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Time-dependent contraction of the SARS-CoV-2–specific T-cell responses in convalescent individuals

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Background: Adaptive immunity in severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection is decisive for disease control. Delayed activation of T cells is associated with a worse outcome in coronavirus disease 2019 (COVID-19). Although convalescent individuals exhibit solid T-cell immunity, to date, long-term immunity to SARS-CoV-2 is still under investigation.

Objectives: We aimed to characterize the specific T-cell response on the basis of the *in vitro* recall of IFN-γ–producing cells to *in silico*–predicted peptides in samples from SARS-CoV-2 convalescent individuals.

Methods: The sequence of the SARS-CoV-2 genome was screened, leading to the identification of specific and promiscuous peptides predicted to be recognized by $CD4^+$ and $CD8^+$ T cells. Next, we performed an *in vitro* recall of specific T cells from PBMC samples from the participants. The results were analyzed according to clinical features of the cohort and HLA diversity.

Results: Our results indicated heterogeneous T-cell responsiveness among the participants. Compared with patients who exhibited mild symptoms, hospitalized patients had a significantly higher magnitude of response. In addition, male and older patients showed a lower number of IFN-γ–producing cells. Analysis of samples collected after 180 days revealed a reduction in the number of specific circulating IFN-γ–producing T cells, suggesting decreased immunity against viral peptides.

Conclusion: Our data are evidence that *in silico*–predicted peptides are highly recognized by T cells from convalescent individuals, suggesting a possible application for vaccine design. However, the number of specific T cells decreases 180 days after infection, which might be associated with reduced protection against reinfection over time. (J Allergy Clin Immunol Global 2022;1:112-21.)

Key words: *SARS-CoV-2, COVID-19, T lymphocyte, adaptive immunity*

Since early 2020, the world has been facing an unprecedented challenge. The pandemic unleashed by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) emerged in Wuhan, China, in 2019, and by November 2021, it had infected more than 259 million people worldwide and taken more than 5.1 million lives (https://covid19.who.int/). SARS-CoV-2 infection can be asymptomatic or present mild-to-moderate symptoms in 81% of cases; however, it can progress to severe cases, leading to severe acute respiratory syndrome and death.[1]

Adaptive immunity in coronavirus disease 2019 (COVID-19) plays a critical role in disease outcome and resolution. In this scenario, $CD4^+$T cells, $CD8^+$T cells, and neutralizing antibodies contribute to the control of SARS-CoV-2 burden,[2,3] but uncoordinated T cells and delayed and/or excessive antibody responses, together with an exacerbated and prolonged innate inflammatory response, modulate disease severity.[4,5] Furthermore, memory T and B cells generated by infection and/or vaccination can be activated following reexposure to SARS-CoV-2 and are key in preventing and controlling infection.[6] T-cell activation after SARS-CoV-2 infection has been characterized only recently, and so far, several studies have demonstrated a robust differentiation of specific $CD4^+$ and $CD8^+$T cells that recognize multiple regions of structural and nonstructural proteins of the virus.[7-9] T-cell immunity against SARS-CoV-2 antigens is frequently found in samples from seronegative convalescent patients, and in some cases, this seems to be independent of the severity of COVID-19, nonetheless suggesting an essential role of the cellular immune response in disease control.[2] In addition, SARS-CoV-2–specific T-cell response also plays an important role during infection

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Abbreviations used

AIM: Activation-induced marker
COVID-19: Coronavirus disease 2019
DMSO: Dimethyl sulfoxide
ELISPOT: Enzyme-linked immunospot
OR: Odds ratio
SARS-CoV-2: Severe acute respiratory syndrome coronavirus-2
SFU: Spot-forming unit
VOC: Variant of concern

by variants of concern (VOCs), being less affected by the mutations than neutralizing antibodies are.[10,11]

Convalescent individuals exhibit solid T-cell immunity, although the generation and persistence of long-term immunity remains poorly understood. To date, only a few longitudinal studies have explored the longevity of T-cell immunity. So far, studies dedicated to the analysis of promiscuous peptides in the SARS-CoV-2 genome have described different patterns of immunodominance.[12] Responsive polyfunctional $CD4^+$ and $CD8^+$ T cells can be found at least 6 months after the onset of COVID-19; however, the responsiveness is reduced compared with that at the first sampling right after the infection.[13] The decrease in T-cell immunity to SARS-CoV-2 that occurs over time is a concern to be addressed, because the incidence of reinfection is increasing worldwide, mostly because of the emergence of VOCs.[14] In this sense, an in-depth analysis of long-term immunity to different antigens found in SARS-CoV-2 is crucial for the development of promising new vaccine candidates. Herein, we report a comprehensive analysis of the magnitude and breadth of T-cell response to SARS-CoV-2 antigens encompassing *in silico*–predicted peptides from SARS-CoV-2 in COVID-19 convalescent individuals on days 30 and 180 after infection.

METHODS

Study participants

We designed a double-center study and carried it out at the Heart Institute–University of São Paulo and at the Federal University of São Paulo in Brazil. Blood samples were collected from recovered (as confirmed by PCR) COVID-19 convalescent donors (n = 121) and unexposed healthy donors (n = 18); the samples from the recovered convalescent donors were collected in 2020, which was before emergence of the SARS-CoV-2 VOCs and vaccines, and the samples from the unexposed donors were collected in 2015. All participants provided informed consent, and the study was approved and carried out according to the guidelines of the local ethics committee (Certificado de Apresentação para Apreciação Ética [CAAE] identifier 30155220.3.0000.0068).

Sample collection

Peripheral venous blood samples were collected by using ethylenediamine tetraacetic acid Vacutainer blood collection tubes. Each blood sample was diluted 1:2 (vol:vol) in Hank's balanced salt solution (Gibco, Waltham, Mass). The PBMCs were isolated by using Ficoll Paque-Plus (GE Healthcare, Chicago, Ill) density gradient, cryopreserved in 90% heat-inactivated FBS (Gibco, Waltham, Mass) plus 10% dimethyl sulfoxide (DMSO) (Sigma, St Louis, Mo) and stored in liquid nitrogen until further use. Samples from 121 COVID-19 convalescent individuals were collected for the study. The infection was confirmed after quantitative RT-PCR testing. All of the selected individuals were symptomatic; 82.64% were treated at home for mild disease, whereas 17.36% required hospitalization. Of the 121 participants, 62.81% were female and 77.69% were younger than 50 years.

