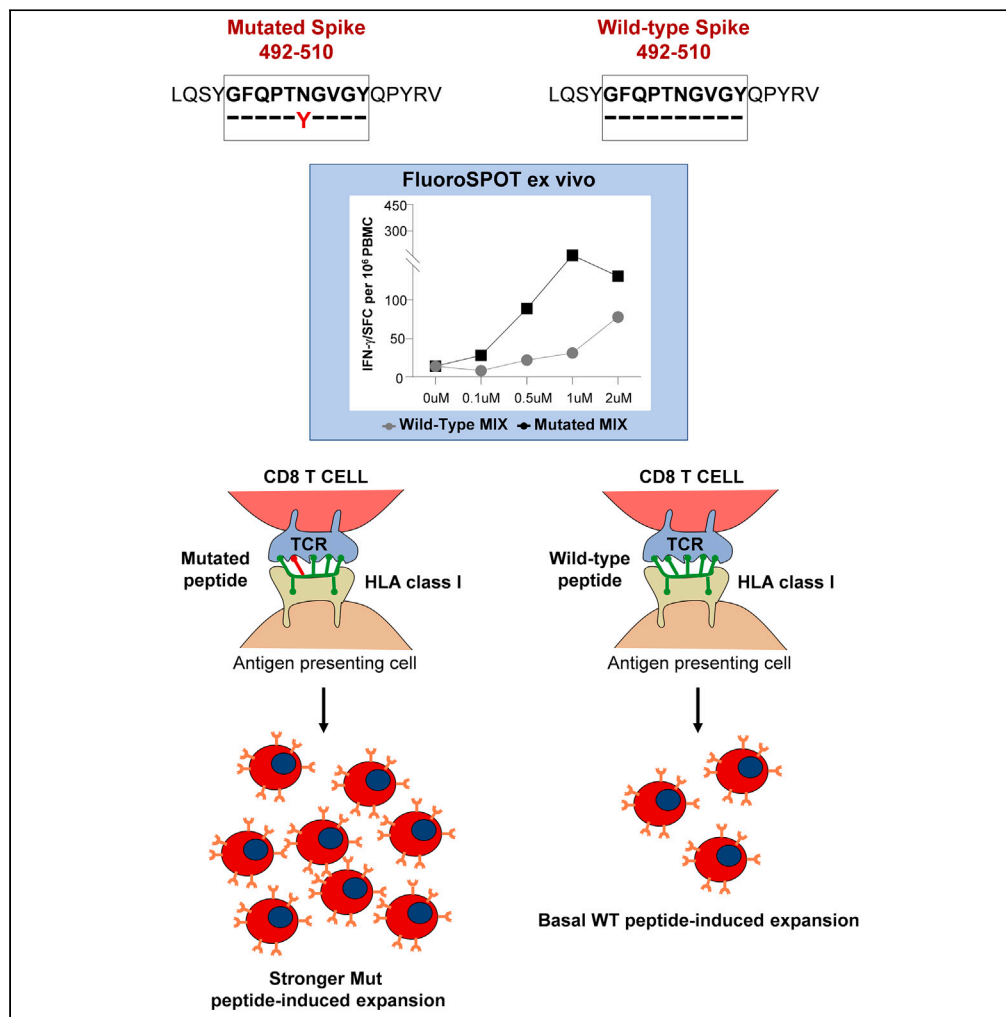


Article

Natural heteroclitic-like peptides are generated by SARS-CoV-2 mutations



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Highlights
SARS-CoV-2 mutations
can have inhibitory or
enhancing effects on CD8
T cell responses

CD8-mediated responses
to SARS-CoV-2 are
generally broad and multi-
specific

In some individuals
inhibitory mutations are
associated with restricted
CD8 responses

When the CD8 repertoire
is narrow, mutations may
affect CD8 T cell
surveillance

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Article

Natural heteroclitic-like peptides are generated by SARS-CoV-2 mutations

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SUMMARY

Humoral immunity is sensitive to evasion by SARS-CoV-2 mutants, but CD8 T cells seem to be more resistant to mutational inactivation. By a systematic analysis of 30 spike variant peptides containing the most relevant VOC and VOI mutations that have accumulated overtime, we show that in vaccinated and convalescent subjects, mutated epitopes can have not only a neutral or inhibitory effect on CD8 T cell recognition but can also enhance or generate *de novo* CD8 T cell responses. The emergence of these mutated T cell function enhancing epitopes likely reflects an epiphenomenon of SARS-CoV-2 evolution driven by antibody evasion and increased virus transmissibility. In a subset of individuals with weak and narrowly focused CD8 T cell responses selection of these heteroclitic-like epitopes may bear clinical relevance by improving antiviral protection. The functional enhancing effect of these peptides is also worth of consideration for the future development of new generation, more potent COVID-19 vaccines.

INTRODUCTION

RNA viruses tend to accumulate mutations more rapidly and extensively than DNA-based pathogens.^{1,2} The main cause of this enhanced mutability is low-fidelity replication, which leads to genetically diverse but related virus variants populations. Such variants may progressively acquire better tropism as well as higher infectivity and pathogenicity, with possible acquisition of resistance to vaccines and antiviral compounds. As an RNA virus, evolution of the SARS-CoV-2 genome resulted in multiple variants (so-called “Variants of Concern”, VoC and “Variants of Interest” Vol) that have progressively acquired an increased transmission capacity.^{3–12} Understanding the effect of mutations on protective immune responses is key to the development of new generation vaccines and antivirals.

As a general rule, new mutations tend to be fixed either because of the acquisition of an enhanced transmissibility or because of escape from immunological pressure. Both mechanisms are thought to be causally involved in the selection of SARS-CoV-2 variants. In the absence of pre-existing immunity, as in the case of the early SARS-CoV-2 outbreak, escape from immune responses is expected to contribute very little to the rate of virus spread,¹³ which should be primarily determined by a direct effect of mutations on virus tropism, efficiency of replication, and infection. However, as protective immunity becomes more widespread escape from neutralizing antibodies and cytotoxic CD8 T cell responses can provide a more relevant contribution to virus transmissibility. Indeed, the establishment of new variants, such as beta (B.1.351), gamma (P.1), delta (B.1.617.2), and omicron (B.1.1.529), has been associated with a progressively growing impact of virus mutations on neutralizing antibody responses.^{14–19} Remarkably, the impact of SARS-CoV-2 variability seems to be instead much weaker on CD8 T-cell-based immunity.^{14,15,20–25} This lower susceptibility of cell-mediated responses to mutational inactivation may dampen evolution of infection toward severe symptomatic disease given the key role of CD8 T cells in limiting the spread of infection within the infected host through their capacity to recognize and cure virus-infected cells.^{26,27}

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Considering this important role of CD8 T cell responses in antiviral protection,^{28–31} we performed a comprehensive investigation of the interplay between SARS-CoV-2 variation and spike vaccine-induced CD8 T cells reactivity. We discovered an unexpectedly enhanced immune responsiveness to a specific subset of mutated spike CD8 T cell epitopes. The possibility of enhancing the immune stimulatory activity of previously identified CD8 T cell epitopes by single amino acid (AA) changes, with generation of variant peptides named heteroclitic, has widely been explored *in vitro*, as a possible strategy to improve the antiviral protection of T cell-based vaccines and therapies.^{32–35} Instead, here we report the natural generation of variant peptides able to ameliorate responsiveness to the corresponding prototype sequences contained in the Wuhan-based vaccine. These findings offer additional perspectives on the breadth of protection that may be conferred by CD8 T cells and on their role in the evolutionary biology of SARS-CoV-2.

RESULTS

Impact of variant mutations on SARS-CoV-2-specific T cell responses

To examine the effect of SARS-CoV-2 variant mutations on CD8 T cell reactivity, we studied 36 vaccinated donors, naive to infection, 1–2 months after the second dose of the Pfizer/BioNTech vaccine. All subjects showed high levels of serum anti-spike neutralizing antibodies detected by a SARS-CoV-2 Wuhan-Hu-1 pseudovirus neutralization assay (Table 1). To study the CD8 T cell response to SARS-CoV-2 spike (S), we used an *ex vivo* FluoroSPOT assay based on Peripheral blood mononuclear cells (PBMC) stimulation with 10 mer peptides (overlapping by 9 residues) spanning VOC- and VOI-containing S regions harboring all the main mutations that emerged since the initial appearance of SARS-CoV-2 in Wuhan until June 2021. For each S mutation, two peptide pools were synthesized in order to cover both the Wuhan prototype and the subsequently mutated SARS-CoV-2 variants and to accommodate each individual mutation at each position of the overlapping peptides (Tables S1 and S2). The ability of each peptide pool to simultaneously elicit IFN- γ , TNF- α , and IL-2 production by CD8 T cells was then measured and stringent criteria were applied in order to identify statistically significant SARS-CoV-2 spike-specific T cell responses (see ‘STAR Methods’ for details).

