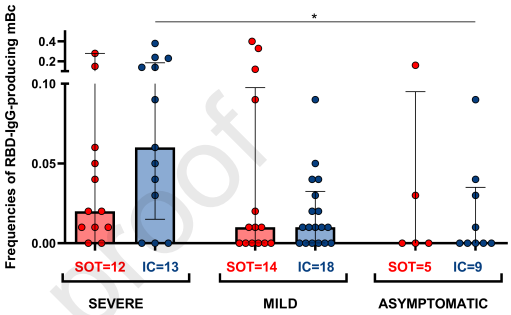
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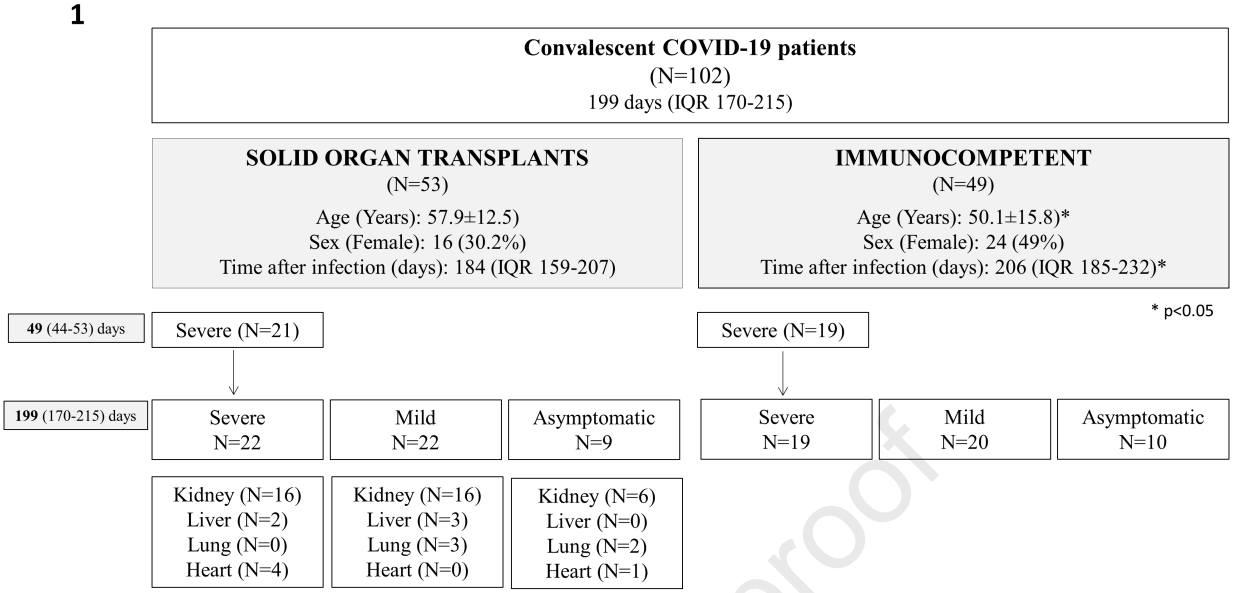


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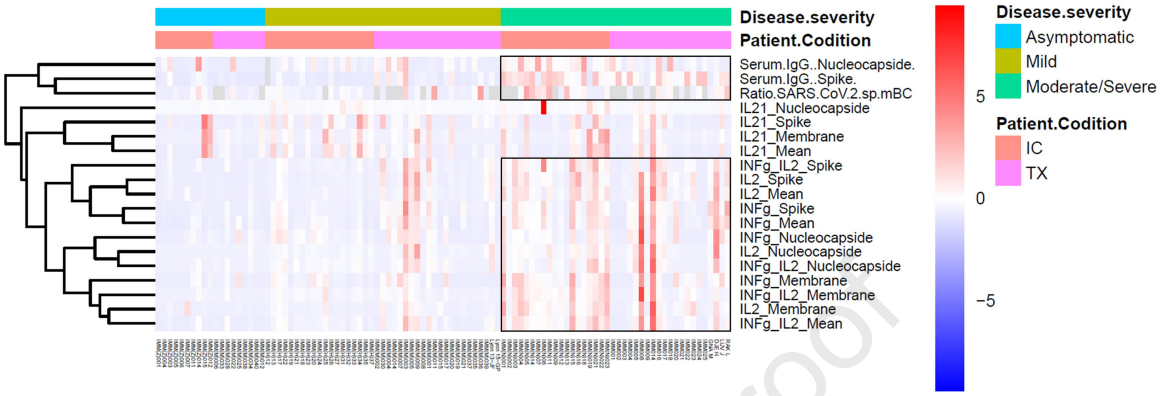
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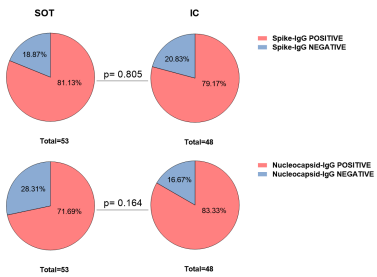
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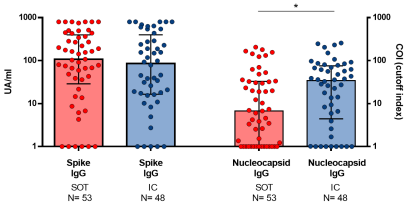