DNA extraction and HLA typing

DNA extraction was performed by using a commercial kit (FlexiGene DNA Kit, QIAGEN, Hilden, Germany) according to the manufacturer's instructions. HLA-A, HLA-B, HLA-C, and HLA-DRB1, and HLA-DDB1 typing was performed using LABType SSO typing kits (One Lambda, Canoga Park, Calif). The amplified product was hybridized with microbeads linked to specific oligonucleotide probes for HLA alleles. The resulting products were analyzed by using a Luminex flow cytometer LABScan3D (Luminex FLEXMAP 3D), and the results were interpreted by using HLA Fusion, version 4.2 (One Lambda, Canoga Park, Calif).

T-cell epitope prediction

The whole genome proteome of SARS-CoV-2 (reference sequence NC_045512.2) was subjected to *in silico* prediction using the Immune Epitope Database[15,16] (www.iedb.org) and ProPred.[17] We identified a set of 20 promiscuous $CD4^+$ T-cell peptides (15- to 20-mer) derived from various SARS-CoV-2 proteins. In parallel, we also selected 26 $CD8^+$ T-cell peptides (9- and 10-mer) that were known to stably bind to HLA I (www.immunitrack.com) or to be recognized[18] in the context of the 10 most frequent HLA class I alleles. The sequence of each peptide is displayed in Tables E1 and E2 (see the Online Repository at www.jaci-global.org). In the end, 46 peptides were synthetized (Genscript, Piscataway, NJ) with greater than 90% purity.

SARS-CoV-2–specific IFN-γ–producing cells by ELISPOT assay

Enzyme-linked immunospot (ELISPOT) assay was performed by using a human IFN-γ ELISPOT assay (BD Biosciences, Franklin Lakes, NJ) according to the manufacturer's instructions. PBMCs were rapidly thawed in a 37°C water bath and washed in R10 (RPMI medium supplemented with 10% of FBS, 2 mM l-glutamine, 1% vol/vol vitamin solution, 1 mM sodium pyruvate, 1% vol/vol nonessential amino acid solution, 40 μg/mL of gentamicin, and 5×10^{-5} M 2-mercaptoethanol (all from Gibco/Invitrogen), as well as 20 μg/mL of ciprofloxacin (Ciprobacter, Isofarma) and 30 U/mL of recombinant IL-2 (Proleukine, Zodiac). Cells were counted and viability was assessed by using the Countess Automated Cell Counter system (Invitrogen, Carlsbad, Calif). Only samples with 80% or more viable cells were used and resuspended (concentration 2×10^6 cells/mL; 100 μL/well [2×10^5 cells/well]) in R10 medium. We then evaluated their ability to secrete IFN-γ after *in vitro* stimulation with SARS-CoV-2 peptides (5 μg/mL) or DMSO as negative control or phorbol 12-myristate 13-acetate and ionomycin (50 ng/mL and 1 μg/mL, respectively, Sigma) as a positive control. Spots were counted by using an AID ELISPOT Reader System (Autoimmun Diagnostika GmbH, Straßberg, Germany). The number of IFN-γ–producing cells/10^6 PBMCs was calculated after subtracting the negative control values. The cutoff was defined as the mean spot-forming unit (SFU) plus 3 times the SD obtained in the analysis of the healthy control group. The cutoff values were 130 SFU/10^6 cells for the megapools for both CD4 (MCD4 [n = 20 peptides]) and CD8 (MCD8 [n = 26 peptides]) and 105 SFU/10^6 cells for individual peptides.

Identification of $CD4^+$- and $CD8^+$-specific T-cell responses

We used activation-induced marker (AIM) assays to identify SARS-CoV-2–specific $CD4^+$ and $CD8^+$ T-cell responses as previously described.[3,18] PBMCs were cultured for 24 hours in the presence of 5 μg/mL of the spike pool (12 peptides for $CD4^+$ and $CD8^+$ T cells) or the nonspike pool (34 peptides for $CD4^+$ and $CD8^+$ T cells) or a pool containing all MCD4 (n = 20) and MCD8 (n = 26) peptides separately. As a negative control, cells were stimulated with DMSO alone. Following culture, cells were stained with anti-CD3 allophycocyanin-cyanine 7 (clone SK7), anti-CD4 BB515 (clone RPA-T4), anti-CD8 peridinin-chlorophyll-protein (clone SK1), anti-OX40 phycoerythrin–cyanine 7 (clone ACT35), anti-CD69 allophycocyanin (clone FN50), and anti-CD137 (clone 4B4-1) (all from BD Biosciences). A total of 2 million events in a live lymphocyte gate were acquired on a FACSCanto

II flow cytometer (BD Biosciences) and then analyzed by using FlowJo software (version 10, Tree Star, Ashland, Ore). $CD4^+$ T-cell AIM ($CD4^+AIM^+$) was defined as $CD3^+CD4^+OX40^+CD137^+$, and $CD8^+$ T cells ($CD8^+AIM^+$) were defined as $CD3^+CD8^+CD69^+CD137^+$. The values of the control DMSO-stimulated cells were subtracted from the peptide-pulsed cells, and the cutoff was defined on the basis of samples from unexposed subjects.

