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Research Article

SARS-CoV-2-specific T cell responses wane profoundly in convalescent individuals 10 months after primary infection

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ABSTRACT

A key question in the coronavirus disease 2019 (COVID-19) pandemic is the duration of specific T cell responses against the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) post primary infection, which is difficult to address due to the large-scale COVID-19 vaccination and re-exposure to the virus. Here, we conducted an analysis of the long-term SARS-CoV-2-specific T cell responses in a unique cohort of convalescent individuals (CIs) that were among the first to be infected worldwide and without any possible antigen re-exposure since then. The magnitude and breadth of SARS-CoV-2-specific T cell responses correlated inversely with the time that had elapsed from disease onset and the age of those CIs. The mean magnitude of SARS-CoV-2-specific CD4 and CD8 T cell responses decreased about 82% and 76%, respectively, over the time period of ten months after infection. Accordingly, the longitudinal analysis also demonstrated that SARS-CoV-2-specific T cell responses waned significantly in 75% of CIs during the follow-up. Collectively, we provide a comprehensive characterization of the long-term memory T cell response in CIs, suggesting that robust SARS-CoV-2-specific T cell immunity post primary infection may be less durable than previously expected.

1. Introduction

Antigen-specific T and B cell responses play fundamental roles in the clearance of most viral infections. Additionally, the establishment of T and B cell memory after recovery is essential for protecting the host against disease re-occurrence. Faced with the unprecedented medical and socioeconomic crisis caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and the associated coronavirus disease 2019 (COVID-19), the scientific community has undertaken tremendous efforts to uncover the correlates of protection as well as determinants of immunity against SARS-CoV-2 (Vabret et al., 2020). Increasing evidence suggest that T cells may play a fundamental role in the resolution of COVID-19 (Canete and Vinuesa, 2020; Chen and John Wherry, 2020). The current dogma is that SARS-CoV-2-specific CD4 and CD8 T cell responses, recognizing multiple epitopes across the viral proteome, are evident in most individuals both during acute COVID-19 and convalescence (Braun et al., 2020; Le Bert et al., 2020; Ni et al., 2020; Peng et al., 2020; Thieme et al., 2020; Weiskopf et al., 2020). The magnitude of SARS-CoV-2-specific T cell responses during the early phase was reported to correlate with the magnitude of antibody responses, and more severe and protracted disease usually drives more vigorous and, in terms of epitope coverage, broader T cell responses (Ni et al., 2020; Peng et al., 2020; Thieme et al., 2020). However, it has also been observed that cellular and humoral immune responses can become uncoupled in some SARS-CoV-2-exposed individuals, who showed strong specific T cell immunity but lack detectable antibody responses (Sekine et al., 2020). It was assumed that this results from antibody responses waning more quickly than T cell responses and that SARS-CoV-2-specific antibody

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responses are rather short-lived, while T cell memory seems to be more long-lasting (Altmann and Boyton, 2020). However, all available data on T cell memory were mainly generated from individuals recovering from COVID-19 during a relatively limited follow-up period for no longer than eight months after infection (Peng et al., 2020; Dan et al., 2021; Jiang et al., 2021; Zhao et al., 2021). It is not yet known whether memory T cell responses generated from natural primary infection with SARS-CoV-2 ancestral strain last and how they change in the long-term post recovery. However, this issue is hard to address due to the large-scale of COVID-19 vaccination and re-exposure to the virus.

Wuhan was the very first city hit by SARS-CoV-2. Accordingly, all patients who experienced the longest phase of convalescence following COVID-19 reside here or close by. Wuhan also installed rigorous mitigation strategies which board the epidemic spread quickly under control. As indicated by a thorough SARS-CoV-2 RNA surveillance covering every Wuhan resident in May 2020, virtually no autochthonous virus circulation occurred afterwards. This situation enabled us to characterize the long-term memory T cell responses in a cohort of 81 COVID-19 convalescent individuals (CIs) with an unprecedented observation time up to 443 days post disease onset (DPDO) in the absence of any possible antigen re-exposure. Our results suggest that SARS-CoV-2 memory T cell responses wane significantly in the majority of CIs during the long-term period after recovery.

2. Materials and methods

2.1. Subjects

Eighty-one convalescent individuals who resolved their SARS-CoV-2 infection were recruited at the Department of Infectious Diseases, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology and the Department of Gastroenterology from April 2020 to April 2021. The diagnosis of COVID-19 was based on the Guidelines for Diagnosis and Treatment of Corona Virus Disease (2019) issued by the National Health Commission of China (7th edition). Mild cases were defined as follows: (1) showing mild clinical symptoms; (2) no sign of pneumonia on chest imaging. Moderate cases were defined as follows: (1) showing fever and respiratory symptoms; (2) radiological findings of pneumonia. Confirmed patients meeting any of the following criteria were defined as severe cases: (1) respiratory distress ($\geq$ 30 breaths/min); (2) oxygen saturation $\leq$ 93% at rest; (3) alveolar oxygen partial pressure/fraction of inspiration (PaO_2/FiO_2) $\leq$ 300 mmHg (1 mmHg = 0.133 kPa). The defining criteria for COVID-19 convalescence were as follows: being afebrile for more than three days, resolution of respiratory symptoms, substantial improvement of chest CT findings, and two consecutive negative RT-PCR tests for SARS-CoV-2 RNA in respiratory tract swab samples obtained at least 24 h apart. CIs were stratified according to the severity of disease into asymptomatic (ACs: 13.58%, 11/81), mild or moderate (MCs: 61.73%, 50/81), and severe COVID-19 cases (SCs: 24.69%, 20/81). Seventeen SARS-CoV-2-unexposed individuals (UIs) who lacked a history of COVID-19 symptoms, RT-PCR positivity, and IgM as well as IgG antibodies recognizing the spike and the nucleocapsid protein were also recruited. For the convalescents, no reinfection of SARS-CoV-2 occurred and all the participants did not receive COVID-19 vaccine inoculation during the sampling period.

2.2. Preparation of PBMCs

Peripheral blood mononuclear cells (PBMCs) of SARS-CoV-2-unexposed individuals and convalescents were isolated using Ficoll density gradient centrifugation (DAKEWE Biotech, China) and were rapidly assessed by flow cytometry analysis without intermittent cryo-preservation.

2.3. Detection of SARS-CoV-2 S/N-specific antibodies

As described previously (Padoan et al., 2020; Wu et al., 2021), SARS-CoV-2 specific IgM and IgG antibodies recognizing the RBD of S or N protein were quantified using capture chemiluminescence immunoassays (CLIA) by MAGLUMITM 4000 Plus (Snibe, Shenzhen, China). The cut-off value was 0.7 AU/mL for anti-S IgM and 1 AU/mL for anti-N IgM, anti-S IgG, and anti-N IgM.

2.4. Analysis of effector T cell responses

Three pools of lyophilized peptides, consisting mainly of 15-mer sequences with 11 amino acids (aa) overlap, either covering the immunodominant sequences of the surface glycoprotein (S) or the complete sequences of the nucleocapsid phosphoprotein (N) or the membrane glycoprotein (M) of SARS-CoV-2 were used for cell stimulation (PepTivator® Peptide Pools, Miltenyi, Germany). On day one, PBMCs were cultivated in complete medium [RPMI 1640 containing 10% (v/v) fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 100 μmol/L 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer] with recombinant interleukin (IL)-2 (20 U/mL; Hoffmann-La Roche, Italy). Cells without anti-CD3, anti-CD28 and peptide stimulation served as negative control. Cells with anti-CD3 (1 μg/mL; Invitrogen, USA) and anti-CD28 (1 μg/mL; Invitrogen, USA) stimulation served as positive control. Cells stimulated with S, N, or M peptide pools (1 μg/mL) in the presence of anti-CD28 served as peptide stimulation groups. Fresh medium containing IL-2 was added on day 4 and 7. On day 10, cells were restimulated for 5 h with the same peptide pool in the presence of brefeldin A (BD Biosciences, San Diego, CA). Cells were then tested for IFN-γ, IL-2, and TNF-α expression by intracellular cytokine staining. Specific cytokine responses were calculated by subtracting the background activation (the percentage of cytokine-positive cells in the unstimulated control) before further analysis. T cell responses were defined as being detectable in the case that the frequency in the specifically stimulated culture exceeded the unstimulated control at least twofold (stimulation index >2). Samples with responseless positive controls were excluded from further analyses.

