Decline in neutralising antibody responses, but sustained T-cell immunity, in COVID-19 patients at 7 months post-infection

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Received 8 February 2021; Revised 27 April, 3 June and 6 July 2021; Accepted 7 July 2021
doi: 10.1002/cti2.1319

Clinical & Translational Immunology 2021; e1319. doi: 10.1002/cti2.1319
www.wileyonlinelibrary.com/journal/cti

INTRODUCTION
The coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has led to over 190 million cases and more than 4.1 million deaths to date. Therefore, there is an urgent need to develop an effective vaccine that can be used to immunise the global population to halt the transmission of the virus. There is considerable
interest in understanding the nature of the immune response to SARS-CoV-2 in patients who have recovered from COVID-19 to shed light on the requirements and likelihood of achieving durable protection from SARS-CoV-2 infection.

The immune system comprises several components that work together to develop protective immunity. Adaptive immune responses, which comprise both humoral and T-cell responses specific to SARS-CoV-2, are important for protection against viral infections. Neutralising antibodies against SARS-CoV-2, especially the surface spike protein that mediates viral entry, have been identified in acute and convalescent COVID-19 patients.5–9 These neutralising antibodies are currently under development as promising therapeutic options.10,11 Most COVID-19 vaccines that induce the production of neutralising antibodies also target the spike protein of SARS-CoV-2.12 However, a concern has been raised regarding the longevity of the antibody response to the spike protein in convalescent COVID-19 patients. Although recent studies have shown that neutralising antibodies last for at least 3 months, some earlier studies have also shown that the level of SARS-CoV-2 IgG declines over time and may become undetectable in a substantial proportion of patients.5,13–15 Helping B cells generate neutralising antibody responses and maintain durable antibody responses is a major function of CD4+ T cells. In addition, recent studies have suggested that T-cell response could be induced by SARS-CoV-2 in the absence of humoral immune responses.16 Therefore, the balance between humoral and cellular immune responses might be important for protection from COVID-19 and avoidance of vaccine-enhanced disease.8,17 Consequently, several COVID-19 vaccines have been designed to elicit robust CD4+ or CD8+ T-cell responses based on neutralising antibodies.18–20

However, there is a lack of longitudinal studies that conduct a combined examination of neutralising antibodies and CD4+ T-cell and CD8+ T-cell responses against SARS-CoV-2 in the same patient population. Addressing these fundamental questions is important in understanding the natural protective immune responses, which may facilitate the development of COVID-19 vaccines. In this study, we aimed to perform a combined assessment of changes in neutralising antibody levels and SARS-CoV-2-specific T-cell responses over time in patients at 7 months after infection.

RESULTS

Declined, but still detectable, humoral response against SARS-CoV-2

The levels of IgG and IgM against spike receptor-binding domain (RBD), as well as surrogate markers of neutralising antibodies, were measured in all collected samples. In the 11 samples from healthy controls, the spike-RBD IgM, IgG and neutralising antibodies were undetectable. Spike-RBD IgM was detected in 48.1% (13/27) of the patients, while high titres of spike-RBD-specific IgG were detected in all patients at their first visit (Figure 1a). By contrast, the titres of neutralising antibodies ranged from low to robust (Figure 1b). Despite the detectable levels of neutralising antibodies in all patients, the 50% inhibitory dilutions were below 1:100 in 29.6% (8/27) of the patients. Disease severity was associated with the titres of neutralising antibodies, but not with those of spike-RBD IgG and IgM (Figure 1c). Patients with previous severe diseases had higher titres of neutralising antibodies than those with mild diseases. Other demographic characteristics, including age and sex, did not affect the titres of neutralising antibodies, spike-RBD-specific IgG or IgM.

IgM titres decayed rapidly as they became undetectable in 96.3% (26/27) of the patients at their second visit. The only patient who had detectable IgM was followed up at 63 days post-onset of symptoms (POS). Spike-RBD IgG titres also decreased sharply in most patients since their first visit (Figure 1a, d). However, spike-RBD IgG remained detected in 92.6% of patients (25/27). Similarly, the titres of neutralising antibodies also decreased significantly at the patient’s second visit compared with that at the first visit (Figure 1b). However, the speed of decay of neutralising antibodies was slower than that of IgG (Figure 1d, e). Despite the detectable levels of neutralising antibodies in the majority (92.6%, 25/27) of the patients at this time point, the 50% inhibitory dilutions higher than 1:100 were only observed in 25.9% (7/27) of these patients. The two patients whose samples were obtained at 214 and 222 days POS, respectively, had loss of neutralising antibodies. The 50% inhibitory dose values of samples obtained from these patients at their first visit were 1:34 and 1:112, respectively. The titres of neutralising antibodies were comparable between patients with different...
The titres of neutralising antibodies were positively correlated with the IgG titres (Figure 1f).