SARS-CoV-2 spike-specific CD8 T cell responses were detected against at least 1 peptide pool in 17 out of the 36 tested vaccinees (Figures 1A and S1). All peptide pools were able to stimulate significant T cell responses in at least one responder individual, showing that each tested mutation was contained within CD8 T cell epitopes. As expected, some mutations had a neutral effect, meaning that prototype (gray bars) and mutated (black bars) peptides were similarly capable of stimulating CD8 T cell responses. Other mutations negatively affected T cell responses because the variant peptide exhibited a reduced stimulatory activity compared to the corresponding prototype sequence. However, the opposite effect was observed with a subset of mutations, which conferred to the corresponding variant peptides a significantly enhanced stimulatory potency compared to the original prototype peptide (Figures 1A and S1).

To unambiguously classify enhancing and inhibitory effects of individual mutations, we calculated the fold-change for each T cell response stimulated by a mutant peptide pool relative to the corresponding unmutated pool (mutated/wild-type) and then applied an *effect size* filter to quantitatively evaluate the relationship between variables (see Figure 1B and ‘STAR Methods’ for details). In addition, to provide an even more stringent definition of the modulatory effects caused by individual mutations, only responses featuring a concordant modulation of at least two of the three tested cytokines were considered as reliable indicators of a positive or a negative effect.

In some cases, negative or positive effects were clearly appreciable even without statistical analysis because the mutated peptides either induced a response not observed with the prototype sequence, or failed to induce any detectable response as observed with the corresponding prototype peptide (in these cases only a gray or a black bar is displayed in Figure S1). Overall, a statistically supported neutral effect was detected in 25% (25/98) of the total responses (Figure 1A). A markedly decreased or no response to mutated spike variant peptide pools were observed and statistically validated in 45 out of 98 cases (46%, Figure 1A) distributed among 13 of the 17 responder vaccinated individuals (76%) (Figure S1). In contrast, the opposite phenomenon, i.e., significantly enhanced response induced by a subset of mutated spike peptides, was observed in 11 of the 17 vaccinated responders (64%), accounting for 29% (28/98) of all significant responses induced by peptide pool stimulation (Figure 1A). As further shown in Figure S2 (right panel), enhanced responses to variant peptides were particularly evident for IFN- γ and IL-2 production.

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Table 1. Demographic and immunological parameters of the vaccine recipient population

	ID patient	Ethnicity	Gender	Age	Days after 2 nd vaccine dose	LOCUS A	LOCUS B	LOCUS C	SARS-CoV-2 serum neutralization assay after 2 nd vaccine dose (NT50/mL)
1	VAX 008*	Caucasian	F	43	17	11:01 24:02	51:01 –	01:02 04:01	≥ 20480
2	VAX 009	Caucasian	F	62	46	01:01 03:01	35:02 40:02	02:02 04:01	≥ 20480
3	VAX 010	Caucasian	F	40	21	30:01 30:04	35:02 51:08	04:01 16:02	≥ 20480
4	VAX 011	Caucasian	M	48	39	02:01 26:01	18:01 35:01	04:01 07:01	≥ 5120
5	VAX 012	Caucasian	F	29	39	02:01 25:01	18:01 –	07:01 12:03	≥ 20480
6	VAX 013	Caucasian	F	51	14	02:01 26:01	14:01 44:02	05:01 08:02	≥ 20480
7	VAX 015*	Caucasian	F	25	30	24:02 –	18:01 44:02	02:02 12:03	≥ 20480
8	VAX 016*	Caucasian	F	28	30	02:01 32:01	18:01 40:02	02:02 07:01	≥ 20480
9	VAX 018	Caucasian	F	29	42	02:01 68:01	37:01 39:01	06:02 12:03	≥ 20480
10	VAX 019	Caucasian	F	51	23	03:01 33:01	13:02 14:02	07:04 08:02	≥ 20480
11	VAX 020	Caucasian	F	50	21	23:01 68:01	07:02 50:01	06:02 07:02	≥ 20480
12	VAX 021	Caucasian	F	53	38	02:01 11:01	15:17 51:01	07:01 07:01	≥ 20480
13	VAX 023*	Caucasian	F	49	29	01:01 24:02	45:01 52:01	07:02 12:02	≥ 10240
14	VAX 024	Caucasian	F	62	26	02:01 32:01	35:03 44:02	04:01 05:01	≥ 2560
15	VAX 025	Caucasian	M	37	41	02:01 26:26	13:02 27:05	02:02 06:02	≥ 20480
16	VAX 026	Caucasian	F	41	52	03:01 29:02	14:02 44:03	08:02 16:01	≥ 20480
17	VAX 027	Caucasian	M	48	29	02:01 30:02	18:01 39:01	05:01 12:03	≥ 20480
18	VAX 028*	Caucasian	F	39	69	01:01 26:01	08:01 38:01	07:01 12:03	≥ 10240
19	VAX 031	Caucasian	F	57	27	01:01 03:01	14:02 37:01	06:02 08:02	≥ 10240
20	VAX 033	Caucasian	F	54	27	02:01 30:02	35:02 44:02	04:01 05:01	≥ 20480
21	VAX 034	Caucasian	F	53	19	03:01 32:01	13:02 50:01	06:02 12:03	≥ 20480
22	VAX 035	Caucasian	F	57	26	30:01 33:01	13:02 14:02	06:02 08:02	≥ 20480

(Continued on next page)

Table 1. Continued

ID patient	Ethnicity	Gender	Age	Days after 2 nd vaccine dose	LOCUS A	LOCUS B	LOCUS C	SARS-CoV-2 serum neutralization assay after 2 nd vaccine dose (NT50/mL)
23 VAX 036	Caucasian	F	61	28	01:01 23:01	15:01 44:03	04:01 07:01	≥ 20480
24 VAX 039	Caucasian	F	62	23	02:01 03:01	44:02 57:01	05:01 07:01	≥ 20480
25 VAX 041*	Caucasian	F	27	15	03:01 24:02	07:02 51:01	07:02 15:02	≥ 20480
26 VAX 042	Caucasian	F	44	55	01:01 26:01	37:01 39:01	06:02 12:03	≥ 20480
27 VAX 043	Caucasian	F	50	31	01:01 02:01	27:02 38:01	02:02P 12:03P	≥ 20480
28 VAX 044	Caucasian	M	65	38	01:01 26:01	08:01 44:05	02:02 07:01	≥ 20480
29 VAX 053	Caucasian	F	26	43	03:01 23:01	07:02 44:03	04:01 07:02	≥ 20480
30 VAX 055	Caucasian	F	27	58	11:01 30:01	13:02 35:01	04:01 06:02	≥ 20480
31 VAX 058	Caucasian	M	31	53	02:01 23:01P	49:01 51:01	07:01 15:02	≥ 5120
32 VAX 060	Caucasian	F	28	59	02:01 03:01	35:02 51:01	04:01 16:02	≥ 20480
33 VAX 061	Caucasian	F	28	59	02:01 03:01	07:05 56:01	01:02 15:05	≥ 10240
34 VAX 065*	Caucasian	F	46	11	24:02 26:01	35:01 35:02	04:01 04:30	≥ 10240
35 VAX 066	Caucasian	M	58	58	02:05 11:01	35:01 58:01	04:01 07:18	≥ 2560
36 VAX 076*	Caucasian	F	26	31	03:01 24:02	07:02 35:08	04:01 07:02	≥ 10240

* The asterisk indicates the vaccinated subjects who have also been tested before vaccination, as a control for the stimulatory activity of the SARS-CoV-2 peptides in the absence of previous SARS-CoV-2 exposure. Three additional subjects, named as VAX 123, VAX 129 and VAX 130 have been enrolled to increase the number of control samples.

Representative examples of the responses elicited by individual peptides and by the corresponding peptide pools are shown in [Figures 1C](#) and [1D](#).

Moreover, as revealed by simultaneous detection of IFN- γ , TNF- α , and IL-2 at a single T cell level, enhanced cytokine production was sustained not only by an increased frequency of the overall population of cells producing individual cytokines upon stimulation (sum of single, double, and triple positive cells) but also by polyfunctional T cells capable of producing IFN- γ , TNF- α , and IL-2 simultaneously ([Figure S3](#)).