Statistical analysis

Statistical analysis was performed by using GraphPad Prism 8 software (GraphPad Software, San Diego, Calif). Data were represented as means plus or minus SEMs. For comparison between 2 groups, we performed a 2-tailed unpaired *t* test. To analyze the relationship between categoric variables, we performed chi-square tests. For 3 or more groups, 1-way ANOVA was conducted. The Wilcoxon matched-pair test was applied for comparison between the first and second PBMC samplings. HLA allele frequency analysis was determined by direct counting. The frequency of the alleles was considered as the total number of copies of the allele in the population sample (alleles/2n). The significance of differences between studied groups was evaluated by chi-square test or 2-tailed Fisher exact test. Odds ratios (ORs) with 95% CIs were also calculated to evaluate the risk association. *P* values less than .05 were considered significant. Because of multiple comparisons, allele frequencies were analyzed after Bonferroni correction (P_c). Only P_c values less than .05 were considered statistically significant. Analysis of Hardy-Weinberg equilibrium was assessed for HLA-A, HLA-B, HLA-C, HLA-DRB1, and DQB1 alleles by using ARLEQUIM software (cmpg.unibe.ch/software/arlequin3).[19]

RESULTS

Prediction of T-cell epitopes and SARS-CoV-2–specific T-cell responses

The protein-coding genome sequence of SARS-CoV-2 was scanned, and the resulting peptides were selected on the basis of prediction of binding to multiple HLA-DR/DQ and HLA-A, HLA-B, and HLA-C molecules. The rates of population coverage of HLAs predicted to bind to the $CD4^+$ and $CD8^+$ T-cell epitopes were 99.6% and 94%, respectively, according to the Immune Epitope Database epitope database.

To analyze the promiscuity of the predicted peptides, we evaluated the specific response by IFN-γ ELISPOT assay in PBMC samples from day 30 after onset of COVID-19 symptoms. For this purpose, the HLA I– and HLA II–restricted peptides were combined into 2 megapools (MCD4 [n = 20] and MCD8 [n = 26]) and used to stimulate PBMCs samples *in vitro* (see Fig E1, *A* [in the Online Repository at www.jaci-global.org]). As a control study group, PBMCs from healthy individuals (samples collected in 2015) were incubated in the presence of the same antigens. On the basis of the results obtained from the healthy control group, the cutoff was established as 130 SFU × 10^6 cells. In total, 76.23% of the convalescents presented a detectable response (above the cutoff) against the MCD4 pool and 78.69% against the MCD8 pool (see Fig E1, *B* and *C*). Although the PBMC samples from most convalescent individuals recognized essentially all peptides, the magnitude of IFN-γ production was markedly different among subjects (see Fig E1, *D-G*). Next, to determine the potential immunodominance of the peptides, we stimulated the PBMCs with each peptide individually. All peptides induced IFN-γ–producing T cells, indicating that the *in silico*–predicted peptides were highly recognized by cells from convalescent individuals (Fig 1, *A* and *B*). It is noteworthy that the samples from unexposed individuals exhibited low IFN-γ production in response to the peptides (see Fig E1, *H*). Furthermore, we did not observe a pattern of immunodominance in response to peptides derived from different regions of the virus. Each individual peptide was recognized by at least 70% of convalescent individuals. The most frequently recognized CD4 peptide was the membrane peptide NRFLYIIKLIFLWLLWPVTL (recognized by 81.3% of the participants), whereas the least frequently recognized CD4 peptide was in the spike protein (TECSNLLLQYGSFCTQL) and was recognized by 70.8% of the participants. For CD8, the most frequently recognized peptide was the membrane peptide TLACFVLAAV (recognized by 82.2% of the participants), and the least frequently recognized peptide was the peptide located on the exonuclease protein (TYACWHHSI), which was recognized by 71% of the participants (see Tables E1 and E2).

Specific IFN-γ production and cohort clinical characteristics

Next, we analyzed whether differential IFN-γ T-cell responses to specific SARS-CoV-2 peptides were associated with different clinical and/or demographic features (see Fig E2, *A* in the Online Repository at www.jaci-global.org). The samples were considered positive when the response was greater than 105 SFU × 10^6 cells based on analysis using PBMCs from unexposed healthy individuals. The median frequencies of IFN-γ positivity above the cutoff for each CD4- and CD8-specific peptide (see Figs E2, *B-D* and E3, *A-C*, respectively, in the Online Repository at www.jaci-global.org) were not significantly different from one another when the results were compared according to age (aged >50 years vs aged <50 years), sex, and clinical outcomes (hospitalized vs nonhospitalized). However, the median magnitude of the IFN-γ T-cell response directed to CD4 peptides, was significantly higher in individuals who had been hospitalized (Fig 2, *A*). Likewise, the magnitude of the IFN-γ response was greater in individuals younger than 50 years and in females (Fig 2, *B* and *C*). The CD8-specific response exhibited the same profile and the same differences (Fig 2, *D-F*). These data indicate that the IFN-γ T-cell response directed against SARS-CoV-2 peptides is significantly more robust in female, younger individuals (aged <50 years), and previously hospitalized convalescents.

Magnitude of T-cell reponse is not associated with HLA alleles

Next, to characterize the antigenicity of the predicted peptides, we compared the magnitude of response with consideration for HLA typing. The HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1 alleles were in Hardy-Weinberg equilibrium ($P > .05$ [data not shown]). Allele frequencies of HLA-class I (HLA-A, HLA-B, and HLA-C) and class II (HLA-DRB1 and HLA-DQB1*) were compared between COVID-19 convalescent individuals according to the magnitude of CD4 and/or CD8 IFN-γ responses against SARS-CoV-2 peptides. The COVID-19 convalescents were also grouped according to low (<5000 SFU × 10^6) and high (≥5000 SFU × 10^6) responses with consideration of the sum of IFN-γ spots detected in response to CD4- and/ or CD8-predicted peptides.

The most common HLA-A allele for both the low- and high-responder groups was A*02 (20.2% and 21.4%, respectively). The HLA-A*24 allele frequency was higher in the group with a