**Sustained SARS-CoV-2-specific T-cell responses during follow-up**

SARS-CoV-2-specific CD4⁺ T-cell and CD8⁺ T-cell responses were measured by quantification of T-cell receptor activation-induced markers (AIM) after in vitro stimulation with two SARS-CoV-2 spike peptides. The data were obtained after subtracting the background control from the DMSO-negative control (Figure 2a). The cumulative SARS-CoV-2-specific CD4⁺ T-cell and CD8⁺ T-cell measurements were calculated as the sum of the two peptides specific CD4⁺ T cells and CD8⁺ T cells, respectively. Adequate living cells were collected from 26 and 25 patients during the first and second visits, respectively.

During their initial visits, SARS-CoV-2-specific CD4⁺ T cells were detected in 96.2% (25/26) of the patients (Figure 2b). Of them, 24 had robust levels of CD4⁺ SARS-CoV-2-specific T cells in the circulation. Despite the low proportion of these cells, spike-specific CD4⁺ T cells were identified in 72.7% (8/11) of healthy controls. The proportion of SARS-CoV-2-specific CD4⁺ T cells in COVID-19 patients was significantly higher than that of healthy controls (Figure 2b). Spike-specific CD8⁺ T cells were also observed in 92.3% (24/26) of the patients. However, the proportions of spike-specific CD8⁺ T cells in COVID-19 patients were statistically comparable to those detected in healthy controls (0.46 [0.13–0.90] % vs 0.14 [0.01–0.38] %, P = 0.052, Figure 2c). In patients without detectable circulating SARS-CoV-2-specific CD4⁺ T cells, 0.14% of SARS-CoV-2-specific CD8⁺ T cells could be identified. Therefore, all patients had measurable SARS-CoV-2-specific CD4⁺ T cells or CD8⁺ T cells.

Both SARS-CoV-2-specific CD4⁺ T cells and CD8⁺ T cells decayed slowly during the follow-up period (Figure 2d, e). SARS-CoV-2-specific CD4⁺ T cells were detectable in 96% (24/25) of the patients at their second visit, which occurred at 212 days POS. Robust SARS-CoV-2-specific CD4⁺ T-cell responses could still be identified in 68.0% (17/25) of the patients. When compared to the levels of SARS-CoV-2-specific CD4⁺ T cells among these patients...
at the first visit, no significant change was observed. The proportion of SARS-CoV-2-specific CD4\(^+\) T cells at this time point was persistently higher than that detected in healthy controls (Figure 2b). Similarly, SARS-CoV-2-specific CD8\(^+\) T-cell responses were identified in 88% (22/25) of the patients, with relatively constant levels compared with those identified in the first visit (Figure 2c). No significant differences were observed in the proportion of spike-specific CD8\(^+\) T cells between patients who had recovered from COVID-19 and healthy controls ($n = 26$ at the first visit and $n = 25$ at the second visit) and healthy controls ($n = 11$). (d) Decay of the proportions of the spike-specific CD4\(^+\) T cells and (e) CD8\(^+\) T cells over time. (f) The disease severity does not have an effect on the changes in spike-specific CD4\(^+\) T-cell and (g) CD8\(^+\) T-cell response. The dashed line indicates the limit of detection.

Correlation between SARS-CoV-2-specific T-cell responses and antibody titres

We then evaluated the association between SARS-CoV-2-specific T-cell responses and antibody titres. We pooled the data from patients who had recovered from COVID-19. A moderate positive correlation was observed between these two cell populations ($r = 0.54$, $P < 0.01$). A similar trend was also observed in the changes in SARS-CoV-2 specific CD4\(^+\) and CD8\(^+\) T-cell levels during these two visits (Figure 2d, e). In this cohort, the differences in the levels of SARS-CoV-2-specific T cells were not significantly associated with age, sex and disease severity (Figure 2f, g).
CD4+ T-cell responses and neutralising antibody titres \( (r = 0.47, P < 0.01, \text{Figure 3a}) \). The titres of spike-RBD IgG were also positively associated with the proportion of SARS-CoV-2-specific CD4+ T cells \( (r = 0.54, P < 0.001, \text{Figure 3b}) \). Moreover, weak correlations were found between the levels of circulating SARS-CoV-2-specific CD8+ T cells and titres of spike-RBD IgG, as well as between the levels of circulating SARS-CoV-2-specific CD8+ T cells and titres of neutralising antibodies (Figure 3c, d).