The enhancing or inhibitory effects associated with individual mutations detected in the initial screening ([Figures 1A](#) and [S1](#)) were confirmed by dose titration experiments using different concentrations of wild-type and variant peptide pools ([Figure 2A](#)). For example, a clear dose-dependent response was obtained with wild-type MIX 1 tested on cells from VAX 055, whereas a barely detectable response was induced by the corresponding mutated sequences. In contrast, mutated MIX 6 induced much stronger responses in VAX 055 than the corresponding wild-type pool. Of note, the varying steepness of the dose-response curves obtained with different vaccinees is likely explained by quantitative differences in peptide affinity

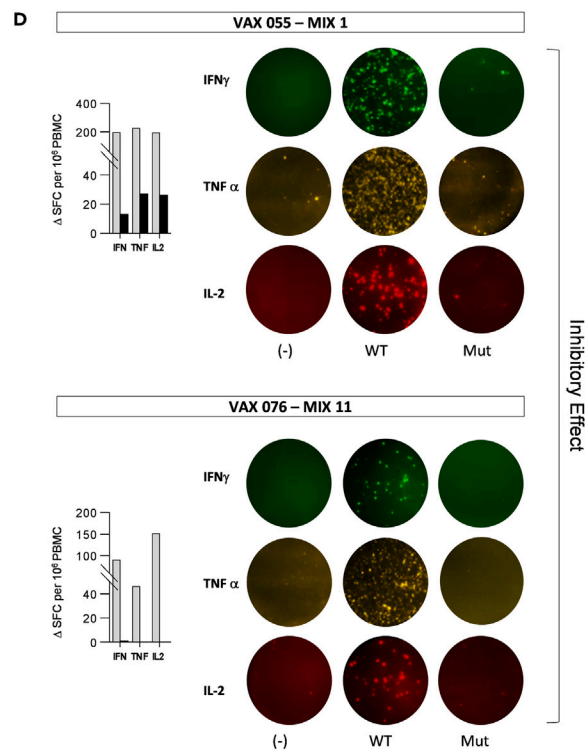
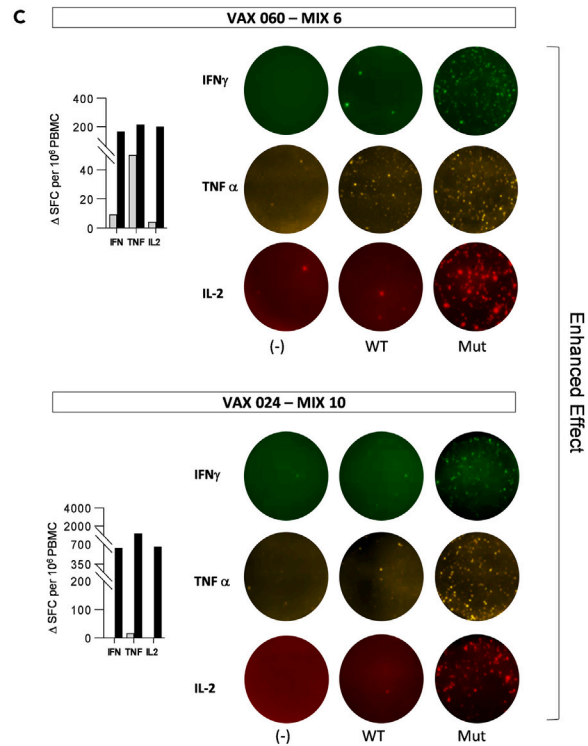
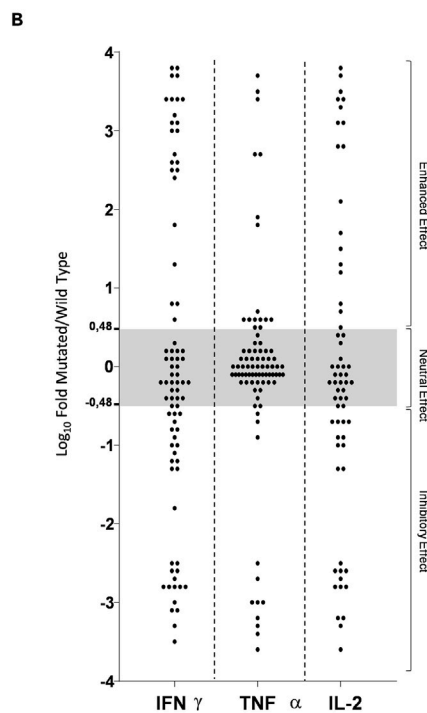
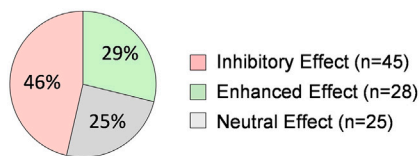
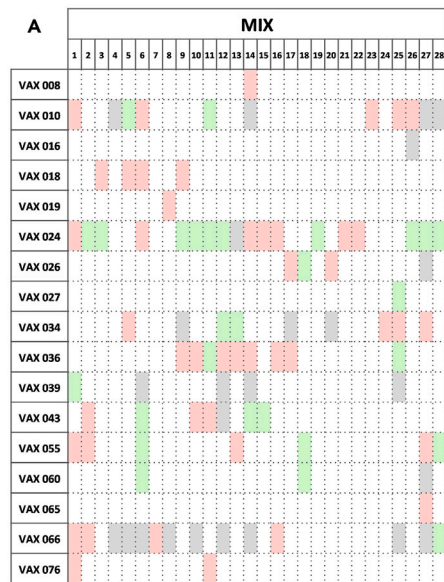


Figure 1. Impact of VOC and VOI mutations on spike-specific T cell responses measured in BNT162b2 vaccinated donors

(A) Schematic summary of enhanced, inhibitory and neutral responses detected in each vaccinated subject tested with 28 wild-type (WT) and mutated peptide pools (top) and percentage of each type of response calculated on the total cases (pie chart; bottom). Red- and green-shadowed areas indicate significantly diminished or increased T cell responses induced by mutated compared to wild-type peptide epitopes, respectively; gray-shadowed areas represent neutral responses (see details and raw data in [Figure S1](#)).

(B) Each dot indicates the fold-change difference between ex vivo IFN- γ -, TNF- α - and IL-2- secreting T cell responses induced by mutated and wild-type (Wuhan type) peptides detected by FluoroSPOT assay in vaccinated individuals ($n = 17$). Only positive responses (according to FluoroSPOT criteria) against an individual peptide mixture are represented. For some positive responses to peptide pools only two out of the three tested cytokines were detectable upon stimulation with both prototype and mutated peptides; for this reason, the total number of dots represented for each cytokine is lower than the total number of positive responses to peptide pools ($n = 98$) illustrated in [Figure S1](#). Keeping the large-effect threshold as a discriminant, only T cell responses below or above the large-effect size values were considered as significantly decreased or increased (< -0.48 or $> +0.48$, respectively). The area delimited by a gray background (comprised between $+0.48$ and -0.48 ; $|0.48| =$ large-effect size threshold) corresponds to neutral CD8 T cell responses (i.e., spot forming cells (SFC) values induced by variant mutated and WT peptide stimulation are not significantly different).

(C and D) Representative ex vivo FluoroSPOT results with peptide pools containing enhancing (C) or inhibitory (D) mutations from 2 vaccinated donors, respectively.

recognition by T cells from different vaccinated subjects ([Figure 2A](#)). Specifically, while for certain pools, peptide concentrations as low as 0.1–0.5 μM were sufficient to induce maximal cytokine production (e.g., VAX 055-MIX 1), concentrations of at least 1 μM were required for other peptides (e.g., VAX 036-MIX 12; VAX 076-MIX 11; VAX 055-MIX 6; VAX 036-MIX 11; VAX 060-MIX 6).

Epitope identification and characterization of the responder T cell subset

To dissect the responses elicited by the different peptide pools and map the specific AA sequences responsible for T cell response induction, individual peptides from pools inducing significant T cell responses were tested for their PBMC stimulation capacity by ex vivo FluoroSPOT. Specific pools (MIX 1, MIX 5, MIX 6, MIX 8, MIX 11, MIX 12, MIX 18, MIX 25, and MIX 27) were chosen according to the best response strengths and donor cell availability. This analysis allowed to deconvolute the individual epitopes within most of the tested pools (8 out of 9). All individual peptides reproduced the same enhancing or inhibitory effects displayed by the corresponding peptide pool ([Figure 2B](#)) and are listed in [Figure 2C](#). The above results, obtained with different concentrations of overlapping peptide pools and individual peptides of optimal length for CD8 T cell recognition, confirmed that while some naturally acquired SARS-CoV-2 spike mutations have either no effect or negatively influence epitope immunogenicity, a subset of mutated epitopes is endowed with an enhanced T cell activation capacity.