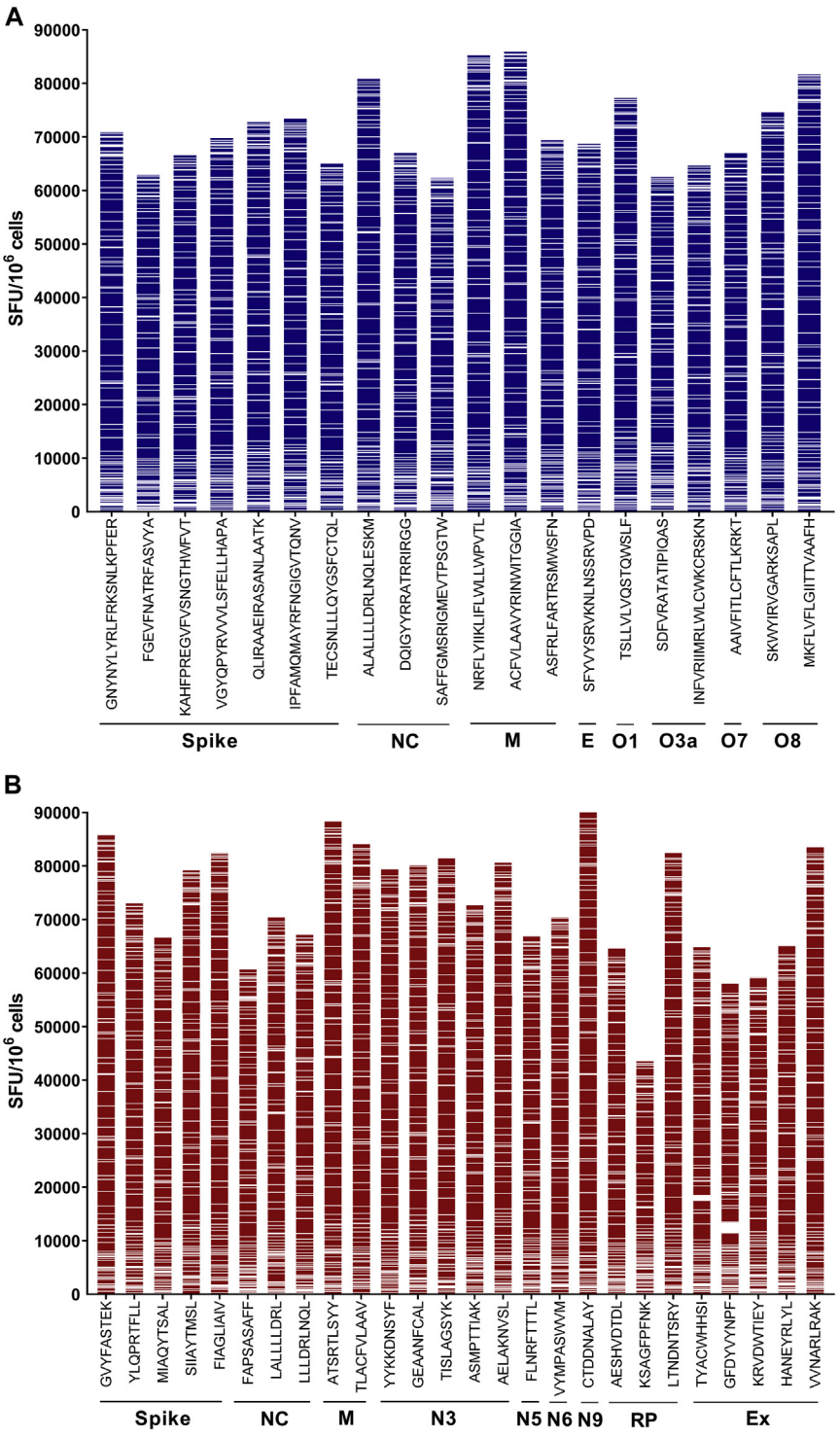

FIG 1. Overall magnitude of T-cell response to the predicted SARS-CoV-2 peptides. Each column represents the sum of IFN-γ–producing T cells (SFU/10^6) of the 121 samples in response to the individual peptides from SARS-CoV-2. **A,** Specific $CD4^+$ T-cell response. **B**, Specific $CD8^+$ T-cell response. *E*, Envelope protein; *Ex*, exonuclease protein; *M*, membrane protein; *N3*, nonstructural protein (NSP) 3; *N6*, NSP6; *N9*, NSP9; *NC*, nucleocapside protein; *O1*, open reading frame (ORF) 1; *O3a*, ORF3a; *O7*, ORF7; *O8*, ORF8; *RP*, RNA polymerase.

low IFN-γ response than in the group with a high response (14.9% vs 8.6%), but with no statistical difference ($P = .114$ [chi-square test]) (see Table E3 in the Online Repository at www.jaci-global.org). When HLA-B allele frequencies were considered, the most common alleles for both groups were HLA-B*35 (15.5% for the low responders 14.3% for the high responders) and HLA-B*44 (15.5% for the low responders vs 14.3% for the high responders). No difference was observed for HLA-B alleles between the high