2.5. Flow cytometry

Surface and intracellular staining for flow cytometry analysis were performed as described previously (Liu et al., 2013; Wang et al., 2018). For surface staining, cells were incubated with relevant fluorochrome-labeled antibodies (eFluor 780-anti-CD3, PE-Cy7-anti-CD8, and PerCP-Cy5.5-anti-CD4) for 30 min at 4 °C in the dark. For intracellular cytokine staining, cells were fixed and permeabilized using the Intracellular Fixation & Permeabilization Buffer Set (Invitrogen, USA) and subsequently stained with FITC-anti–IFN–γ, PE-anti-IL-2 and APC-anti-TNF-α (Invitrogen, USA). Approximately 100,000 PBMCs were acquired for each sample using a BD FACS Canto II flow cytometer. Data analysis was performed using the FlowJo software V10.0.7 (Tree Star, Ashland, OR, USA). Cell debris and dead cells were excluded from the analysis based on scatter signals and Fixable Viability Dye eFluor 506.

2.6. Statistical analysis

Statistical analyses were performed using the SPSS statistical software package (version 22.0, SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk method was used to test for normality. Mann-Whitney U test, Spearman correlation test, and Chi-square test were used where appropriate. All reported P values were two-sided, and a P value less than 0.05 was considered statistically significant (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

3. Results

3.1. Characteristics of the study cohort

To characterize SARS-CoV-2-specific memory CD4 and CD8 T cell responses in individuals who had recovered from COVID-19, 115 blood samples derived from 81 CIs together with 17 UIs were assessed. The demographic profiles of all individuals are shown in Table 1. The median period between disease onset and blood sampling was 330 days (range: 83–443 days). Among the COVID-19 cases, 71.23% (52/73) were hospitalized and 52.54% (31/59) received oxygen inhalation support. Leukopenia and lymphopenia were observed in 52.63% (10/19) and 72.22% (13/18) of tested cases, respectively. Increased C-reactive protein and interleukin (IL)-6 levels were apparent in 63.16% (12/19) and 86.67% (13/15) of tested patients, respectively. Abnormal radiological findings consistent with pneumonia were evident in 69.44% (25/36) CIs by chest computed tomography scans (CT). Forty-one CIs (50.62%) had been tested positive for SARS-CoV-2 RNA by RT-PCR. All patients were confirmed for past infection by SARS-CoV-2-specific IgM and IgG seropositivity. At the time of the last blood sampling, 75.31% (61/81) were IgG single-positive, 13.58% (11/81) were IgM and IgG double-positive, and 11.11% (9/81) were IgG seronegative. At the time of recruitment, all CIs were negative for SARS-CoV-2 RNA and had no medical conditions related to COVID-19.

3.2. Characterization of the long-term memory T cell response against SARS-CoV-2

PBMCs of UIs and CIs were re-stimulated with three panels of overlapping peptides spanning the SARS-CoV-2 proteins S, N, and M, respectively, to determine SARS-CoV-2-specific T cell responses *in vitro*. We used an intracellular cytokine staining flow cytometry assay (Supplementary Fig. S1), and the percentages of cytokine [interferon (IFN)-γ, IL-2, and tumor necrosis factor (TNF)-α] producing CD4 and CD8 T cells of all participants are shown in Fig. 1A. Additionally, the frequencies of the IFN-γ, IL-2, or TNF-α-positive CD4 and CD8 T cells are also shown individually in Fig. 1B and C. Consistent with previous reports (Braun et al., 2020; Le Bert et al., 2020), a proportion of T cells weakly responded to SARS-CoV-2 peptides in UIs (both CD4 and CD8 T cells: 58.82%, 10/17), but with much lower frequencies compared to the responses observed in CIs (Fig. 1A–C). In general, SARS-CoV-2-specific T cell responses considerably varied in magnitude between individual CIs. The frequencies of IFN-γ, IL-2, or TNF-α responses against S, N and M of CD4 and CD8 T cells were positively correlated (Supplementary Fig. S2).

Next, we analyzed the correlation between the magnitude and breadth (to how many peptide pools the T cells responded) of the overall SARS-CoV-2-specific T cell responses and the time after disease onset. The CIs were studied up to 14 months after disease onset and we combined the data from MCs and SCs for the analysis. For CD4 T cells, both the magnitude of SARS-CoV-2-specific responses against S, N, or M and the breadth of the responses showed a significant inverse correlation with days post disease onset (DPDO) (Fig. 2A). For CD8 T cells, the magnitude of SARS-CoV-2-specific responses against N, but not the breadth of the responses, was significantly inversely correlated with DPDO (Fig. 2B). As a strong association between COVID-19 severity and antigen-specific immune responses has been reported (Rydyznski Moderbacher et al., 2020), we separately analyzed MCs and SCs for their changes in SARS-CoV-2-specific T cell responses over time. In MCs, the magnitude of SARS-CoV-2-specific CD4 T cell responses against S, N, or M, the magnitude of SARS-CoV-2-specific CD8 T cell responses against N, as well as the breadth of CD4 T cell responses showed a significant inverse correlation with DPDO (Fig. 2C and D). In SCs, however, only a negative correlation between the magnitude of SARS-CoV-2-specific CD4 T cell responses against S and DPDO was observed, no significant correlations were observed for the breadth of SARS-CoV-2-specific CD4 and CD8 T cell responses and DPDO (Fig. 2E and F), which is probably due to the limited numbers of severe cases we were able to enroll in the study. Similar results for MCs and/or SCs were observed when the magnitude of SARS-CoV-2-specific T cell responses was analyzed by single effector cytokine (IFN-γ, IL-2, or TNF-α) expression (Supplementary Fig. S3). Taken together, these results indicated that the SARS-CoV-2-specific CD4 and CD8 T cell responses in recovered COVID-19 patients waned significantly overtime during a period of 14 months.

Next, we stratified MCs and SCs into two groups according to their recovery time (more or less than 300 DPDO), since a characterization of SARS-CoV-2-specific T cell responses in CIs with a recovery time over 10 months has been seldomly reported (Cromer et al., 2021). The magnitude of specific CD4 and CD8 T cell responses against S, N, or M was significantly lower in CIs with DPDO >300 compared to those with DPDO <300 (Fig. 3A). The mean frequencies of specific CD4 T cells against S, N, and M in CIs with DPDO >300 declined by 87% (3.06 versus 23.18), 82% (4.26 versus 23.78), and 83% (4.65 versus 26.64) compared to CIs with DPDO <300, respectively. The mean frequencies of cytokine-producing memory CD8 T cells against S, N, and M in CIs with DPDO >300 declined by 75% (3.41 vs 13.86), 82% (2.52 vs 13.93), and 75% (2.20 vs 8.95) compared to CIs with DPDO <300, respectively (Fig. 3A). Significant decreases in the magnitude of SARS-CoV-2-specific CD4 and CD8 T cell responses were also observed in CIs with DPDO >300 when MCs and SCs were separately analyzed (Fig. 3B and C). Similar results were observed when the magnitude of SARS-CoV-2-specific T cell responses was measured by single effector cytokine (IFN-γ, IL-2 or TNF-α) expression (Supplementary Fig. S4). Specific CD4 T cell responses against S, N, and M were undetectable in 44.44% (24/54) of CIs with DPDO >300, but only in 6.45% (2/31) of

Table 1
Baseline characteristics of the study cohort.

Parameters	Unexposed individuals	Convalescent individuals
N	17	81
Gender (male/female)	8/9	26/55
Age (year)	37.3	52.2
Asymptomatic cases %	/	13.58% (11/81)
Mild or moderate cases %	/	62.73% (50/81)
Severe cases %	/	24.69% (20/81)
Days from disease onset	/	330 (83–443)
Clinical parameters		
Fever %	/	60.26% (47/78)
Respiratory symptoms %	/	62.82% (49/78)
Hospitalized %	/	71.23% (52/73)
Oxygen therapy %	/	52.54% (31/59)
Laboratory parameters[a]		
Leukopenia %	/	52.63% (10/19)
Lymphopenia %	/	72.22% (13/18)
Increased CRP %	/	63.16% (12/19)
Increased ferritin %	/	40.00% (4/10)
Increased LDH %	/	40.00% (6/15)
Abnormal liver function %	/	53.33% (8/15)
Abnormal renal function %	/	0 (0/16)
Increased CK %	/	20.00% (3/15)
Abnormal blood coagulation %	/	6.25% (1/16)
Increased IL-6%	/	86.67% (13/15)
CT scan		
Normal %	/	30.56% (11/36)
Viral pneumonia %	/	69.44% (25/36)
Virological markers		
RNA positive %	/	50.62% (41/81)
IgG single-positive %	/	75.31% (61/81)
IgM & IgG positive %	/	13.58% (11/81)
IgG negative %	/	11.11% (9/81)

CT, computed tomography; CRP, C-reactive protein; LDH, lactate dehydrogenase; CK, creatine kinase; IL, interleukin.
[a] Increased: above the upper limit of normal.