Additional experiments were then performed by ex vivo intracellular cytokine staining (ICS) to further verify FluoroSPOT assay results and to better define the specific T cell sub-populations targeted by SARS-CoV-2 spike peptides. To this end, PBMCs from FluoroSPOT-positive vaccinated donors were stimulated by overnight incubation with mutated or wild-type peptide pools, followed by flow-cytometric measurement of IFN- γ , TNF- α , and IL-2 production as well as CD107a degranulation. As suggested by the length of the peptides utilized for this analysis, antiviral cytokine production and CD107 degranulation were entirely sustained by SARS-CoV-2-specific CD8 T cells ([Figure 3A](#)). As shown in [Figure 3B](#), FluoroSPOT and ICS results were mostly concordant. Parallel measurements of CD107 degranulation also gave largely concordant results with only a few exceptions; namely, VAX 060/MIX 6, VAX 066/MIX 1, and VAX 076/MIX 1 ([Figure 3B](#)) where a discordance between cytokine production and degranulation was observed.

The enhancing or inhibitory effects detected by ex vivo FluoroSPOT and ICS assays were reproduced by T cell lines generated upon 8–10 days stimulation of PBMCs with wild-type and mutated peptide pools ([Figures 3C](#) and [S4](#)). In keeping with ex vivo results, responses measured after *in vitro* T cell expansion were exclusively mediated by CD8 cells, with no detectable contribution by CD4 T cells ([Figure 3D](#)).

CD8T cell reactivity against wild-type and mutated SARS-CoV-2 epitopes in convalescent subjects

To find out whether the immune potentiation effect of natural stimulatory mutations, we observed in vaccinated subjects also occurs under natural SARS-CoV-2 infection, we tested PBMCs from 10 convalescent donors that were infected between April and October 2020, i.e., at a time when the variant viruses we selected for this study had not yet been detected in Italy. SARS-CoV-2 spike-specific CD8 T cell responses were detected 1–2 months after the acute phase of infection in 6 out of 10 convalescent subjects ([Table S3](#)). Thirteen (45%, 13/29) and nine (31%, 9/29) of the positive peptide pools elicited inhibitory or enhanced

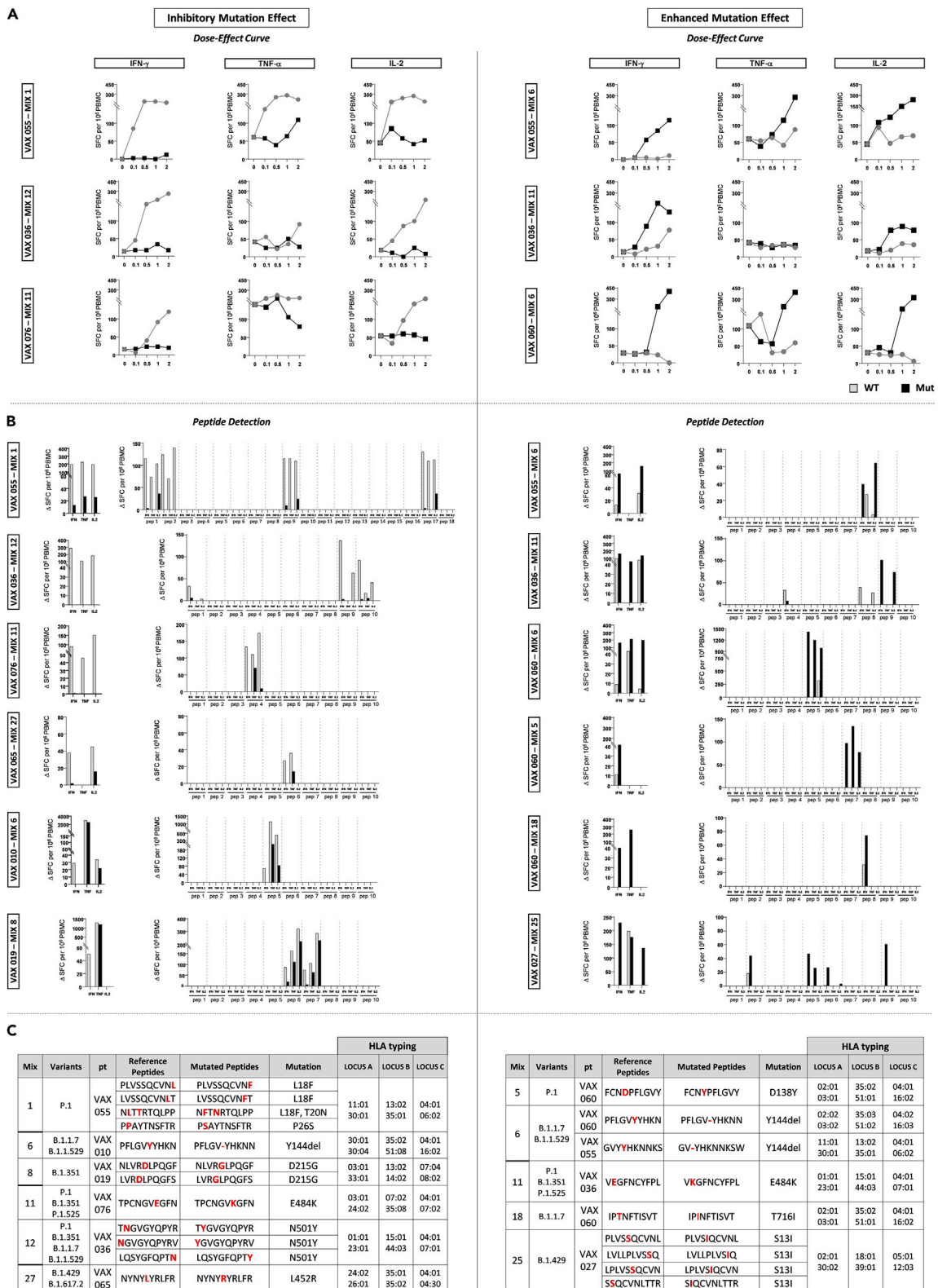


Figure 2. Dose-dependent response to wild-type and variant peptides and identification of individual T cell epitopes

(A) Dose-response curves to the different peptide pools determined by *ex vivo* FluoroSPOT using the indicated increasing concentrations of wild-type and variant peptide pools. Gray and black lines and symbols represent T cell responses induced by wild-type (WT) and mutated peptide pools, respectively. (B) Responses to most of the pools were dissected by using individual component peptides. Individual epitopes were identified by *ex vivo* FluoroSPOT assays performed on PBMCs from vaccinated subjects (n = 8). Gray and black bars represent the responses induced by wild-type and mutated peptides, respectively. (C) List of mutated CD8 epitopes, that negatively ('Inhibitory') or positively ('Enhancing') modulate the CD8 T cell response compared to the corresponding prototype ('Wuhan') epitopes, identified by *ex vivo* FluoroSPOT assay in vaccinated subjects (n = 8).

responses, respectively (Figure 4A). As in the case of vaccinated subjects, we were able to identify the optimal T cell epitope sequences within the peptide pools in all 3 convalescent subjects that we had the opportunity to test (Figure 4B), which yielded concordant enhancing or inhibitory effects of individual mutations on T cell responses as assessed by FluoroSPOT or ICS assays both *ex vivo* (Figure 4C) and after *in vitro* expansion (Figure 4D). As indicated by the results of IFN- γ production and CD107a degranulation measurements (Figures 4C and 4D), and in agreement with the analysis of T cell responses in vaccinees, immune responses were sustained by CD8 T cells.

To gain initial insight into the possible mechanisms underlying the enhanced CD8 T cell reactivity properties, the nine peptides identified as "enhancing epitopes" were analyzed by two different prediction tools (IEDB and Prime 2.0) to predict HLA class-I binding, TCR recognition propensity and immunogenicity.

As shown in Figure S5, when compared with the corresponding prototype peptides, most of the experimentally validated enhancing 10-mer peptides were not recognized as highly immunogenic epitopes or high-affinity HLA/TCR binders, possibly because of their 10-mer length rather than the 9-mer length on which both prediction algorithms have been calibrated. In at least three cases (peptides #5, #6, #8; Figure S5), however, most or all prediction scores consistently indicated better HLA binding and TCR recognition properties for the mutated compared to the corresponding prototype peptides. In at least a case (#4) the intervening mutation affected the P2 anchor thus, determining a change in the HLA restriction element that co-occurred with a higher TCR functional avidity.

The use of these prediction scores allowed to explain the increased CD8 T cell reactivity by a random acquisition of superior HLA/TCR recognition properties only for a subset of the enhancing peptides. This is, however, not totally surprising, first of all because both prediction algorithms have been calibrated on 9-mer rather than 10-mer peptides as those used in our analysis.