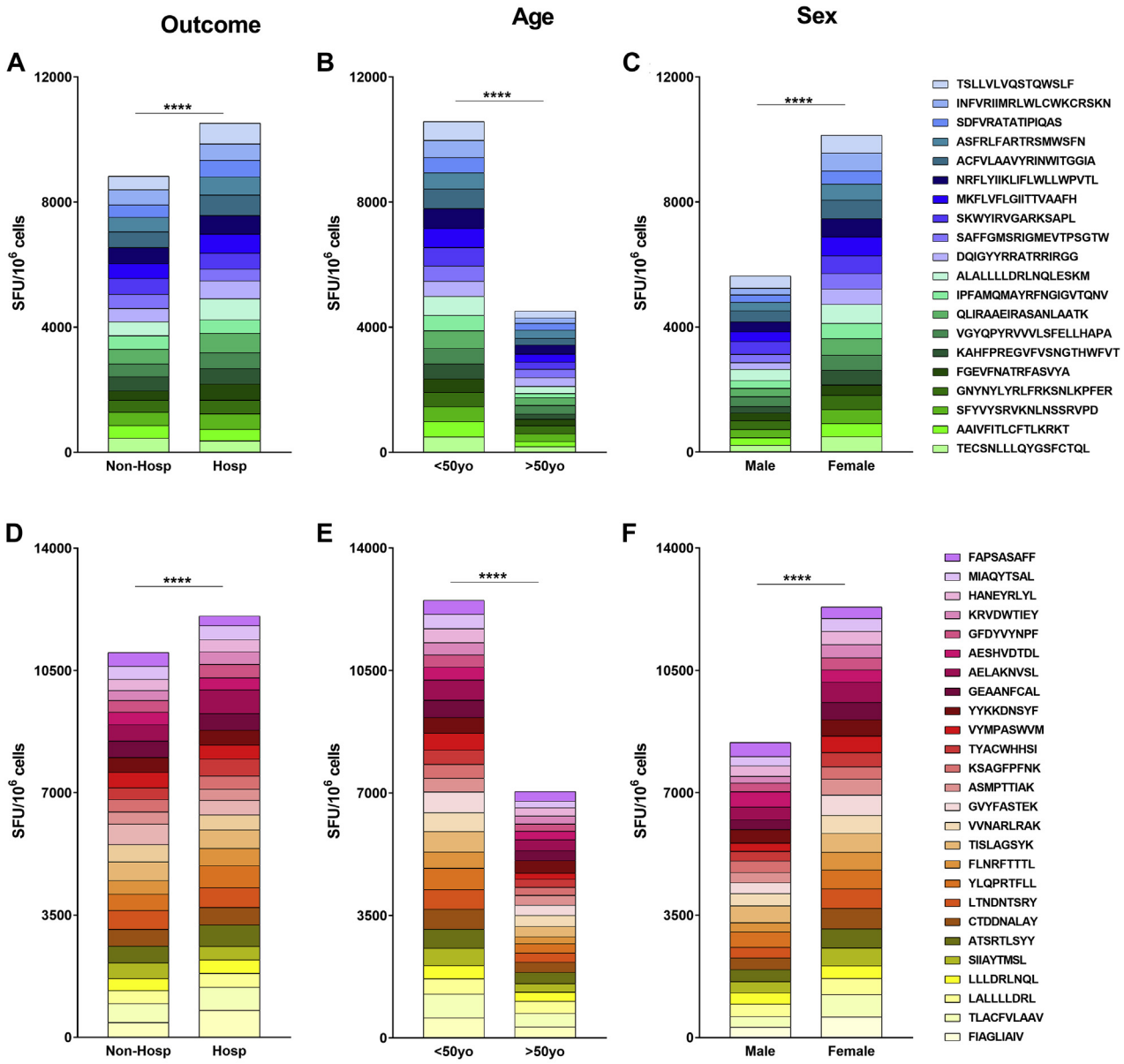

FIG 2. Magnitude of the specific IFN-γ production based on clinical characteristics. Each colunm represents the summed mean of the specific IFN-γ production (SFU × 10^6) of the study participants in response to each peptide, grouped according to clinical charatcteristics as COVID-19 outcome, age, and sex. **A-C,** Specific $CD4^+$ T-cell response. **D-F,** Specific $CD8^+$ T-cell response. The relationship between categoric variables was analyzed by using the chi-square test. ****$P < .0001$.

and low responders (see Table E4 in the Online Repository at www.jaci-global.org). The most common HLA-C allele for both groups was HLA-C*04 (20.9% for the low responders and 21.0% for the high responders). Again, no significant differences were observed for HLA-C alleles between groups displaying a lower or higher IFN-γ response (see Table E5 in the Online Repository at www.jaci-global.org).

For HLA class II (HLA-DRB1 and HLA-DQB1) allele frequencies, we first considered only responses to CD4 peptides, comparing individuals presenting with low (<5000 IFN-γ SFU × 10^6) or high (≥5000 SFU × 10^6) responses. We initially found a risk for lower responses for HLA-DRB1*10 ($P = .017$; OR= 9.7; 95% CI = 1.1-82), but the significance was lost after the Bonferroni correction ($P_c = .221$). The same was observed for HLA-DRB1*12 ($P = .024$; $P_c = .312$; OR = 14; 95% CI = 0.76-269). We should point out that the large 95% CI for this analysis was possibly due to the small sample size (see Table E6 in the Online Repository at www.jaci-global.org). The HLA-DQB1 allele frequency did not show any difference between the low and high IFN-γ responders to CD4 SARS-CoV-2 peptides. Nevertheless, we detected a lower frequency of DQB1*02 in the low-response group than in the high-response group (15.95% vs 27.4%), but the difference did not reach significance ($P = .0528$;

OR = 0.5; 95% CI = 0.24-1.0) (see Table E7 in the Online Repository at www.jaci-global.org).

Likewise, we found no significant differences in HLA allele frequency when we considered IFN-γ responses to both CD8 and CD4 SARS-CoV-2 peptides together, comparing individuals who showed low (<5000 SFU) and high responses (≥5000 SFU) (data not shown). Therefore, peptide promiscuity appears to be independent of HLA alleles.

Long-term immunity to SARS-CoV-2–predicted peptide epitopes

Next, we sought to evaluate long-term immunity to SARS-CoV-2 in response to the previously pooled peptides (MCD4 and MCD8) by analyzing a late convalescent period (days after the initial symptoms). This late-period analysis was possible for 52 of the initial 121 participants. Less than 25% of these individuals displayed IFN-γ responses to MCD4 and MCD8 peptides above the cutoff (Fig 3, *A*). When we compared IFN-γ responses at the early (day 30) and late (day 180) time points for these 52 individuals, we found a marked decrease in the number of circulating IFN-γ–producing T cells specific for MCD4 (Fig 3, *B*) and MCD8 peptides (Fig 3, *C*). However, the decrease in IFN-γ T-cell responses to MCD4 (Fig 3, *D*) and MCD8 (Fig 3, *E*) SARS-CoV-2 peptides did not show significant differences in relation to age, sex, or disease severity (requiring hospitalization or not). Together, these results suggest that the frequency of specific T cells in the bloodstream after virus exposure is time dependent. Although the response is perceptible in some of the samples 180 days after the onset of infection, the magnitude of the response is aproximately 80% lower for CD4 and CD8 peptides.

Activation-induced marker expression in SARS-CoV-2–specific $CD4^+$ and $CD8^+$ T cells

To further characterize the T-cell recall response to SARS-CoV-2 antigens, we used flow cytometry to analyze expression of the AIMs on $CD4^+$ and $CD8^+$ T cells stimulated *in vitro* in 12 participants at the early (30 days after the initial symptoms) and late (180 days after the initial symptoms) time points. The peptides were grouped into 2 different pools containing only spike protein–derived peptides or peptides outside the spike sequence (nonspike), specific for CD4 and CD8 T cells. Additionally, PBMC samples were also incubated in the presence of a pool containing all MCD4 and MCD8 peptides. $CD4^+$ and $CD8^+$ AIM^+ T-cell populations were identified on the basis of surface markers (Fig E4, *A* and *B*). The $CD4^+$ AIM^+ ($OX40^+CD137^+$) and $CD8^+$ AIM^+ ($CD69^+CD137^+$) cells were selected on the basis of the fluorescence minus one (FMO) gate strategy (Fig E4, *C*). We established a cutoff based on the analysis of PBMC samples from individuals not exposed to SARS-CoV-2 and stimulated with the same antigens.