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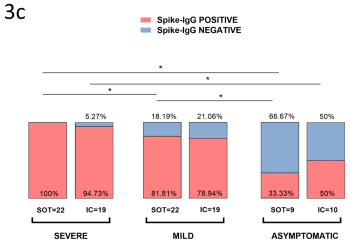
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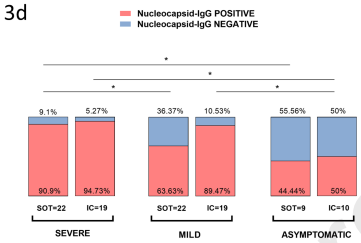
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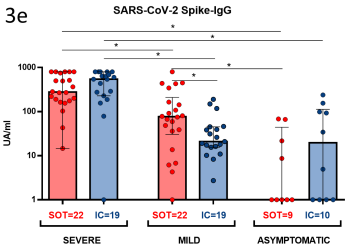
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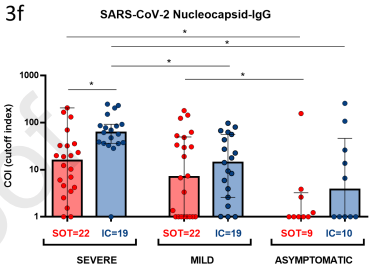
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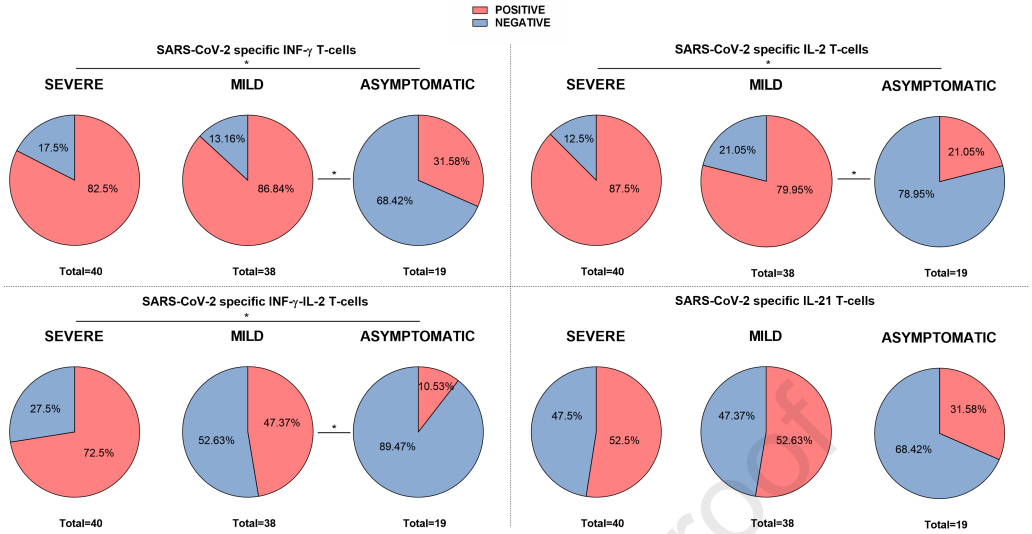


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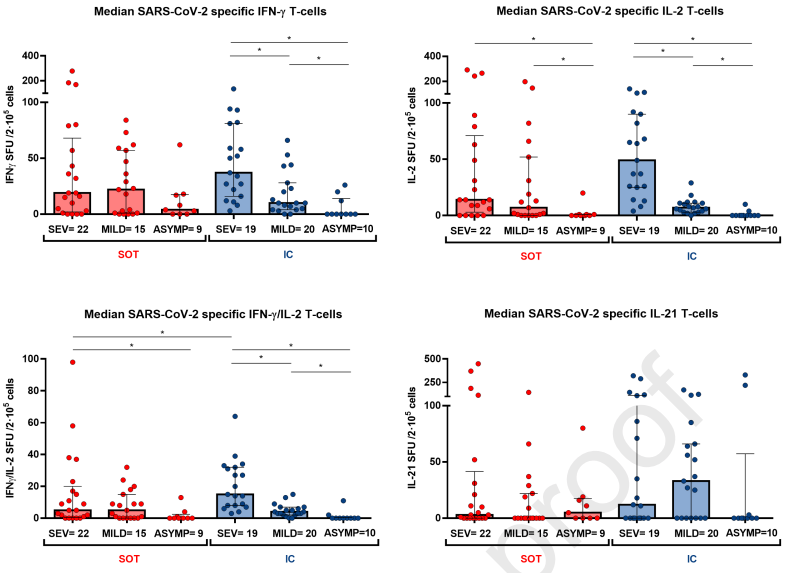
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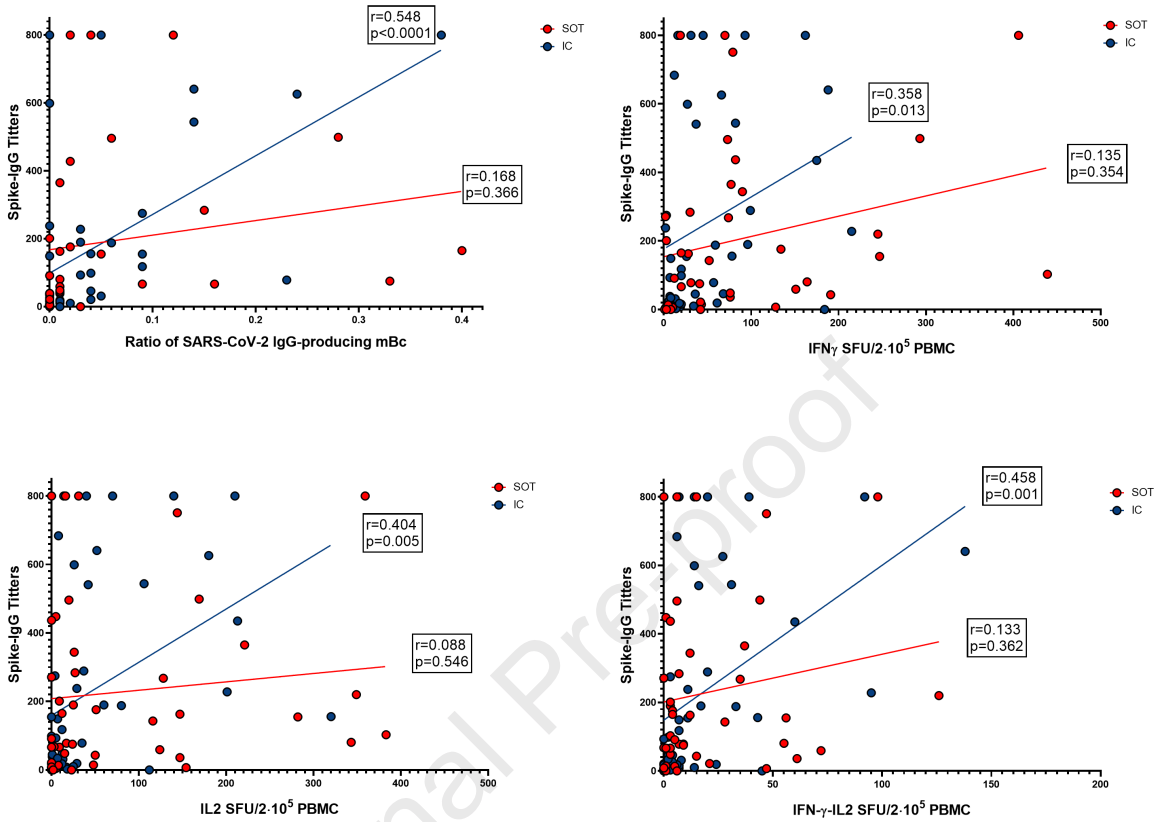
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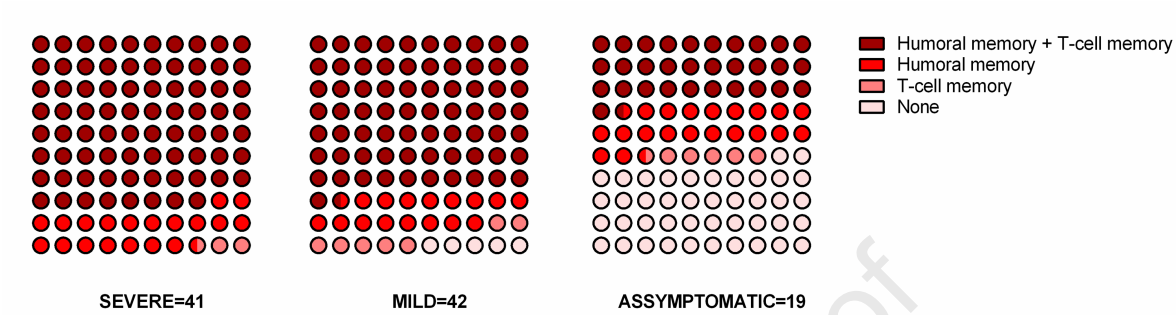