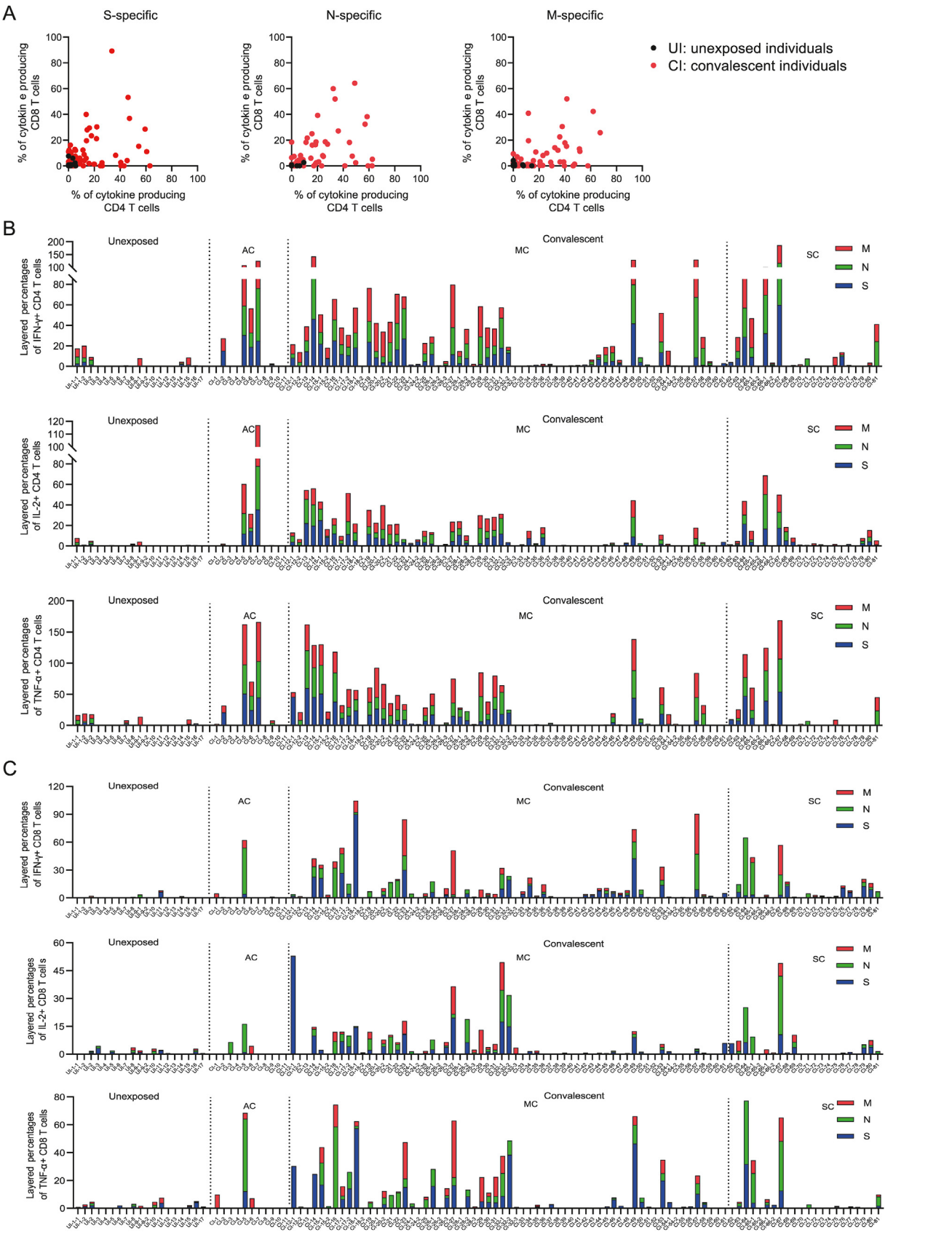

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Fig. 1. The magnitude and breadth of long-term SARS-CoV-2 memory T cell responses are heterogeneous in COVID-19 convalescent individuals. PBMCs of SARS-CoV-2-unexposed individuals (UI) and COVID-19 convalescent individuals (CI) were tested for responses to three panels of overlapping peptides spanning the SARS-CoV-2 S, N, and M, respectively, using intracellular cytokine staining flow cytometry assay. **A** The magnitude of overall cytokine responses of CD4 and CD8 T cells against S, N, and M of SARS-CoV-2 of all participants are shown. A T cell which produced any one or more of the three cytokines (IFN-γ, IL-2, and TNF-α) in response to the stimulation was defined as a "cytokine producing" T cell. **B**, **C** The magnitude of IFN-γ, IL-2, and TNF-αresponses of CD4 (**B**) and CD8 (**C**) T cells specific to S, N, and M of SARS-CoV-2 of all participants are also shown individually. Each colored segment represents the source protein corresponding to peptide pools eliciting T cell responses. Bars superimpose percentages of separate T cell culture experiments individually stimulated with indicated antigens. Unexposed individuals are arranged randomly. Convalescent individuals are arranged from the shortest days post disease onset (DPDO) to the longest DPDO in each group. Data of individuals who took multiple detections of memory T cell responses at different points are all included. AC: asymptomatic case; MC: mild or moderate case; SC: severe case. S: surface glycoprotein; N: nucleocapsid phosphoprotein; M: membrane glycoprotein; IFN: interferon; IL: interleukin; TNF: tumor necrosis factor.

CIs with DPDO <300 ($P < 0.001$, Fig. 3D). 64.52% (20/31) of MCs with DPDO <300 showed detectable specific CD4 T cell responses to three of the viral proteins, which was observed in only 14.81% (8/54) of MCs with DPDO >300 ($P < 0.001$, Fig. 3D). Specific CD4 T cell responses against three of the viral proteins were more frequently observed in CIs with DPDO <300 than CIs with DPDO >300 no matter MCs and SCs were analyzed separately or not ($P < 0.001$, Fig. 3D; $P = 0.001$, Fig. 3E; $P = 0.001$, Fig. 3F). Similar differences in the breadth of SARS-CoV-2-specific CD8 T cell responses were also observed between the two groups when MCs and SCs were analyzed together or separately while no statistical difference was tested (Fig. 3D–F).

To further characterize the kinetics of SARS-CoV-2-specific memory T cell responses, the magnitude of T cell responses were longitudinally examined in 12 individual CIs. Strong and broad CD4 (in all 12 individuals) and CD8 (10 out of 12 individuals) T cell responses against S, N, or M were detected at the first sampling time point (83–358 DPDO, Supplementary Fig. S5). In 9 out of 12 CIs, decreases in the magnitude of SARS-CoV-2-specific CD4 and CD8 T cell response were observed over time, which was most pronounced for the response against the S peptide pool (Fig. 4A–C, Supplementary Fig. S5). CI-17 and CI-20 showed sustained SARS-CoV-2-specific CD4 and CD8 T cell responses over time, however, the last sampling time points of the two individuals were rather early (Supplementary Figs. S5C and S5E). CI-24 only showed sustained SARS-CoV-2-specific CD4 and CI-26 showed fluctuation in the magnitude of SARS-CoV-2-specific CD8 T cell responses (Supplementary Figs. S5F and S5G).

Taken together, these results suggested that SARS-CoV-2-specific T cell responses decreased over time and might wane significantly 10 months after disease onset in the majority of CIs.