On day 30 after infection, 7 of 12 participants presented the AIM^+ phenotype for the $CD4^+$ T-cell–specific spike peptide pool (Fig 4, *A* and *B*), whereas the response to peptides outside the spike (the nonspike pool) was found in only 2 of the analyzed samples (Fig 4, *C*). Specific $CD4^+$ activation in the presence of the pool containing all of the peptides was average (Fig 4, *D*). The same pattern was observed for $CD8^+$-specific activation (Fig 4, *E-H*). At 180 days, we detected a 40% decrease in AIM phenotype positivity versus at the earlier time point in response to the same spike peptides (Fig 4, *B* and *F*). Together, these data indicate that as observed in ELISPOT assay, at 180 days after infection the number of specific circulating T cells is considerably lower, which may be a predictor of impaired long-term response; however, it is important to mention that despite the tendency, we did not observe signicant statistical differences. Furthermore, for AIM expression on $CD4^+$ and $CD8^+$ T cells, peptides present in the spike protein appear to exert greater immunodominance when compared with antigens outside the spike protein.

DISCUSSION

We identified a set of SARS-CoV-2 promiscuous $CD4^+$ and $CD8^+$ T-cell peptides recognized by convalescent individuals. We detected an overall positivity higher than 70% against the pooled peptides in samples collected 30 days after the onset of symptoms. Moreover, the characterization of the IFN-γ–producing cells in response to individual peptides showed that most of the participants recognized more than 80% of the 46 potentially promiscuous $CD4^+$ and $CD8^+$ peptides; this indicates promiscuous recognition, which is consistent with the absence of HLA associations in T-cell responders to any peptide. According to our selection strategy of choosing highly promiscuous peptides following the HLA-binding prediction analyses, the capacity of peptides to induce IFN-γ T-cell response was independent of the HLA alleles of the participants. This finding suggests that the peptides described in this study may be a potential target for the evaluation of SARS-CoV-2 immunity and vaccine studies. Accordingly, it has been reported that the combination of natural acquired immunity after infection and a further immunization protocol with similar antigens may induce a higher differentiation of memory B and T lymphocytes.[20] Considering the prevalence of COVID-19, it is possible that from now on, the SARS-CoV-2 vaccine may be administrated seasonally in the general population. In this sense, the analysis of samples from previously infected and immunized individuals is an efficient strategy to identify promiscuous peptides. This evaluation allows selection of the most promising antigens capable of inducing the generation of responsive T cells useful for the elaboration of new vaccine constructs.

Our results did not indicate a specific immunodominant profile of the T-cell response against epitopes presented in different regions of the viral genome, although the magnitude of the response was markedly variable among the cohort. Variability of response among a diverse cohort is expected, but in addition, the peptides were selected on the basis of their high promiscuity to T cells. In this respect, robust T-cell immunity directed toward multiple viral peptides is likely relevant to prevention of infection by VOCs because T cells from infected or vaccinated individuals display a cross-reactive responsiveness against the Alpha (B.1.1.7) and Delta (B.1.617.2) variants.[10] These data suggest potential relevance of T-cell response diversity and cross-reactivity for protection or mitigation of severe symptoms after infection by a VOC.

Although natural infection may induce broader memory cell populations, vaccination protects against severe outcomes.[21] In this sense, the identification of peptides of interest to induce protection after immunization in parallel with boosting of the immunity elicited by natural infection is a concern to be addressed.

On the basis of the heterogeneity of the study participants' T-cell response, we focused on the evaluation of specific subgroups. In this sense, we observed that characteristics of the