CD8 T cell responses to mutated SARS-CoV-2 epitopes in healthy uninfected/unvaccinated subjects

Next, we tested 11 unvaccinated and uninfected ("naive") individuals to assess whether and to what extent cross-reactivity with pre-existing, SARS-CoV-2 unrelated memory T cells may contribute to anti-SARS-CoV-2 specific CD8 T cell responses. Such analysis may thus, reveal whether cross-reactivity might explain the enhanced stimulatory effect exerted by some variant mutated peptides. Overall, 3% of the total stimulatory variant peptide pools induced detectable CD8 T cell responses in PBMCs from naive subjects. More specifically, as shown in Figure S6, a significantly enhanced CD8 T cell response was observed against two different peptide pools in two of the 11 tested subjects.

A possible explanation for the CD8 T cell cross-reactivity observed in naive subjects is previous exposure to peptides from other coronaviruses or unrelated human pathogens that share sequence homology with specific variant SARS-CoV-2 epitopes. We tested this hypothesis by using spike-associated SARS-CoV-2 T cell function enhancing epitopes as queries for a BLASTP search. Hits retrieved from this analysis were first restricted to 10-mer sequences at least 80% identical to specific SARS-CoV-2 peptides and further filtered to include only sequences from known human pathogenic microorganisms. None of the selected hits was found to be 100% identical to the mutated stimulatory SARS-CoV-2 peptides, whereas three mutated spike peptides (IPINFITISVT, LVLLPLVSIQ, and PELGVYHKNN) shared 90% sequence identity with peptides from different bacterial, fungal, and protozoan pathogens (see Table S4). Several cases of 80% identity between additional function enhancing spike variant peptides and SARS-CoV-2-unrelated human pathogens (including two infectious nematodes) also emerged from this analysis (Table S4). Only one peptide (IPINFITISVT) was found to be similar to a subset of

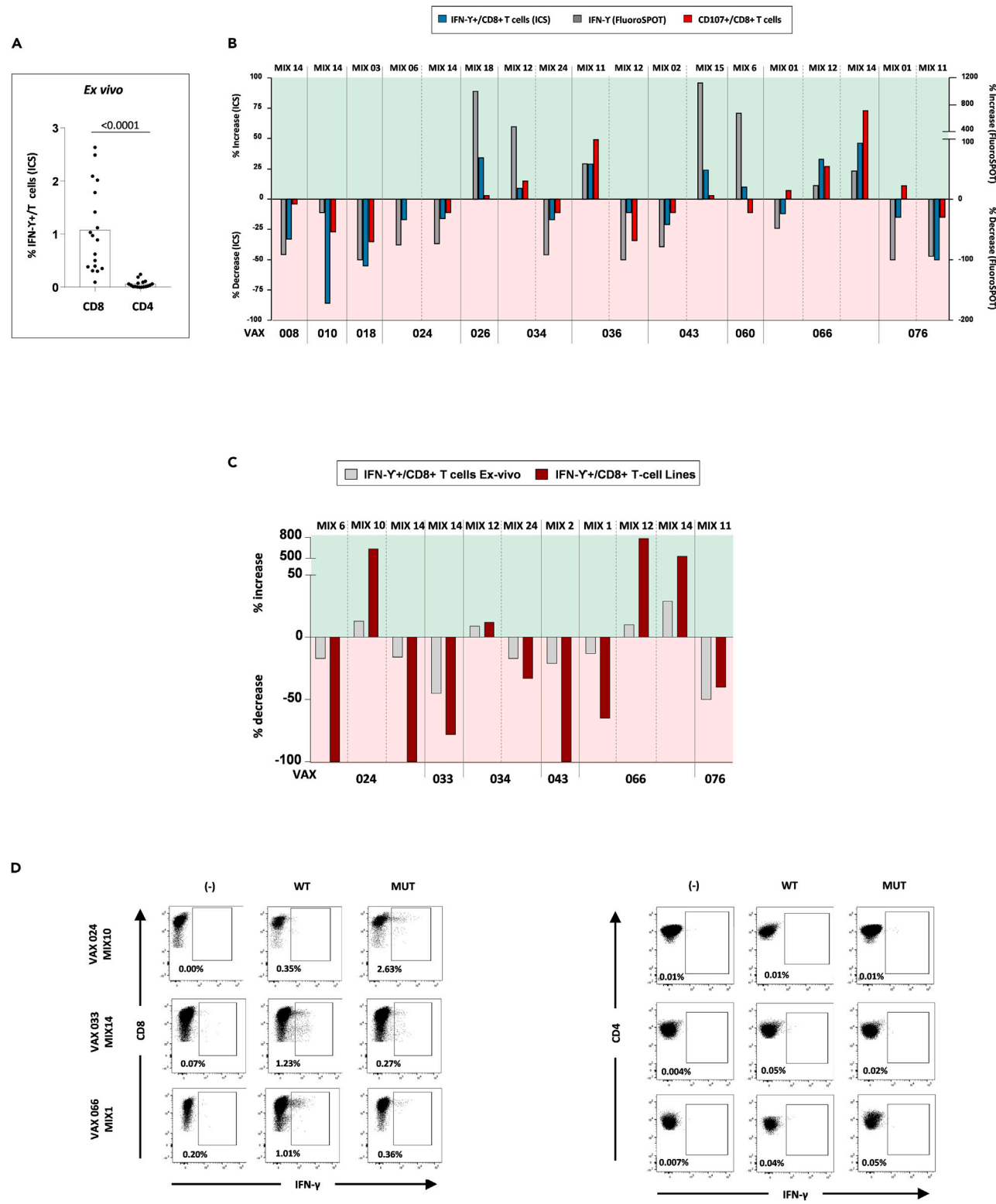


Figure 3. Comparative *ex vivo* and *in vitro* analysis of the variant peptide effect on T cells
(A) Dots represent the frequency of spike-specific IFN- γ -producing CD8 and CD4 T cells reactive to wild-type and variant spike peptide pools determined by flow cytometry after 18 h stimulation; background-subtracted data were analyzed statistically by the Mann-Whitney test.

Figure 3. Continued

(B) Bars represent decreased or increased (red- and green-shadowed areas, respectively) responses to variant versus wild-type peptide pools measured by IFN- γ production (FluoroSPOT and ICS assays) and CD107 degranulation in 11 vaccinated subjects.
(C) Enhancing and inhibitory effects of mutated versus wild-type peptides measured by ICS on CD8 T cells *ex vivo* (gray bars) or on *in vitro* expanded C8 T cell lines (red bars); T cell lines were generated by wild-type and variant peptide pool stimulation for 8 days of PBMCs from six vaccinated donors.
(D) Representative dot-plots derived from cytofluorimetric analysis of *in vitro* expanded T cells, confirming that the stimulatory and inhibitory effects of variant peptides on IFN- γ production are totally sustained by CD8 T cells.

animal coronaviruses (canine, ferret, raccoon, feline, and porcine coronaviruses), while none of the common cold coronaviruses (CCCoV) reached the 80% identity threshold.

Multispecificity of the CD8 T cell responses

Depending on the breadth and multispecificity of the whole repertoire of SARS-CoV-2-specific CD8 T cells, the overall protective antiviral CD8 activity may be affected to different extents by the loss or functional improvement of individual CD8-mediated responses caused by SARS-CoV-2 mutations. We addressed this issue by using two different peptide mixtures for PBMC stimulation: the first one composed of seven pools of 33–39 15-mer peptides overlapping by 10 residues and spanning the entire spike sequence; the second one comprising nine pools of peptides varying in length from 9 to 13 AA, previously described in the literature as CD8 T cell epitopes with different degrees of immunodominance and CD8 T cell stimulatory capacities (Tables S5 and S6, respectively, and supplemental information). A fraction of the latter CD8 epitopes is located within conserved Spike regions that are expected not to tolerate mutational changes because of structural/functional constraints. In addition, some of those epitopes are degenerate, i.e., able to associate with different HLA class I molecules and to be simultaneously presented to CD8 T cell populations of different HLA restriction.³⁶ Both mixtures were used in parallel to test the multispecificity of the response in all vaccinated and convalescent subjects that displayed decline or enhancement of spike-specific responses induced by variant mutations. In line with previous observations,^{37–39} most vaccinated (85%, 22 out of 26) and convalescent (80%, 8 out of 10) donors exhibited multi-specific and powerful CD8-mediated T cell responses with simultaneous IFN- γ , TNF- α , and IL-2 production stimulated by all seven 15-mer peptide pools (Figure 5). Since each pool contains more than 30 peptides, this likely translates into dozens of CD4 and CD8 epitopes simultaneously recognized by T cells. In addition, most individuals also recognized a sizable fraction of the immunodominant CD8 T cell epitopes previously identified within highly conserved spike regions. In contrast, a few subjects (n = 4) recognized a more limited number of peptide pools (3–5) and displayed weaker responses to them. Collectively, these data indicate that in most, but probably not all subjects the presence of a vigorous and multi-specific CD8-mediated T cell response can largely compensate for the mutational loss of single responses.