3.3. Correlation between the long-term SARS-CoV-2-specific T cell response and disease severity

Next, we examined the differences in the magnitude and breadth of SARS-CoV-2-specific CD4 and CD8 T cell responses in CIs according to their different degrees of COVID-19 severity. ACs, MCs, and SCs showed no significant difference in age and their time periods after infection were similar (Supplementary Fig. S6A). In general, the magnitude of T cell responses against S, N, or M, either for the overall or individual cytokine production, was comparable in ACs, MCs, and SCs (Fig. 5A and B, Supplementary Figs. S6B and S6C). Also, no significant correlations were observed between the magnitude of SARS-CoV-2-specific T cell responses and clinical parameters collected during hospitalization such as white blood cell and lymphocyte numbers, IL-6, C-reactive protein, D-dimer, lactate dehydrogenase (LDH), alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, serum creatinine, fibrinogen (FIB), and blood urea nitrogen levels (Supplementary Fig. S7). However, CD4 T cell responses against S, N, and M became undetectable in 54.55% (6/11) of ACs, but only in 30.00% (15/50) of MCs at the last time point of sampling (Fig. 5A). CD8 T cell responses against S, N, and M became undetectable in 72.73% (8/11) of ACs, but only in 30.00% (15/50) of MCs ($P = 0.014$, ACs v. s. MCs) and 35.00% (7/20) of SCs, respectively (Fig. 5B). No AC showed SARS-CoV-2-specific CD8 T cell responses against multiple peptide pools, while 36.00% (18/50) of MCs and 45.00% (9/20) of SCs showed SARS-CoV-2-specific CD8 T cell responses to at least two different peptide pools at the last time point of sampling (Fig. 5B).

Elderly people are predisposed to develop severe COVID-19 and mortality increases dramatically with age (Liu et al., 2020). We have previously shown that the cytotoxic CD8 T cell response is impaired in elderly COVID-19 patients (Westmeier et al., 2020). Here, we observed that the magnitude, but not the breadth, of SARS-CoV-2-specific CD4 T cell responses against S or M was inversely correlated with the age of CIs (Fig. 6A). No significant correlation between the magnitude and breadth of SARS-CoV-2-specific CD8 T cell response and the age of CIs was observed (Fig. 6B). Elderly CIs (>60-year-old) showed significantly weaker magnitude of SARS-CoV-2-specific T cell responses compared with the young and middle-aged CIs (Supplementary Fig. S8). Moreover, we also observed that male CIs showed weaker magnitude of SARS-CoV-2-specific T cell responses compared with female CIs, and the difference in M-specific CD4 T cell responses was statistically significant (Supplementary Fig. S9).

3.4. SARS-CoV-2-specific T cell responses in individuals who lost their IgG response to SARS-CoV-2

During the acute phase of COVID-19, T cell responses positively correlate with the magnitude of antibody responses (Ni et al., 2020; Peng et al., 2020; Thieme et al., 2020). However, to our knowledge, it is not clear whether this association is maintained during long-term convalescence. To this end, we compared SARS-CoV-2-specific T cell responses and antibody responses in CIs from 83 to 443 DPDO. As shown in Supplementary Fig. S10, the magnitude of specific CD4 and CD8 T cell responses against S and N showed no significant correlation with titers of the corresponding IgG against S and N. Moreover, we were interested if patients who had lost their SARS-CoV-2-specific IgG still kept their SARS-CoV-2-specific T cells. In our cohort, nine CIs, including one AC and eight MCs, were IgG-seronegative at the last sampling time point (range: 97–312 days; median: 127 days). Only 11.11% (1/9) of these IgG-seronegative CIs showed undetectable SARS-CoV-2-specific CD4 T cell responses to the three peptide pools, while 88.89% (8/9) CIs showed SARS-CoV-2-specific CD4 T cell responses to at least two peptide pools (Fig. 7A). Loss of CD8 T cell responses against all three peptide pools was observed in 33.33% (3/9) of these CIs, and 33.33% (3/9) showed SARS-CoV-2-specific CD8 T cell responses to at least two peptide pools (Fig. 7B). In total, only one IgG-seronegative CI with mild disease course showed undetectable SARS-CoV-2-specific T cell responses for both CD4 and CD8 T cells. Taken together, our data showed that immune memory in at least one compartment of adaptive immunity was measurable in most CIs within 14 months post-infection.

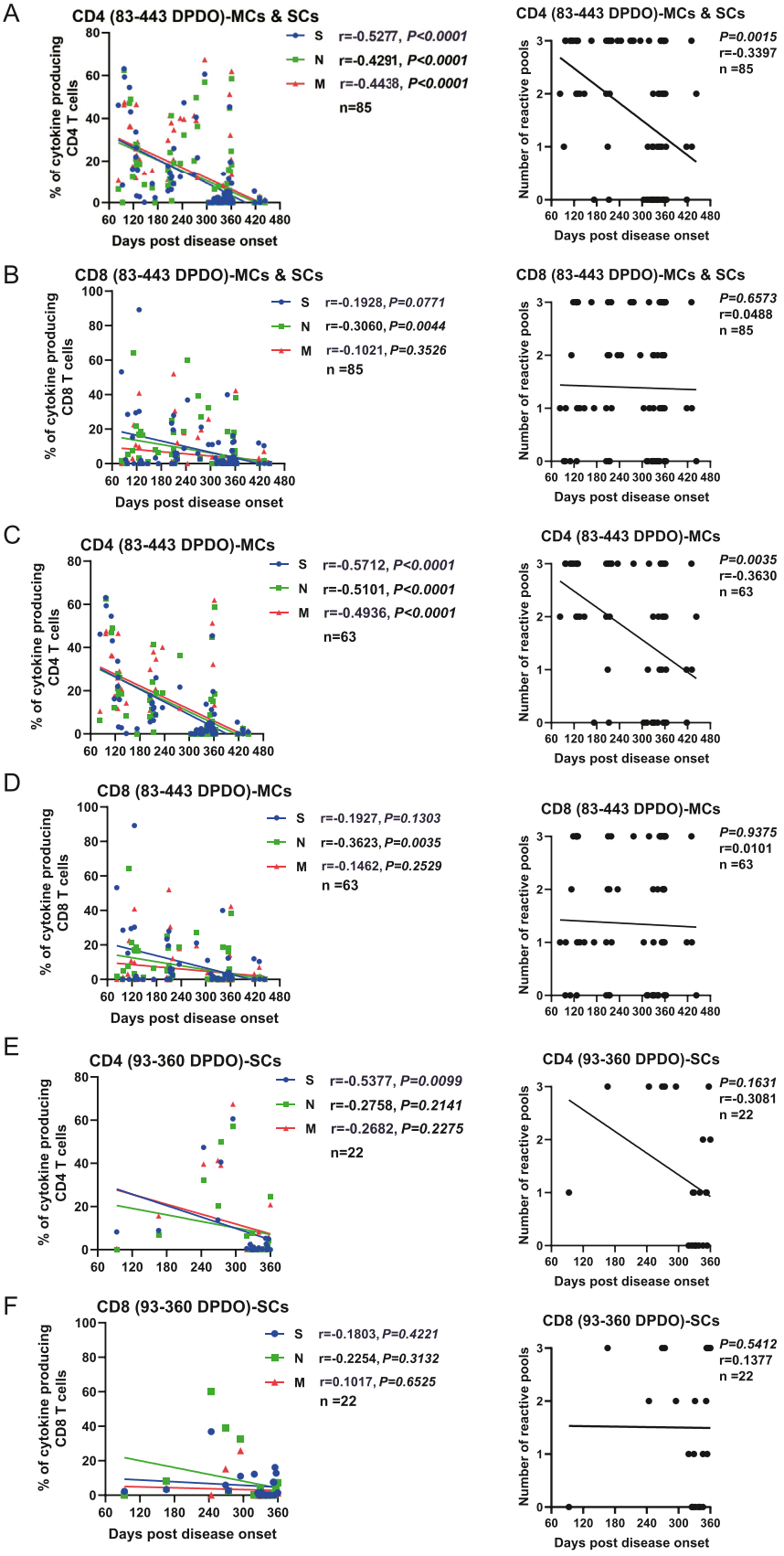

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Fig. 2. Correlation between the frequency and breadth of SARS-CoV-2 memory T cell responses and the time that had elapsed from disease onset. The correlation between the magnitude and breadth of memory CD4 and CD8 T cell responses specific to S, N, and M and days post disease onset (DPDO) up to 443 days in MCs and SCs (**A**, **B**), MCs (**C**, **D**) and SCs (**E**, **F**) are shown. Asymptomatic cases were not included due to the undefined DPDO and data of individuals who took multiple detections of memory T cell responses at different points are all included. A T cell that produced any one or more of the three cytokines (IFN-γ, IL-2, and TNF-α) in response to the stimulation was defined as a "cytokine producing" T cell. Pearson product-moment correlation coefficient test was used to test the significance and *P* value and *r* value (correlation coefficient) are indicated in each panel. MC: mild or moderate case; SC: severe case; S: surface glycoprotein; N: nucleocapsid phosphoprotein; M: membrane glycoprotein.