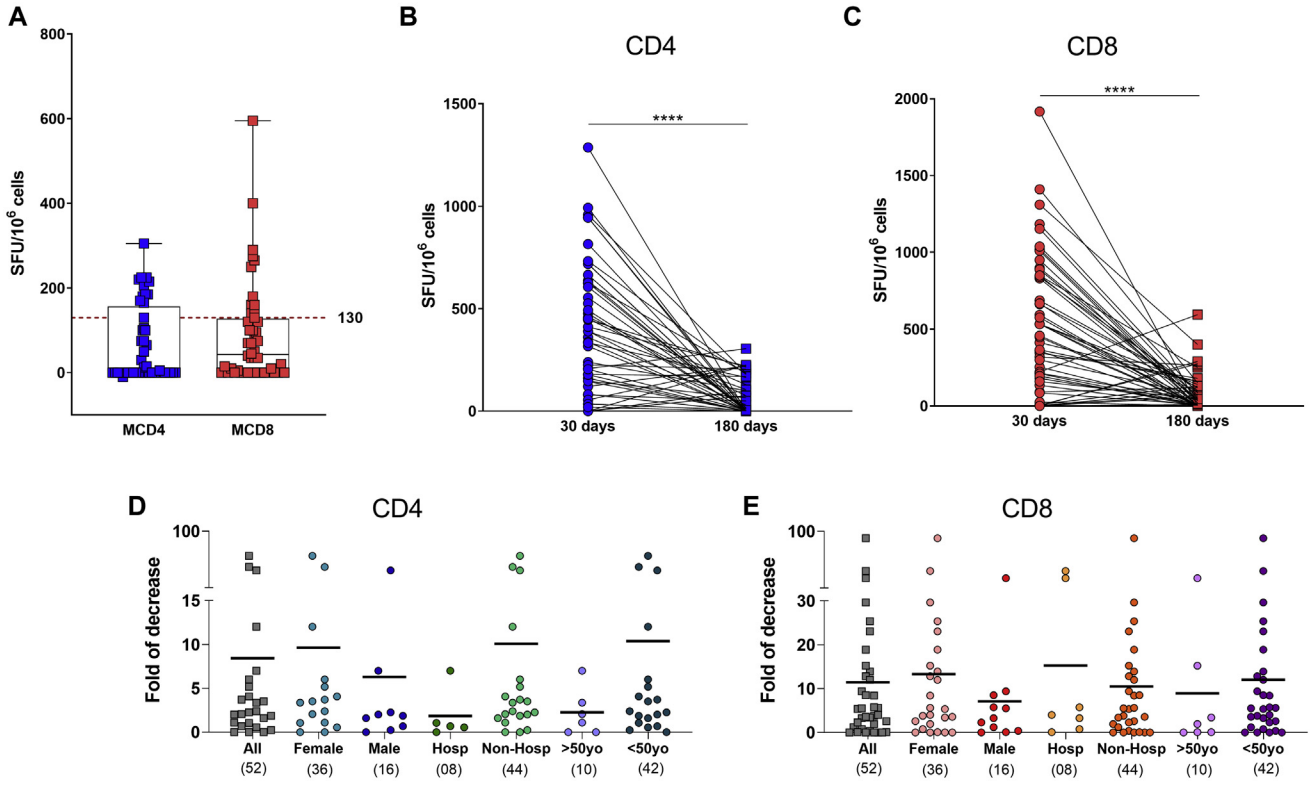

FIG 3. Long-term immunity to SARS-CoV-2 peptides. PMBC samples collected at 2 different time points (30 and 180 days after symptom onset) were stimulated with megapools containing 20 CD4 peptides (MCD4) and 26 CD8 peptides (MCD8). **A,** T-cell recall (IFN-γ production) 180 days after sympton onset. **B** and **C,** Paired analysis of the specific $CD4^+$ and $CD8^+$ T-cell IFN-γ production after 30 and 180 days, respectively. **D** and **E,** Overall fold of response decrease after 180 days according to clinical charatecteristics of the cohort. Matched-pair analysis was performed with the Wilcoxon test. ****$P < .0001$.

participants, such as sex, age, and disease outcome were associated with the magnitude of response. The entire cohort was composed of symptomatic subjects; however, those who developed severe symptoms and required hospitalization displayed a significantly stronger IFN-γ response directed toward both CD4 and CD8 peptides. Corroborating our results, evidence suggests that those individuals who display the severe outcome do in fact develop increased T-cell responses.[22] Nevertheless, higher numbers of IFN-γ–producing $CD4^+$ and $CD8^+$ T lymphocytes in response to viral peptides were found in patients who showed severe symptoms and were able to survive the COVID-19.[6] Likewise, all of the hospitalized participants in our cohort survived. The higher magnitude of SARS-CoV-2–specific T-cell clones reactive to SARS-CoV-2 proteins may be a result of prolonged exposure to a high viral load, possibly owing to impaired early T-cell differentiation. Viral burden induces increased production of cytokines and chemokines, which promotes an inflammatory environment that could possibly elicit T-cell activation.[23]

We found a significantly higher magnitude of $CD4^+$ and $CD8^+$ IFN-γ T-cell responses to SARS-CoV2 in females. In line with our data, increasing evidence indicates that disease severity and mortality rates are higher in male patients.[24,25] Accordingly, compared with female patients, male patients have been reported to show marked innate cytokine production (IL-8 and IL-18) after infection. Unlike females, male patients also exhibited a lower T-cell response that is associated with disease severity.[24,25] An early expansion of T-cell immunity leads to less severe COVID-19,[26] which is also found in other coronaviruses such as Middle East respiratory syndrome.[27] In line with this idea, our results demonstrated that the number of epitope-specific IFN-γ–producing $CD4^+$ and $CD8^+$ lymphocytes is lower in older individuals (aged >50 years). This reduction is consistent with immunosenescence and is in line with findings from our group, which focused on the study of subjects vaccinated with an inactivated SARS-CoV-2 vaccine.[28] Nevertheless, we had no deaths in our cohort. Most studies to date suggest that this phenotype is related to insufficient adaptive immunity against the virus and an increased innate inflammatory response. Age-induced immunity impairment is mainly associated with thymic aging, decaying of the diversity of the T-cell receptor repertoire, and senescent T cells.[29] Exacerbated inflammation is likely associated with the low-grade chronic inflammation usually that is found in the elderly.[30,31] We should mention that although the magnitude of response was significantly higher in female, younger, and hospitalized individuals, the overall frequency of recognition was similar in all groups.

A different concern that must be considered for vaccine design or diagnostic purposes is long-term immunity to SARS-CoV-2 and the prevalence of circulating responsive T cells. The longevity of the specific response to SARS-CoV-2 antigens is not fully understood. Although follow-up studies suggest that