DISCUSSION

Virus-neutralizing antibodies induced by prophylactic anti-SARS-CoV-2 vaccination are essential to prevent infection,¹⁸ while elimination of virus infected cells and cytokine-mediated purging of intracellularly hidden virus is the main role of CD8 T cells which is key to curb severe disease evolution.^{28,29,38,40} Compared to short-lived antibody responses, CD8-mediated memory T cell immunity is long-lasting and remains detectable well after antibody waning.^{22,41} Recognition of multiple epitopes per antigen and HLA allele, presentation of individual peptides by multiple HLA molecules and location of target epitopes in structurally/functionally constrained regions of SARS-CoV-2 antigens represent CD8 T cell features, which are crucial for counteracting SARS-CoV-2 immune evasion.

Several studies have analyzed the effect of SARS-CoV-2 mutations on CD8 T cell-mediated protection by studying responses to selected individual epitopes^{42,43} or by comparing the immune responses elicited by pools of synthetic peptides covering the overall proteome or the entire spike sequence of the parental Wuhan SARS-CoV-2 strain and from the several VOCs/VOIs viral lineages that have emerged since the beginning of the COVID-19 pandemic.^{22–25}

At variance with most previous studies, here, we used several pools of overlapping 10-mer peptides containing the most relevant VOC and VOI mutations that have accumulated overtime within the spike sequence to analyze the effect of AA variations on CD8 T cell activity. In parallel, breadth and quality of the overall CD8 responses were further characterized in the same cohorts of vaccinated and convalescent individuals by two additional peptide panels, one comprised 96 validated CD8 epitopes of varying HLA restriction representing the majority of the CD8

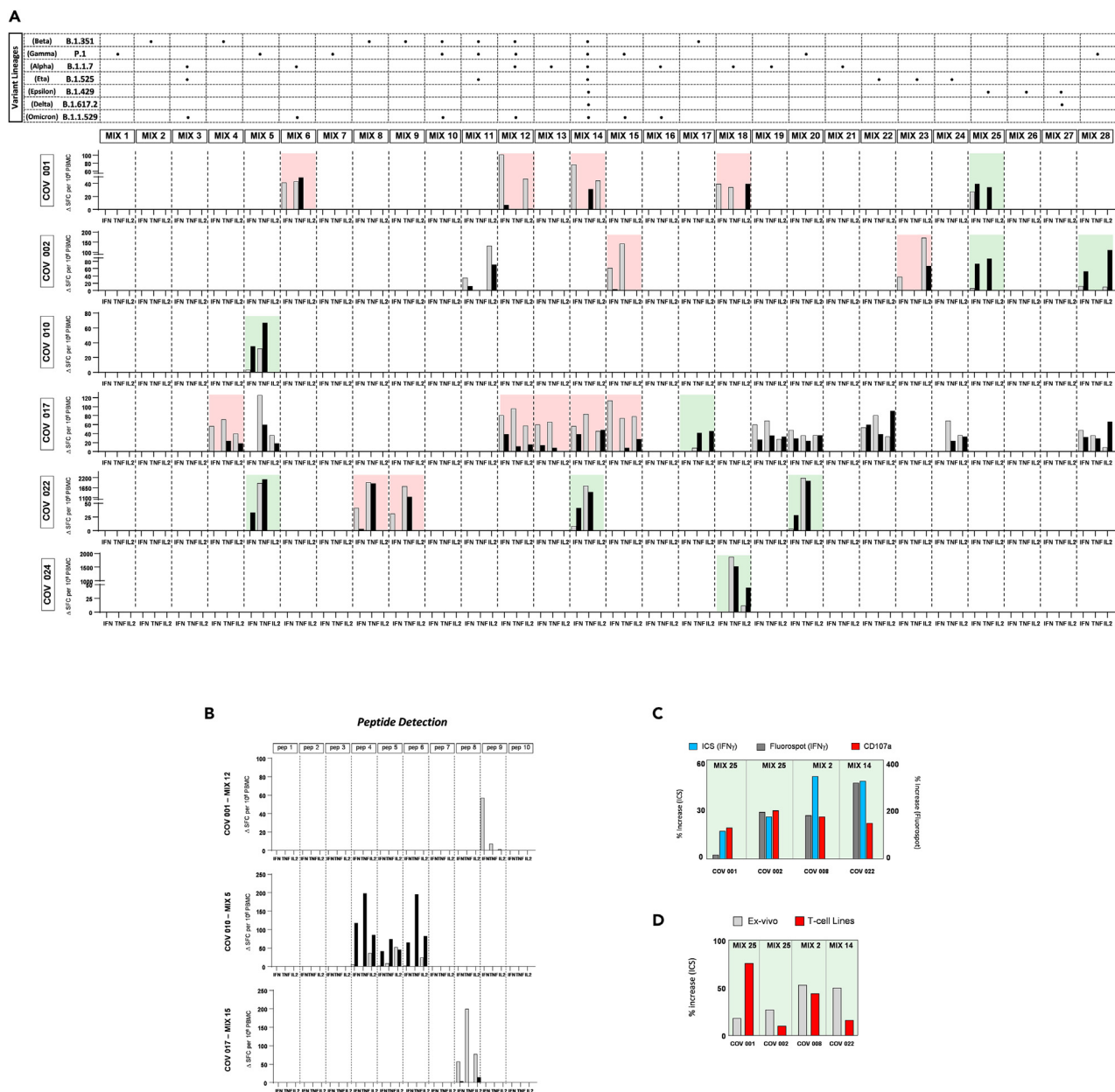


Figure 4. Effect of VOC and VOI mutations on spike-specific T cell responses in Covid-19 convalescent individuals

(A) PBMCs from convalescent patients were stimulated for 18 h by overlapping wild-type and variant peptides. Cytokine-secreting T cells were measured by FluoroSPOT assays. As in Figure S1, only subjects with significant ex vivo SARS-CoV-2 spike-specific CD8 T cell responses for at least two of the three analyzed cytokines (IFN- γ , TNF- α , and IL-2; 6 out of 10 tested patients) are illustrated (see Figure S1 legend for further details).

(B) Individual stimulatory peptides identified by ex vivo FluoroSPOT assays in three convalescent patients; gray and black bars represent the responses induced by wild-type and variant peptides, respectively.

(C) Bars represent the percentage increase of ex vivo spike-specific IFN- γ production detected by FluoroSPOT (gray bars), ICS (blue bars) and CD8 T cell degranulation (red bars) upon PBMC stimulation with mutated vs. wild-type spike peptide pools (n = 4).

(D) Bars represent the percentage increase of IFN- γ detected by ICS either ex vivo on PBMC (gray bars) or *in vitro* on expanded spike-specific CD8 T cell lines stimulated with variant vs. wild-type peptide pools (red bars) (n = 4).

epitopes within the spike region so far described in the literature and the other composed of 15-mer peptides overlapping by 10 residues covering the entire spike sequence. Our assumption was that individual mutations able to abrogate the CD8 T cell function *in vitro* should really have the potential to affect antiviral CD8-mediated protection *in vivo* only in the context of an extremely narrow repertoire of CD8 specificities.