4. Discussion

One of the most important and challenging questions facing medicine today concerns the extent to which immunity develops and persists following COVID-19. Previous studies suggest that the persistence of protective immunity against different coronaviruses varies significantly, since those against seasonal coronavirus are short-lived (Edridge et al., 2020) while those against SARS and middle east respiratory syndrome coronavirus (MERS) are described to last longer (Mo et al., 2006; Choe et al., 2017; Le Bert et al., 2020). Here we provide, to our knowledge, the longest analyses of memory T cell responses against SARS-CoV-2 in a cohort of COVID-19 convalescent individuals up to 14 months following primary SARS-CoV-2 ancestral strain infection. We show that the magnitude and breadth of long-term memory T cell responses to SARS-CoV-2 are heterogeneous. The majority of CIs demonstrate strong and broad SARS-CoV-2-specific T cell responses within 10 months post disease onset, however, a significant proportion of CIs have lost their T cell responses against the studied antigens after 10 months. The magnitude and breadth of SARS-CoV-2-specific CD4 and CD8 T cell response against S, N, and M are inversely correlated with the time that had elapsed from disease onset, suggesting SARS-CoV-2 memory T cell responses wane overtime after primary SARS-CoV-2 infection. Intriguingly, more than half of the asymptomatic cases have lost their SARS-CoV-2-specific CD4 and CD8 T cell responses, suggesting the memory T cell responses might be less durable in asymptomatic cases than in symptomatic cases. The magnitude of SARS-CoV-2-specific CD4 and CD8 T cell responses were inversely correlated with the age of the patients, suggesting the memory T cell responses might also be less durable in elderly individuals. Moreover, while the kinetics of SARS-CoV-2-specific T cell responses are heterogeneous in the herein examined CIs, most of them show a sharp decline of responses over time, especially after 10 months post disease onset. Our results also suggest that the intensity of SARS-CoV-2-specific T cell responses detected in peripheral blood may fluctuate over time in some of CIs. Although the possibility of local spread of the virus in Wuhan and the surrounding area has been precluded by the thorough SARS-CoV-2 RNA test conducted in May 2020 covering virtually every resident, re-exposure to SARS-CoV-2 might still occur in some of the individuals experiencing asymptomatic re-infection and thus boosted the SARS-CoV-2-specific T cell responses. Future studies are needed to closely monitor the SARS-CoV-2 memory T cell responses to address how the intensities of these responses are regulated in CIs.

By using a novel HLA-DRB1*15:01 tetramer, Wragg et al. reported the epitope-specific CD4 T cells were detected in COVID-19 convalescents 15 months after symptom onset (Wragg et al., 2022). Another study has analyzed immunological memory to SARS-CoV-2 in CIs around 10 months post-infection (Yao et al., 2021). The authors claimed that SARS-CoV-2 memory T cell responses persist in most CIs over 9 months post-infection. However, the magnitude of memory T cell responses was only analyzed in a short time window within 9–11 months post-infection, and was not compared with the magnitude of T cell responses from earlier time points. Actually, the study reported the detection of SARS-CoV-2-specific T cell responses in about 70% of CIs 9–10 months post-infection, which is comparable to our current observation that SARS-CoV-2-specific T cell responses were detected in about 78% of CIs 10–14 months post-infection, but in a much lower magnitude than before 10 months. Jennifer Dan et al. performed a follow-up study to analyze immunological memory to SARS-CoV-2 in 188 COVID-19 CIs for up to 8 months post-infection and suggested that SARS-CoV-2 memory CD4 and CD8 T cells declined with a half-life of 3–5 months (Dan et al., 2021). The authors also speculated that T cell memory might reach a more stable plateau, or slower decay phase, beyond the first 8 months post-infection. However, our data from beyond 8 months post-infection suggest that the decline of SARS-CoV-2 T cell memory may rather accelerate 10 months post-infection - at least in absence of reencounters - and the magnitude of the responses maintains at a low level (Fig. 8). It should be noticed that the method we used for analyzing memory T cell response is different from the other two studies in which the PBMCs were stimulated for either 18 h (Jiang et al., 2021) or 24 h (Dan et al., 2021). In our study, PBMCs were stimulated for 10 days to let the SARS-CoV-2 responding T cells to expand and thus become easier for detection. This method has also been employed recently by Jennider R. Habel et al. for analyzing SARS-CoV-2-specific T cell response (Habel et al., 2020). Long-term *in vitro* cultivation of T cells may result in dramatic change of T cell phenotype compared to the short-term *ex vivo* stimulation (personal communication with Dr. Gennadiy Zelinskyy). However, we have also observed that the intensities of SARS-CoV-2-specific CD4 T cell responses detected by 16-h and 10-day stimulation were positively correlated (data not shown), suggesting the results generated from 10-day stimulation method are in comparable to those generated from *ex vivo* stimulation in terms of analyzing the intensity of SARS-CoV-2-specific T cell responses.

So far, the contribution of different aspects of immune memory to the protection against SARS-CoV-2 reinfection in humans remains unclear. Previous animal studies have demonstrated macaques infected with SARS-CoV-2 are resistant to reinfection with the same virus isolate following recovery from their initial infection (Chandrashekar et al., 2020; Deng et al., 2020), and have suggested that both humoral and cellular immunity contribute greatly in protecting against SARS-CoV-2 reinfection (Mcmahan et al., 2021). However, reinfections with SARS-CoV-2 in these studies were carried out only 4 and 5 weeks after the primary infection, close to peak titers of expansion phases of adaptive immunity. In contrast to the observation in the macaque model, there have been increasing numbers of well-documented cases of reinfection with SARS-CoV-2 in humans (Bongiovanni, 2020; Larson et al., 2020; Tillett et al., 2020; To et al., 2020). Several studies have tried to address how likely reinfection is to occur and provided evidence for strong protection from reinfection in seropositive individuals during the first few months after infection (Hansen et al., 2021; Lumley et al., 2021). This is in consistent with the observation from us and others that SARS-CoV-2 primary infection induces relatively sustained humoral immune responses in CIs for over 6 months (Wu et al., 2021; Dan et al., 2021). However, in contrast to this high level of protection following recent infection (<6 months), a recent seroepidemiological study in the Brazilian city of Manaus has demonstrated the ongoing spread of infection in a highly seropositive population at later times, suggesting increasing susceptibility to reinfection beyond 6 months after infection in CIs