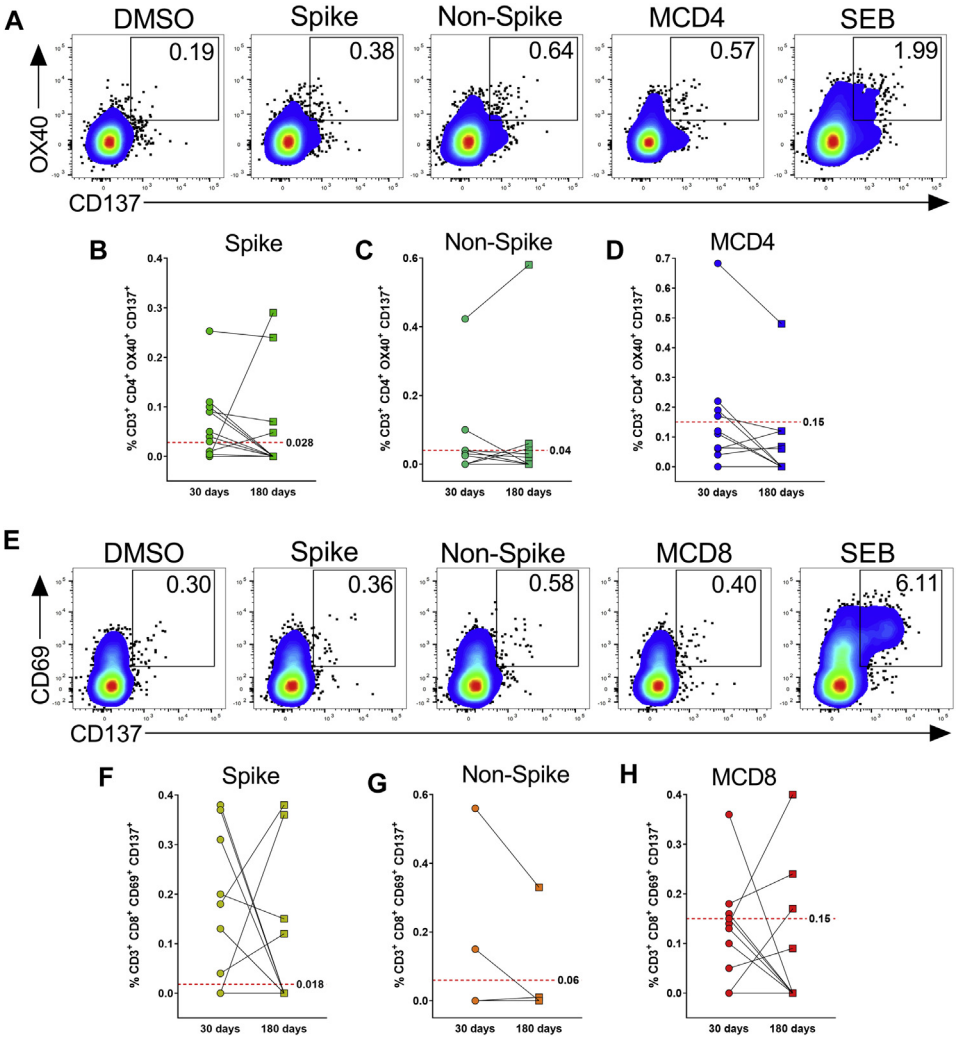

FIG 4. Expression of AIMs in $CD4^+$ and $CD8^+$ T cells. The remaining PMBC samples collected at 2 different time points (30 and 180 days after sympton onset) were stimulated for 24 hours with 3 pools of peptides (peptides found in spike protein only, peptides found outside the spike protein, or a megapool containing all peptides [MCD4]). **A**, Gating strategy for $CD4^+$ AIM^+ T cells. **B-D**, $CD4^+$ AIM^+ T cells stimulated with peptides from spike protein, outside the spike protein, and MCD4, respectively. **E**, Gating strategy for $CD8^+$ AIM^+ T cells. $CD8^+$ AIM^+ T cells stimulated with peptides from spike protein, outside the spike protein, and MCD8, respectively.

antibodies decline over time, in some cases IgG can be detected for up to 7 months after the onset of infection, indicating limited duration.[32-34] Insights derived from studies of SARS-CoV-1 and Middle East respiratory syndrome infection[35] suggest that unlike humoral immunity, cellular immunity against SARS-CoV-2 may be sustained for longer periods. In fact, a recent study reported the presence of SARS-reactive T cells 17 years after infection.[36]

To date, only a few studies have explored longitudinal immunity to SARS-CoV-2 infection, with some of them showing detection of IFN-γ–producing $CD4^+$ and $CD8^+$ T cells responsive to SARS-CoV-2 epitopes 6 months and 1 year after infection.[37,38] In contrast with the data available for other coronaviruses, our results indicate that the numbers of peptide-responsive IFN-γ–producing $CD4^+$ and $CD8^+$ T cells decreased 180 days after infection, although they were still detected in approximately 20% of the participants. The prevalence of a cellular response up to 6 months after infection has been described to be restricted to dominant T-cell epitopes.[39]

In our study, we found a reduced magnitude of responsiveness 180 days after infection. Compared with what we observed in the first PBMC collection, we saw a decrease in the number of circulating responsive T cells. Another longitudinal study recently published by Dan et al reported that longitudinal cellular immunity in convalescent patients decays 6 to 8 months after infection. In this case, the retained specific immunity to SARS-CoV-2 was accessed by AIMs.[40] In agreement with these findings, we observed a 40% decrease in positivity of $CD4^+$ and $CD8^+$ AIM^+ T cells after *in vitro* recall with a pool of spike protein peptides 180 days after infection. As described in the current study, the magnitude of response was measured for the main viral proteins, but only circulating cells were accessed. On the basis of this information, it is important to consider that the

compartmentalization of memory cells in lymphoid organs or the specific local response in the mucosa may be maintained; so far, however, there are no reports regarding such long-term response. Even considering the decline in response over time, a recent study has shown the presence of responsive circulating IFN-γ–producing T cells specific to spike, nucleocapsid, and membrane peptide pools until 10 months after infection.[41] Some recent data reinforce the idea that memory T cells are associated with protection. Even in the absence of viral infection, individuals in close contact with SARS-CoV-2–infected patients can develop memory reactive $CD4^+$ and $CD8^+$ T cells.[42] Memory T cells are recruited for the site of infection during a second exposure to a pathogen. However, in the absence of antigen, tissue-resident memory T cells can be found permanently residing in different tissues, suggesting that the decrease in the number of reactive cells in our study might not be correlated with protection.[43]

One of the limitations of longitudinal studies is the lack of information on the correlates of protection in reinfections. Some evidence suggests that reinfections are poorly related to the potency of the adaptive immune response and the incidence of VOCs is a potential risk of viral evasion.[44] Regarding the report of impaired humoral response effectiviness to VOCs,[45] cellular immunity has been less studied. Recent findings have suggested that $CD4^+$ and $CD8^+$ T-cell responses in convalescent COVID-19 subjects are not substantially affected by mutations found in the VOCs.[11] Indeed, most of the predicted T-cell epitopes described here are highly conserved among the variants. For this reason, a multiepitope-based vaccine may have potential implications for the development of vaccines with broader protective immunity against VOCs. Nevertheless, understanding the immunity profile of a heterogeneous population and determining the durability of the response is imperative to the development of treatment strategies and vaccine design.

Clinical implications: The magnitude of the response to SARS-CoV-2 is influenced by the clinical characteristics of the patients. The response decreased 180 days after infection, suggesting a time-dependent contraction of response.

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