**DISCUSSION**

Ascertaining the magnitude and quality of humoral and T-cell immunological memory against SARS-CoV-2 is critical to understanding durable protection. Doing so could also help in the development of effective vaccines. Although our understanding of COVID-19 is expanding, our knowledge on immunity to SARS-CoV-2 after recovery is still limited. Comprehensive evaluations of SARS-CoV-2-specific humoral and T-cell responses in the same patients who have recovered from COVID-19 are essential to expand our understanding. The results of our exploratory study suggest that although the titres of neutralising antibodies against SARS-CoV-2 decay, immunity mediated by T cells, predominantly SARS-CoV-2-specific CD4+ T cells, is sustained at 7 months after primary infection.

Our results show that the spike-RBD-specific IgM and IgG titres, as well as the neutralising antibody titres, declined significantly within the first 7 months after SARS-COV-2 infection. The results of our study are in line with the reports of previous studies, which showed that SARS-CoV-2-specific IgG decayed sharply in convalescent COVID-19 patients.3,13 Recent studies also revealed that the neutralising antibody waned after the titre peaked, which was detected 3–4 weeks after infection.6,21 Taken together, these results support the notion that the humoral response against SARS-CoV-2 is a typical response after an acute viral infection. Hence, the primary concern is the longevity of the neutralising antibody after an acute SARS-COV-2 infection. Several longitudinal studies have shown that there was little to no decrease in the neutralising antibody titres at 75 days POS and that only modest declines were observed within 5 months POS.5,15 In this study, 92.5% of the patients still had detectable neutralising antibodies approximately 7 months after infection, despite a decrease in titres. However, two patients with relatively low neutralising antibody titres at their first visit lost

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**Figure 3.** Correlations of spike-specific humoral response and T-cell response. (a) Neutralising antibody titres and (b) spike-RBD IgG titres are both positively correlated with the proportions of spike-specific CD4+ T cells and (c, d) CD8+ T cells. The dashed line indicates the limit of detection.
of SARS-CoV were detectable in patients who had recovered from SARS 17 years after the outbreak of this disease in 2003. In the current study, the humoral response to SARS-CoV-2 declined significantly after 7 months, while the levels of specific T-cell responses remained stable, indicating that cellular responses to SARS-CoV-2 may last longer than humoral responses.

Emerging evidence suggests that both humoral and T-cell immune responses are required for effective protection against SARS-CoV-2 infection. In a mouse study, the intravenous adoptive transfer of SARS-CoV-immune splenocytes or in vitro-generated T cells to mice enhanced their survival and reduced the virus titres in the lungs, suggesting that T cells play a crucial role in SARS-CoV clearance. However, it is still unknown whether the persistence of the cellular immune response can prevent SARS-CoV-2 reinfection or reduce the severity of infection when neutralising antibodies are present. In a recent study, higher T-cell responses to SARS-CoV-2 were reported to be associated with a lower risk of developing COVID-19. In addition, a few case reports of laboratory-confirmed SARS-CoV-2 reinfections have also suggested that T-cell responses may prevent reinfection.

Our study has some limitations. First, we used the ACE2-RBD binding inhibition assay, which is a surrogate neutralisation test, and may be less helpful compared with a living virus assay. Second, we only used peptide pools from the spike protein. Although the neutralising antibodies are generally considered against the spike protein, humoral and T-cell responses against other proteins, including the M and N proteins, and full epitope mapping in the future could provide comprehensive information on human coronavirus-specific humoral and T-cell responses. Third, the sample size used in our study was limited by expediency, which may be underpowered for detecting subtle differences. Finally, all COVID-19 patients enrolled in the present study were admitted in the hospital. Therefore, it remains unknown whether these asymptomatic patients have a similar pattern of changes in humoral and T-cell responses.

In conclusion, our study suggests that in patients who have recovered from COVID-19,
SARS-CoV-2-specific T-cell immune responses persist, while the neutralising antibodies are waning. Hence, further studies are needed to determine the longevity of neutralising antibodies and to evaluate whether these T cells are sufficient to protect patients from reinfection.