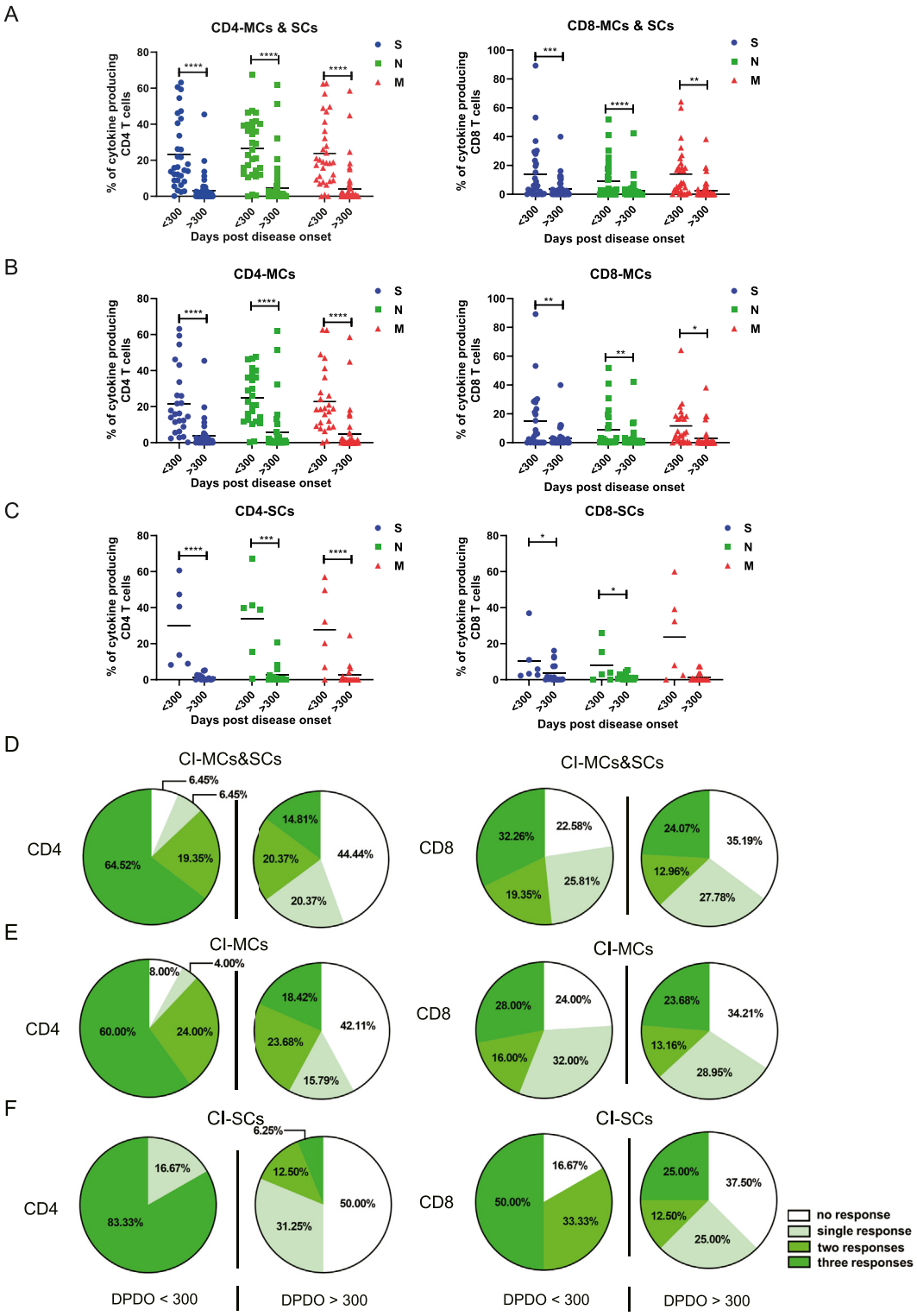

Fig. 3. Comparison of memory T cell responses to SARS-CoV-2 before and after 300 days post disease onset. The magnitude of memory CD4 and CD8 T cell responses are compared in MCs and SCs (**A**), MCs (**B**), and SCs (**C**) with DPDO <300 and DPDO >300. The breadth of memory T cell responses in MCs and SCs (**D**), MCs (**E**), and SCs (**F**) with DPDO <300 and DPDO >300 are also shown respectively. The breadth of T cell responses was calculated by the number of reactive peptide pools of S, N, and M. Data of individuals who took multiple detections of memory T cell responses at different points are all included. The short lines in the scatter plots indicate the mean values. A T cell that produced any one or more of the three cytokines (IFN-γ, IL-2, and TNF-α) in response to the stimulation was defined as a "cytokine producing" T cell. Mann-Whitney *U* test and Chi-square test were used to test the significance. MC: mild or moderate case; SC: severe case; S: surface glycoprotein; N: nucleocapsid phosphoprotein; M: membrane glycoprotein.

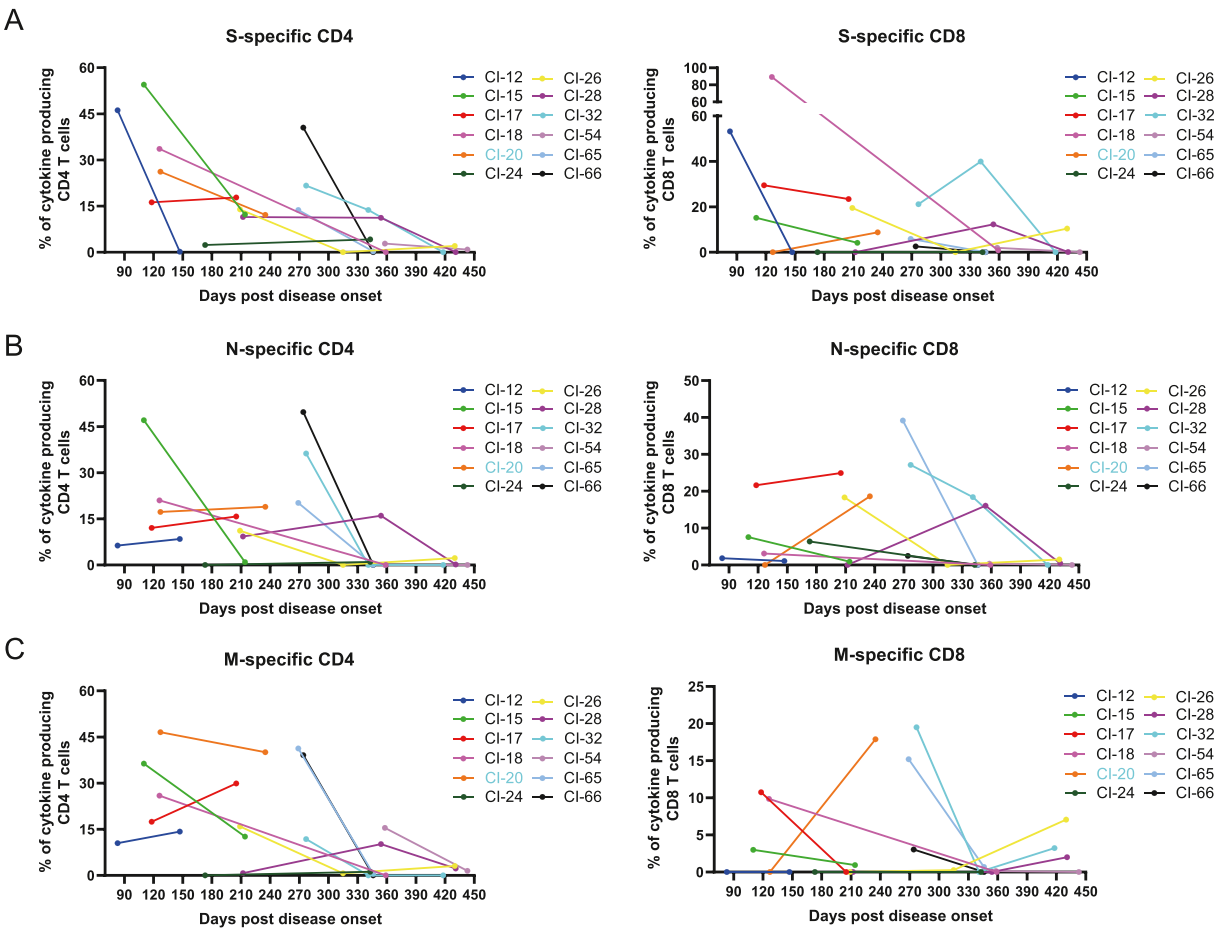

Fig. 4. Kinetics of memory T cell responses to SARS-CoV-2 in COVID-19 convalescent individuals. PBMCs were longitudinally collected from 12 COVID-19 convalescent individuals at indicated time points and were tested for memory T cell responses recognizing SARS-CoV-2 S, N, or M by using intracellular cytokine staining flow cytometry assay. A T cell that produced any one or more of the three cytokines (IFN-γ, IL-2, and TNF-α) in response to the stimulation was defined as a "cytokine producing" T cell. The dynamics of different CIs are exhibited by lines of different colors. CI-12, CI-15, CI-17, CI-18, CI-20, CI-24, CI-26, CI-28, CI-32 and CI-54 are mild or moderate cases; CI-65 and CI-66 are severe cases. CI: convalescent individual; S: surface glycoprotein; N: nucleocapsid phosphoprotein; M: membrane glycoprotein.

(Sabino et al., 2021). In line with this report, our recent data suggest that neutralizing antibodies against SARS-CoV-2 decay rapidly 7–12 months post primary infection (Xiang et al., 2021). It is assumed that in the context of declining neutralizing antibody titers, cellular immunity is required to provide maximal protective immunity against SARS-CoV-2 reinfection (Canete and Vinuesa, 2020; Cromer et al., 2021; Mcmahan et al., 2021). However, our current analysis of memory T cell immunity raises a great concern that the rapid waning of cellular immunity against SARS-CoV-2 may also occur a few months after the decay of neutralizing antibody titers.