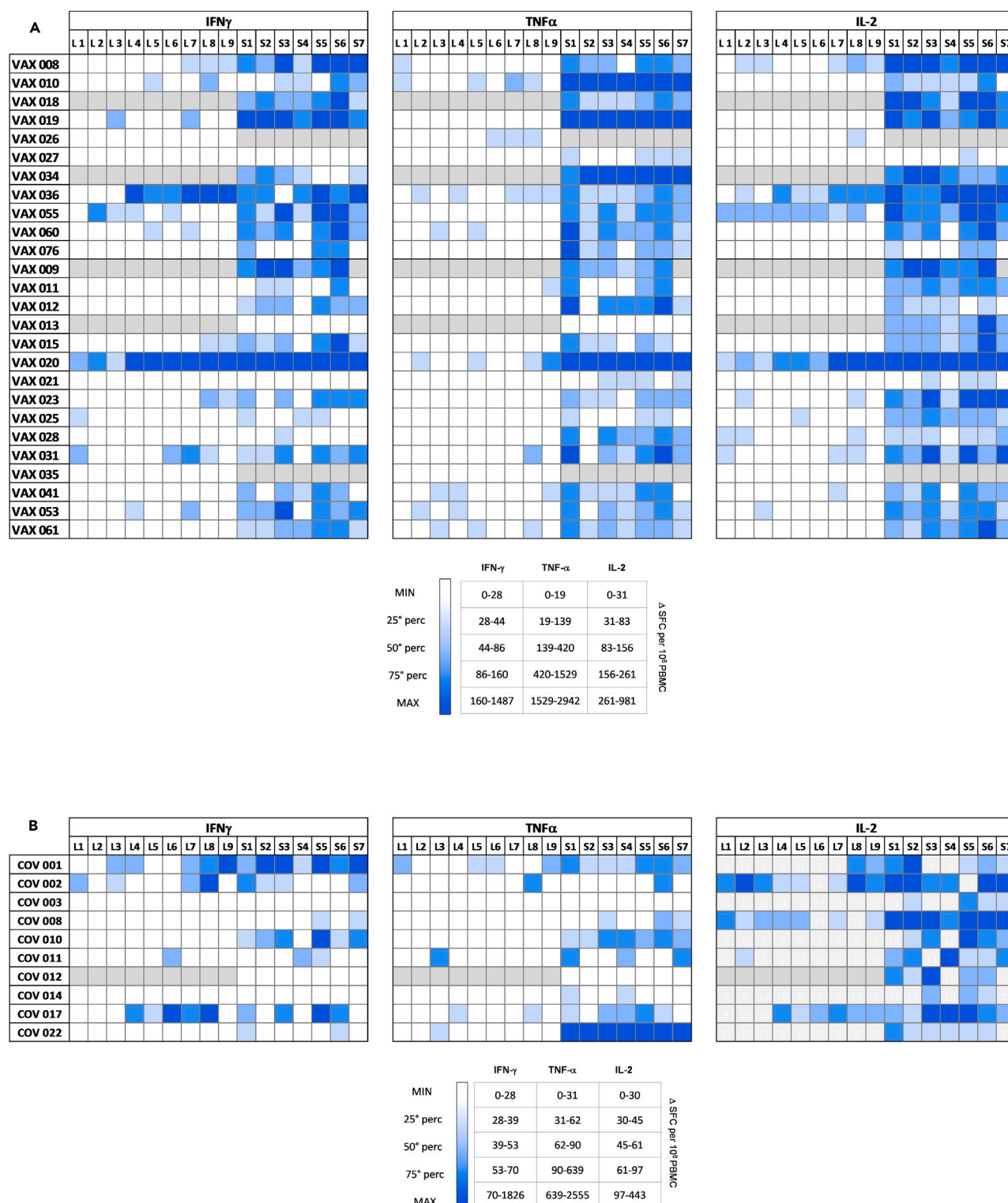


Figure 5. Breadth of the global spike-specific T cell response in vaccinated subjects and in SARS-CoV-2 infection convalescent patients

PBMCs from vaccinated ($n = 26$) and convalescent ($n = 10$) individuals were stimulated for 18 h with seven pools of overlapping 15-mer peptides spanning the entire spike sequence (*right*) and nine pools of 10 peptides representing CD8 T cell epitopes previously described in the literature varying in length from 9 to 13 AA (*left*). Color intensity in the heatmaps indicates IFN- γ , TNF- α or IL-2 production levels measured as SFCs generated upon stimulation with each spike peptide pool (lower than 25th, 25th–50th, 50th–75th, higher than 75th percentile as specified in the inset) of individual vaccinated (panel A) and convalescent (panel B) subjects.

All variant peptide pools were recognized by at least one of the 17 vaccinated responders (out of 36 tested subjects), with some vaccinees reactive to multiple mutated peptide pools. As expected, most SARS-CoV-2 mutations (~70%) were either inhibitory or neutral, meaning that the corresponding peptides elicited CD8 immune responses lower than, or comparable to, those induced by the corresponding prototype epitopes. Interestingly, however, ~30% of the variant epitopes outperformed the CD8 reactivity of the corresponding prototype epitopes or elicited immune responses that were otherwise undetectable with prototype non-mutated epitopes, thus behaving as naturally evolved heteroclitic peptides.

Heteroclitic peptides are typically created artificially, to induce stronger T cell responses by changing specific HLA anchor residues in order to increase HLA binding affinity or to generate new HLA anchor motifs, but also by modifying TCR binding residues in order to increase the affinity of peptide-HLA recognition by TCRs.^{32–35} SARS-CoV-2 mutations within epitopic or flanking sequences able to mediate a more robust stimulation of CD8 T cell responses than their wild-type counterparts have occasionally been reported but never investigated in detail.^{44–47} Of note, we detected function enhancing AA changes in all tested variants and some of the mutated peptides induced opposite (stimulatory or inhibitory) effects in different vaccinated subjects (e.g., the Y144 deletion was inhibitory in VAX 010 but enhancing in VAX 060), an inter-subject variability that is likely explained by the marked individual TCR plasticity and by a possible differential effect of mutations on HLA binding if the same peptide can be presented to CD8 T cells by different HLA molecules in different subjects.

Multiple lines of evidence in our study indicate that the effects of mutated variant epitopes, especially the heteroclitic-like ones, represent a real modulation of T cell functions of potential relevance to antiviral protection, rather than just an *in vitro* phenomenon. First, the effects of individual mutations were classified as stimulatory or inhibitory based on a stringent effect size analysis, using the large-effect threshold as a discriminant to designate T cell responses falling below or above this cutoff as decreased or increased significantly. Moreover, only responses with a concordant modulation of at least two of the three tested cytokines were considered as reliable indicators of a positive or negative effect. Second, the same stimulatory or inhibitory effects detected in the initial screening with peptide pools were reproduced by individual immunogenic peptides identified within each stimulatory peptide pool and were confirmed by dose titration experiments comparing individual variant with the corresponding wild-type peptides. Third, mutated peptide effects initially detected by multi-cytokine FluoroSPOT assays were independently validated by *ex vivo* ICS and CD107 degranulation, which confirmed that the observed responses were indeed CD8 T cell-mediated and that the modulatory effect also affected the cytolytic component of the CD8 function. The fact that these cytotoxic T lymphocyte (CTL) responses are entirely sustained by CD8 T cells with no detectable contribution by CD4 T cells, was further corroborated by the concordant results obtained with *in vitro* expanded T cells stimulated with mutated or prototype peptides. Importantly, similar stimulatory and inhibitory effects were observed in 6 out of 10 convalescent patients, indicating that positive and negative modulation of CD8 responses also occurs during natural infection and may be clinically relevant.

Decline or abrogation of CD8-mediated T cell responses by newly emerged, CD8 epitope-targeting mutations is an expected and well documented event for infections caused by highly variable RNA viruses, which may lead to mutated viral escape if the breadth of the overall antiviral CD8-mediated response is sufficiently narrow to preclude the compensatory role of co-existing non-mutated immunodominant epitopes. Since CTL responses to SARS-CoV-2 have generally been reported to be broadly multi-specific,^{37–39} it is usually thought that virus escape from CD8 T cell control is a rare event requiring the concomitant emergence of multiple inactivating mutations targeting different immunodominant epitopes to make the virus invisible to all or most protective CTL clones. Our data, however, indicate that in a fraction of vaccinated and SARS-CoV-2 infected subjects (e.g., VAX 026, VAX 027, VAX 013, VAX 021, COV 003, and COV 014) CD8 T cell responses appear to be focused on a limited number of spike regions, suggesting the possibility that in this more restricted setting even the abrogation of single CTL responses may negatively impact the overall CTL-mediated antiviral protection. It is also important to note, however, that in natural infection CTL responses are stimulated by other SARS-CoV-2 antigens in addition to the spike protein, thus making CD8 T cell multispecificity even wider and further reducing the possibility of a mutational effect on the overall CD8 T cell-mediated antiviral protection.^{37,39,48–50}

Conversely, it is less clear and counterintuitive the appearance of CD8 T cell function-enhancing mutations that may potentiate protective immunity by facilitating virus elimination rather than its persistence. Since CD8 response-enhancing peptides were also identified in a fraction (2 out of 11) of naive subjects,

suggesting the existence of some cross-reactivity with pre-existing memory CD8 T cells of different specificity, we asked whether the enhancing effect of these mutated peptides might reflect sequence homology with polypeptides from unrelated human pathogens. Interestingly, multiple 10-mer peptides, 80%–90% identical to spike stimulatory peptides, were identified within polypeptide sequences from unrelated human pathogens contained in the NCBI repository. This points to the potential relevance of SARS-CoV-2-specific CD8 T cell cross-reactivity triggered by pre-existing memory responses raised against unrelated microorganisms in shaping the strength and quality SARS-CoV-2-specific CTL responses.^{51,52} These homology data are also in line with the frequent detection of SARS-CoV-2-specific T cells in virus-unexposed individuals.^{37,39,53–57} This potential heterologous priming, however, is not unique to the enhancing peptides, as comparable numbers of unrelated homologous sequences sharing similar levels of AA identity with SARS-CoV-2 epitopes were retrieved by a BLASTP search performed using the inhibitory peptides as queries (data not shown). Furthermore, the enhancing effect per se will necessarily result in a better CD8 T cell reactivity, which would thwart variant virus selection.