METHODS

Ethical statement and clinical definitions

This study was approved by the ethics committee of the Shanghai Public Health Clinical Center. Written informed consent was obtained from all donors. All donors were COVID-19 patients admitted in the hospital between January and February 2020. They were discharged after showing symptom relief and clearance of SARS-CoV-2. They were routinely followed up at the outpatient clinic. Blood samples for antibody tests and cellular analyses were collected and stored at each visit. The samples were collected at a median of 36 (range: 23–77) and 212 (range: 52–235) days after the onset of self-reported symptoms at the first and second visits, respectively. The disease severity of the patients was identified based on the degree of hypoxemia. Eight patients were classified as having a severe case because they had a PaO2/FiO2 ratio lower than 300 and met the criteria for acute respiratory distress syndrome, while 19 were classified as having a mild case. Details regarding the patients’ information are provided in Table 1. Eleven healthy donors were enrolled as controls.

Peripheral blood mononuclear cells (PBMCs) from healthy donors and patients were isolated from fresh blood samples by Ficoll-Paque density gradient centrifugation on the day of blood collection. The majority of the purified PBMCs were used for immune cell phenotyping, whereas the plasma samples were subjected to antibody tests.

| Table 1. Demographic and clinical characteristics of the study population |
|-----------------|-----------------|
| Characteristics  | Value           |
| Age, median (IQR)| 52 (39–64)      |
| Sex, n (%)       | Male 12 (44.4)  |
|                  | Female 15 (55.6) |
| Days post-onset of symptoms, median (IQR) | First visit 36 (31–39) |
|                  | Second visit 212 (70–222) |
| Disease severity, n (%) | Mild 19 (70.4)  |
|                  | Severe 8 (29.6) |
| Comorbidities, n (%)| Hypertension 8 (29.6) |
|                  | Diabetes 4 (14.8) |
|                  | Coronary heart disease 1 (3.7) |
|                  | COPD 3 (11.1) |
| Symptoms, n (%) | Fever 25 (92.6)  |
|                  | Cough 15 (55.6) |
| White blood cells count (× 10^9 L^-1) | < 3.5, n (%) 6 (22.2) |
|                  | Lymphocytes (× 10^9 L^-1) 0.85 (0.59–1.16) |
|                  | Baseline CD4^+ T-cell count, median (IQR), cells μL^-1 316 (211–595) |
|                  | Baseline CD8^+ T-cell count, median (IQR), cells μL^-1 192 (103–281) |
|                  | Baseline CD4/CD8 ratio 1.78 (1.32–2.54) |
|                  | Use of glucocorticoid (n, %) 4 (14.8) |
|                  | Use of intravenous immunoglobulin (n, %) 7 (25.9) |

Surrogate virus neutralisation test

The levels of serum neutralising antibodies against the RBD of SARS-CoV-2 were measured using a novel surrogate virus neutralisation test with a commercial kit provided by GenScript (L00847; GenScript, Nanjing, China). The serum samples were diluted (at 1:10) with twofold serial gradients and incubated with an equal volume of horseradish peroxidase-conjugated RBD (HRP-RBD) at 37°C for 30 min. Then, the serum/HRP-RBD mix (100 μL) was added to each well and incubated at 37°C for 15 min. Unbound HRP-RBD was removed by four washes, the chromogenic substrate TMB was added, and the mixture was incubated at 25°C for 15 min. The colorimetric reaction was terminated by adding a stop solution. The absorbance at 450 nm was measured using a microplate reader (iMark, Bio-Rad, CA, USA). The percentage inhibition for each sample was calculated using the following formula: % reduction = [1 – OD450 (sample)/average OD450 (negative control)] × 100%. The neutralising antibody titre was calculated with a half-maximal inhibitory concentration.

Spike S1-receptor-binding domain-specific IgG and IgM test

The serum titres of IgG and IgM antibodies against SARS-CoV-2 spike S1-RBD were determined using commercial kits (L00845; GenScript, China) according to the manufacturer’s instructions. The serum samples were diluted to 10-fold serial gradients. Briefly, 100 μL of serum sample was incubated at 37°C for 30 min, followed by four washes. Then, 100 μL of HRP-conjugated mouse anti-human IgG or IgM was added and incubated at 37°C for 15 min, after which the chromogenic substrate (TMB) was added and incubated at 25°C for another 15 min. The colorimetric reaction was terminated by adding a stop solution, and the absorbance of each sample at 450 nm was measured with a microplate reader (iMark). The sample/cut-off ratio (S/CO) was calculated according to the manufacturer’s instructions. The serum IgG and IgM antibody titres were calculated as the reciprocal of the dilution factor when S/CO = 1.