Different from the observation during and shortly after the acute phase of SARS-CoV-2 infection (Ni et al., 2020; Peng et al., 2020; Thieme et al., 2020), we observe that the magnitudes of long-term SARS-CoV-2-specific cellular and humoral responses are not positively correlated with each other. In contrast, most IgG-seronegative CIs demonstrate strong SARS-CoV-2-specific memory CD4 T cell responses. A recent study started to investigate the possible mechanisms of short-lived antibody responses observed in COVID-19 patients and has reported that germinal centers in secondary lymphoid organs were largely absent during the acute phase of COVID-19 (Kaneko et al., 2020). The authors speculate that the absence of germinal centers is a result of abundant Th1 cell responses and aberrant extra-follicular TNF-α accumulation (Kaneko et al., 2020). Consistently, our current observation, that CIs with short-lived antibody responses demonstrate robust SARS-CoV-2-specific CD4 T cell responses, provides the first evidence that the above-mentioned effect may extend to a far longer period in the convalescent phase of COVID-19. Our data also demonstrate that most CIs may retain at least one arm of the adaptive immune response against SARS-CoV-2 long-term post recovery——this nourishes the hope for an "immunological division of labor". Further characterization of the protective roles as well as the interaction of cellular and humoral immune responses against SARS-CoV-2 has significant implications for vaccine development and application, especially in terms of the need for booster vaccinations (Xiang et al., 2022). Moreover, our results support the scenario that high-level immunity induced by primary SARS-CoV-2 infection is not long-lasting and is followed by partial immunity. Reinfection during partial immunity may lead to mild infection, low levels of transmission, and immune boosting (Cromer et al., 2021).

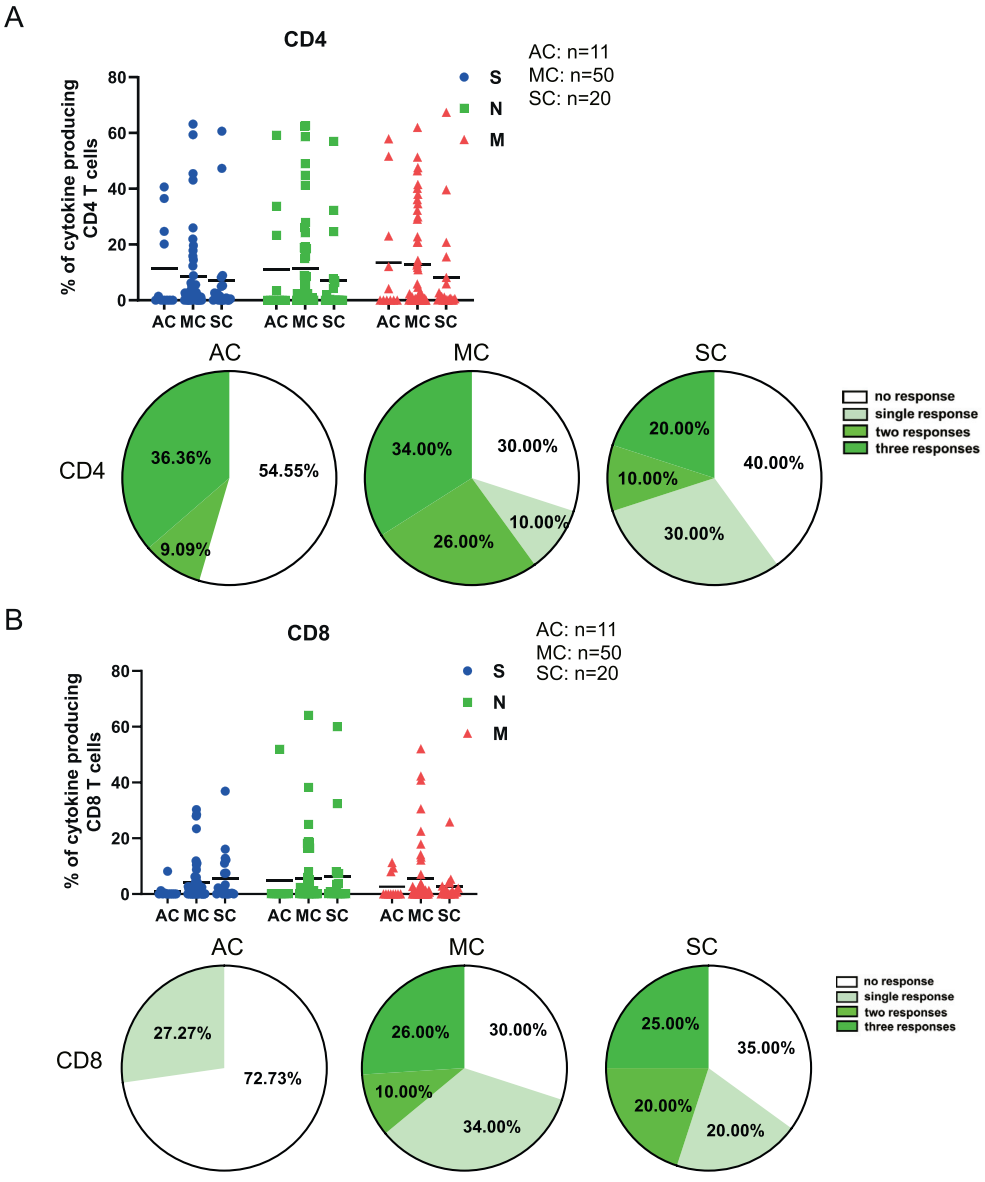

Fig. 5. Loss of SARS-CoV-2 memory CD4 T cell responses is more frequent in asymptomatic cases than symptomatic cases. The magnitude and breadth of memory CD4 (**A**) and CD8 (**B**) T cell responses are compared among the AC, MC, and SC. The last data of individuals who took multiple detections of memory T cell responses at different points are included. A T cell that produced any one or more of the three cytokines (IFN-γ, IL-2, and TNF-α) in response to the stimulation was defined as a "cytokine producing" T cell. The short lines in the scatter plots indicate the mean values. One-way ANOVA and Chi-square test were used to test the significance. AC: asymptomatic case; MC: mild or moderate case; SC: severe case. S: surface glycoprotein; N: nucleocapsid phosphoprotein; M: membrane glycoprotein.

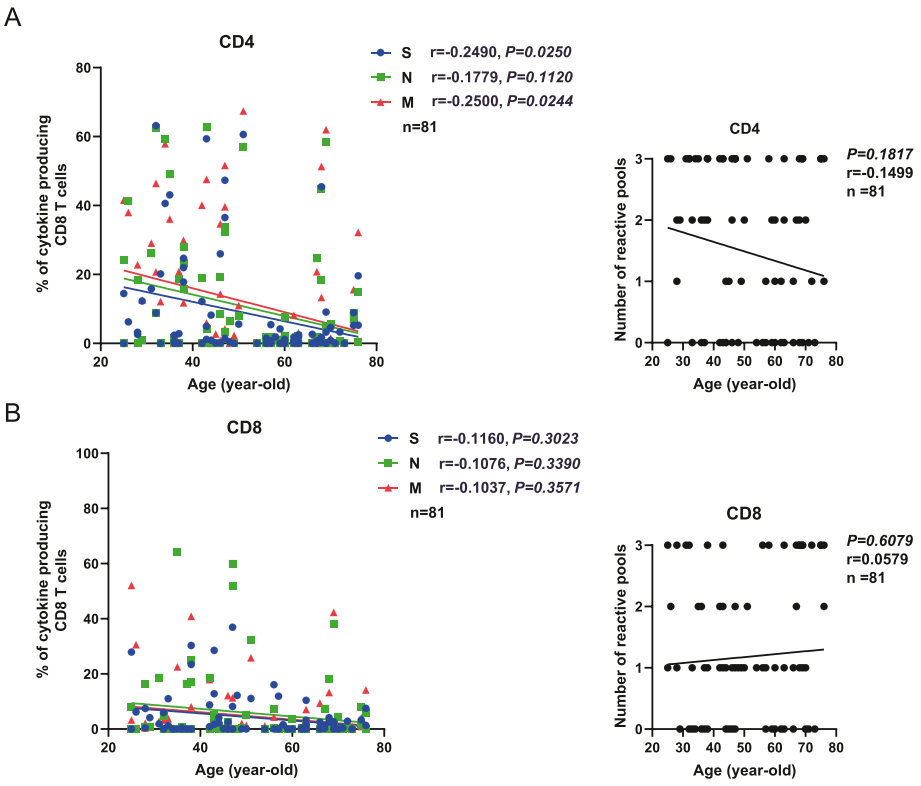

Fig. 6. The magnitude of long-term SARS-CoV-2 memory T cell responses is inversely correlated with the age of COVID-19 convalescent individuals. The correlation between the magnitude and breadth of memory CD4 (**A**) and CD8 (**B**) T cell responses specific to S, N, and M and age are shown. A T cell that produced any one or more of the three cytokines (IFN-γ, IL-2, and TNF-α) in response to the stimulation was defined as a "cytokine producing" T cell. Pearson product-moment correlation coefficient test was used to test the significance and *P* value and *r* value (correlation coefficient) are indicated in each panel. S: surface glycoprotein; N: nucleocapsid phosphoprotein; M: membrane glycoprotein.