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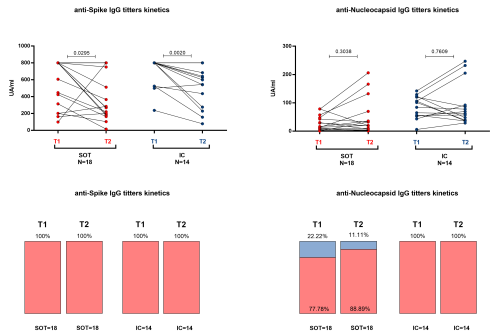
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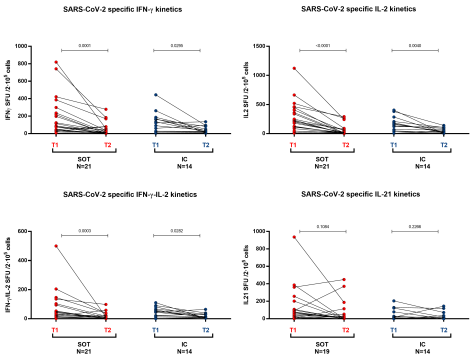


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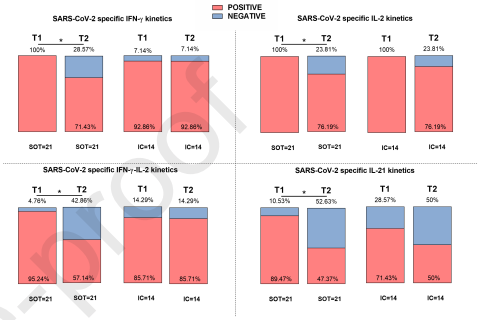
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9b



9c



Journal Pre-proof