Detection of SARS-CoV-2 antigen-responsive T cells

The PBMCs were separated using a Lymphoprep kit (AS1114546; Axis-Shield, Dundee, UK). The PBMCs (1 million) were cultured in RPMI 1640 medium (Gibco, Gaithersburg, MD, USA) supplemented with 10% foetal...
bovine serum (Gibco, Logan, UT, USA) and an antibiotic cocktail containing 100 µg mL⁻¹ penicillin and 100 µg mL⁻¹ streptomycin. Five nanograms of the SARS-CoV-2 peptide pool at a concentration of 5 ng mL⁻¹ was added to the culture media to stimulate the production of T cells for 3 days. The same volume of DMSO and PHA served as the negative control and positive control, respectively, for each sample. The peptide pool, including 316 peptides (delivered in two subpools of 158 and 158 peptides), was derived via a peptide scan (15 mers with 11 aa overlap) through the entire spike glycoprotein (protein ID: PDTC2) of SARS-CoV-2 (GenScript). Cell culture was performed at 37°C in a 5% CO₂ humidified environment. Then, the SARS-CoV-2-specific CD4⁺ T-cell and CD8⁺ T-cell responses were measured by quantification of T-cell receptor AIMs using flow cytometry in living cells. Briefly, the cells were harvested, washed twice with PBS, incubated with fixable viability stain 510 (BDTM Horizon, 564406) to distinguish whether the cells were alive or dead for 30 min and washed once with PBS. Then, the cells were incubated with the APC-H7 mouse anti-human CD8 antibody (clone SK1; BD Bioscience, Franklin Lakes, NJ, USA), BV605 mouse anti-human CD4 antibody (clone RPA-T4; BD Bioscience, Franklin Lakes, NJ, USA), Percp mouse anti-human CD3 antibody (clone SK7; BD Bioscience, Franklin Lakes, NJ, USA), APC anti-human CD137 antibody (clone 4B4-1; BioLegend, San Diego, CA, USA), PE/cyanine7 anti-human CD134 antibody (clone Ber-ACT35; BioLegend) and FITC-CD69 monoclonal antibody (11-0699-42; Thermo Fisher Scientific, Waltham, MA, USA) for 15 min. After washing with PBS, the cells were permeabilised and washed with BD PermWash™ buffer (554723; BD Bioscience, Franklin Lakes, NJ, USA), and flow cytometry analysis was performed using BD LSRFortessa. The AIM⁺ CD4⁺ T cells (CD134⁺CD137⁺) and AIM⁺ CD8⁺ T cells (CD69⁺CD137⁺) were gated. Robust T-cell responses were defined as more than 0.1% of AIM⁺ CD4⁺ or CD8⁺ T cells detected in the circulation.⁸ Flow cytometry data were analysed using the FlowJo version 10 software (FlowJo LLC, Ashland, OR, USA).

Statistical analyses
Statistical analyses were performed using the GraphPad Prism 9 software (GraphPad, San Diego, CA, USA). The data were represented as mean values with SDs or medians (interquartile ranges) depending on their distribution. A Mann-Whitney two-tailed U-test was used to compare variables between the two groups, and a Wilcoxon matched-pairs signed-rank test was used to compare paired non-parametric data. The correlations were calculated using Spearman’s rank correlation coefficient.

ACKNOWLEDGMENTS
The work was supported by the Shanghai Science and Technology Committee (20411950200 and 2021190900), the Development Fund for Shanghai Talents (2020089) and the Shanghai ‘Rising Stars of Medical Talent’ Youth Development Program, Specialist Program (no. 2019-72). We thank Editage (www.editage.com) for English-language editing.

AUTHOR CONTRIBUTIONS
Jun Chen: Conceptualization; Formal analysis; Investigation; Writing-original draft. Xiaomin Li: Investigation. Methodology. Xinyu Zhang: Investigation; Methodology. Yixiao Lin: Investigation; Methodology. Danping Liu: Investigation; Methodology. Jingna Xu: Investigation; Methodology. Zhenyan Wang: Investigation. Ling Gu: Investigation; Methodology. Qian Li: Investigation; Methodology. Dan Yin: Project administration. Junyang Yang: Conceptualization; Funding acquisition; Supervision; Writing-review & editing.

CONFLICT OF INTEREST
The authors declare no conflict of interest.

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