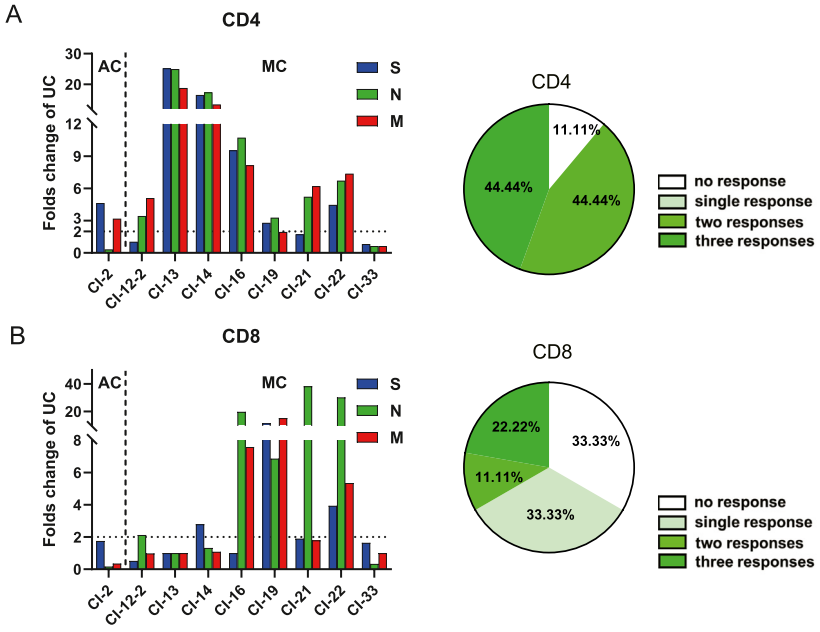

Fig. 7. Characterization of long-term SARS-CoV-2 memory T cell responses in IgG-seronegative COVID-19 convalescent individuals. The magnitude of memory CD4 (**A**-left) and CD8 (**B**-left) T cells specific to S, N, and M in IgG-seronegative convalescent individuals are shown by stimulation index, individually. T cell responses were defined as detectable if stimulation index >2 (as shown by the horizontal dashed line). The breadth of memory CD4 (**A**-right) and CD8 (**B**-right) T cell responses are also shown. UC: unstimulated control; AC: asymptomatic case; MC: mild or moderate case; S: surface glycoprotein; N: nucleocapsid phosphoprotein; M: membrane glycoprotein.

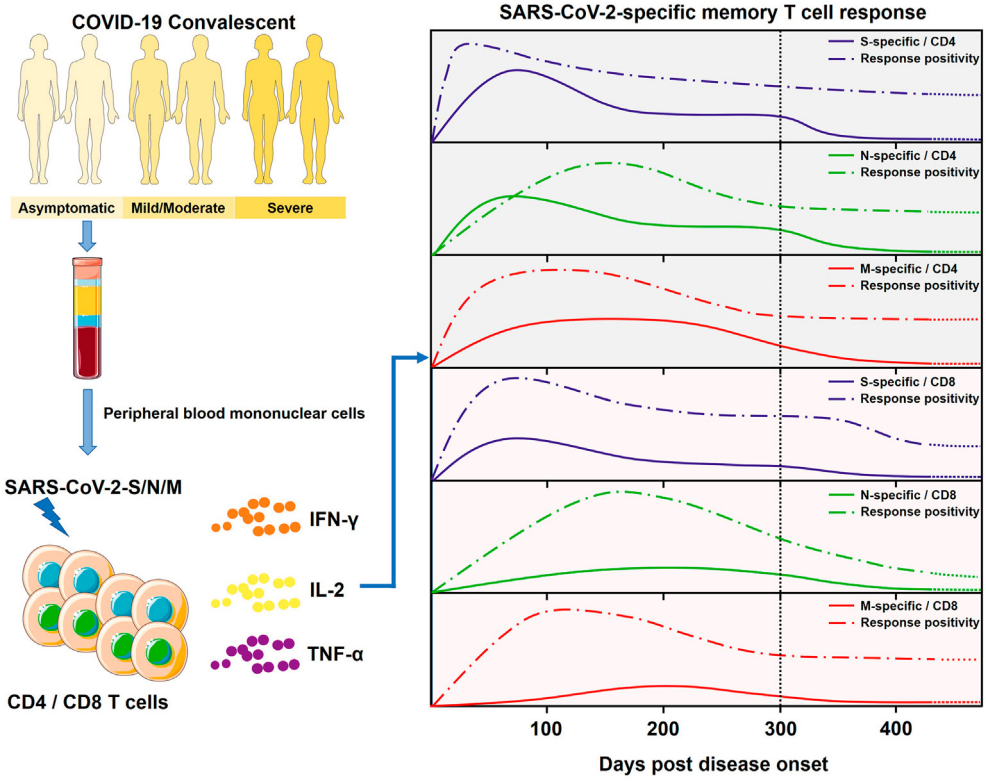

Fig. 8. A schematic representation of memory T cell response following SARS-CoV-2 infection. The x-axis indicates the timeline following disease onset. The fitting curve on the vertical axis reflected the trends of magnitude (continuous line) and positive rate (dot and dash line) of SARS-CoV-2 memory T cell responses with time.

5. Conclusions

Taken together, our data fill important gaps in our basic understanding of cellular immune memory after primary infection of SARS-CoV-2 ancestral strain, and document a low durability of robust SARS-CoV-2-specific T cell responses in COVID-19 CIs 10 months after infection.

Data availability

We support data sharing of the individual participant data. The individual participant data that underlie the results reported in this article, after de-identification will be shared. Researchers who provide a scientifically sound proposal will be allowed to access the de-identified individual participants' data. Proposal should be sent to the corresponding authors, at jialiu77@hust.edu.cn, xinz@hust.edu.cn, or dlyang@hust.edu.cn. The proposals will be reviewed and approved by the funders, investigators, and collaborators on the basis of scientific merit. To gain access, data requestors will need to sign a data access agreement.

Ethics statement

Informed written consent was obtained from each patient and the study protocol was approved by the Local Medical Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology (2020IEC-J-587). The study was conducted in accordance with the guidelines of the Declaration of Helsinki.

Author contributions

Ziwei Li: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing – original, writing-review & editing; Tiandan Xiang: data curation, formal analysis, investigation, methodology; Boyun Liang: data curation, formal analysis, investigation, methodology; Jing Liu: data curation, formal analysis, investigation, methodology, visualization; Hui Deng: data curation, investigation, methodology; Xuecheng Yang: investigation, resources; Hua Wang: investigation, methodology, resources; Xuemei Feng: investigation, resources; Gennadiy Zelinskyy: writing-review & editing; Mirko Thrilling: writing-review & editing; Kathrin Sutter: writing-review & editing; Mengji Lu: writing-review & editing; Ulf Dittmer: writing-review & editing; Baoju Wang: resources, writing-review & editing; Dongliang Yang: funding acquisition, project administration, resources, supervision, writing-review & editing; Xin Zheng: funding acquisition, project administration, resources, supervision, writing-review & editing; Jia Liu: conceptualization, data curation, funding acquisition, investigation, methodology, project administration, resources, visualization, writing – original, writing-review & editing.

Conflict of interest

Prof. Xin Zheng and Prof. Mengji Lu are editorial board members for Virologica Sinica and were not involved in the editorial review or the decision to publish this article. All authors declare that there are no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virs.2023.06.011.

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