The interpretation we favor is that selection of such variant enhancing epitopes can occur through a two-step mechanism. As well documented by available studies,^{14–19} VOC and VOI mutations can favor virus transmissibility by causing specific spike structural/functional alterations or abrogating antibody neutralization. This would allow the virus to gain a selective advantage of general relevance for the overall human population and would thus represent the primary driver of variant virus selection and spreading, regardless of the specific host genetic background and individual CD8 T cell reactivity. If mutations fortuitously fall within CD8 epitopes, only upon infection of individuals carrying the appropriate HLA restriction element for peptide presentation and a narrow CD8 T cell repertoire of SARS-CoV-2 specificities mutations would affect CD8 T cell reactivity. The random positioning of the AA changes within the epitope sequence, and the local chemical modifications imposed by the variant AA would thus determine the positive or negative impact on HLA and/or TCR binding. The possibility that individual AA changes can exert multiple and seemingly divergent effects, with a possible concomitant impact on both antigenicity and cell tropism has previously been reported in other virus infections.⁵⁸

In conclusion, these enhancing mutations might be poorly relevant to the overall antiviral protection in the presence of widely multi-specific CD8 T cell responses, but might bear pathogenetic relevance contributing to improve a weak antiviral protection of individuals with more narrowly focused responses, as we observed in some vaccinated subjects. The potential relevance of our study is the elucidation of an unusual and unexpected mechanism of host/virus interaction that is apparently counterintuitive and thus so far underestimated. In general, virus mutations can allow virus escape from immune surveillance by hiding specific epitopes to antibody neutralization or by abrogating CD8 recognition of infected cells. In addition, however, our data show that mutations can also provide in some specific settings an advantage to the host by improving antiviral CD8-mediated reactivity. Thus, characterization of this mechanism allows us to clarify another unexpected facet of the complex interplay between host immune system and virus with possible implications for the outcome of infection and the development of new generation, more potent COVID-19 vaccines.

Limitation of the study

There are limitations in this study;

- first, the HLA restriction elements for each immunogenic peptide have not been identified and a more detailed characterization of HLA/TCR binding should be performed to elucidate whether mutated peptides with enhanced T cell stimulatory properties can be relevant with respect to next-generation COVID-19 vaccines;
- second, a careful analysis of the intracellular generation of mutated peptides from endogenously synthesized antigen processing would strengthen the pathogenetic relevance of our findings.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.isci.2023.106940>.

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AUTHOR CONTRIBUTIONS

C.T., A.V., M.R.: execution of experiments, acquisition of data, statistical analysis, analysis and interpretation of data. D.C., A.B.: contribution to figure drawing, analysis and interpretation of data, and contribution to the selection of the references to quote. D.L.: administrative support. L.S., F.B., T.M., A.T., A.N.: recruitment and characterization of the patients. G.D.: neutralization assay. P.Z., M.B., S.G.: HLA typing analysis. S.D., A.P., P.F., I.M., C.C.B., V.R., A.M., S.U.: execution of experiments and interpretation of data. G.P.: statistical analysis. G.M., A.M., D.C., S.O.: critical revision of the manuscript. C.F., C.B.: study concept and design, critical revision and editing of the manuscript, obtained funding, study supervision and interpretation of data. All authors contributed to the article and approved the submitted version.

DECLARATION OF INTERESTS

A.T. and D.C. are employees of Vir Biotechnology Inc. and may hold shares in Vir Biotechnology Inc. C.F.: Grant: Gilead, Abbvie. Consultant: Gilead, Abbvie, Vir Biotechnology Inc, Arrowhead, Transgene, BMS. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
APC-H7 anti-human CD3	BD Biosciences, NJ, USA	Cat#560176; RRID: AB_1645475
PE anti-human CD4	BD Biosciences, NJ, USA	Cat#555347; RRID: AB_395752
BV786 anti-human CD8	BD Biosciences, NJ, USA	Cat#563823; RRID: AB_2687487
APC-R700 anti-human IFN- γ	BD Biosciences, NJ, USA	Cat#564981; RRID: AB_2739031
BV650 anti-human TNF- α	Miltenyi	Cat#563418; RRID: AB_2738194
BB700 anti-human CD107a	BD Biosciences, NJ, USA	Cat#566558; RRID: AB_2869782
Chemicals, peptides, and recombinant proteins		
10-mer synthesized peptides	Mimotopes Pty Ltd, AUS	N/A
15-mer SARS-CoV-2 overlapping Spike	Genscript	N/A
GolgiPlug Protein Transport Inhibitor (Containing Brefeldin A)	BD Biosciences, NJ, USA	Cat#555029
GolgiStop Protein Transport Inhibitor (Containing Monensin)	BD Biosciences, NJ, USA	Cat#554724
Lymphocyte separation medium	Biowest, Nuaille, FR	Cat#L0560-500
Critical commercial assays		
Human IFN- γ /TNF- α /IL-2 Three-Color FluoroSPOT	Cellular Technology Limited (CTL), DE	Cat#HT3001F-1M/5
LIVE/DEAD Fixable Viability Stain 575V	BD Biosciences, NJ, USA	Cat#565694
FIX&PERM® Cell Fixation and Permeabilization Kit	Nordic MUBio	Cat#GAS-002
Software and algorithms		
Flowjo 10.8.1	BD Biosciences	N/A
PRIME 2.0	N/A	N/A
IEDB	https://www.iedb.org/	N/A
ImmunoSpot® 7 Software	Cellular Technology Limited (CTL), DE	N/A
Blastp	https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins	N/A
GraphPad prism 7	GraphPad	N/A
Other		
LSR Fortessa	BD Biosciences	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Carolina Boni (cboni@ao.pr.it).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All data reported in this paper will be shared by the [lead contact](#) upon request.
- This paper does not report original code.

- Any additional information required to reanalyze the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND PARTICIPANT DETAILS

Study participants

36 health care workers (HCW) were enrolled into a vaccination study at the Unit of Infectious Diseases and Hepatology of the Parma University Hospital, Italy. PBMCs and serum samples from participants were collected 1–2 months after the second dose of Pfizer/BioNTech BNT162b2 vaccination. At the same time point of sample collection, Nucleocapsid (N) immunoglobulin G (IgG) was tested by CMIA (chemiluminescent microparticle immunoassay; Architect-Abbot) to exclude previous SARS-CoV-2 infection (1 positive donor out of 36). 12 patients were also recruited from April to October 2020 after hospitalization for SARS-CoV-2 infection, as indicated by positive RT-PCR. Samples from convalescents were obtained 1–2 months after disease onset. The study was approved by the competent local Ethic Committee and all patients provided written, informed consent. Details on the vaccinated and convalescent donors are reported in [Tables 1](#) and [S3](#).

Sequence analysis and peptide design

Genome sequences derived from the different VoCs and Vols were compared with the original Wuhan SARS-CoV-2 strain by the GISAID data base in order to map all spike amino acid substitutions and deletions associated with the main Variants of Concern and Variants of Interest, including the UK (Alpha), South-African (Beta), Brazilian (Gamma), Californian (Epsilon), Indian (Delta) VoCs, as well as Nigerian (Eta) Vols (GISAID data base - <https://cov.lanl.gov/content/index>) ([Table S1](#)).

Next, we designed a set of peptides of optimal length for cytotoxic T cell recognition, i.e. 10 AA long, overlapping by nine residues, with variable numbers of flanking AA at both sides of the mutations (Mimotopes Pty Ltd, Australia). For each relevant mutation we synthesized 2 pools of 10 peptides (a wild type and a mutant pool, except for MIX 1 consisting of 18 peptides, and for MIX 3 and 9 consisting of 11 and 14 peptides, respectively), containing individual mutations at each position of the peptides, for a total of 28 couples of pools ([Tables S1](#) and [S2](#)). Our study was already in progress when the delta and omicron variants emerged; thus, most delta and omicron mutations were not included, with the exception of those shared with other variants.

To investigate the breadth of the spike-specific T cell response elicited by vaccination, PBMCs were also stimulated with 15-mer peptides overlapping by 10 amino acids spanning the overall Wuhan spike sequence and consisting of 7 pools, each composed of about 40 peptides ([Table S5](#)).

Finally, to characterize further strength and multispecificity of spike-specific CD8 T cell responses primed by vaccination, we used an additional set of 96 peptides, previously described in the literature as Spike-specific CD8 T cell epitopes, varying in length from 9 to 13 AA ([Table S6](#)). A proportion of these peptides are located within conserved spike regions, as defined by Shannon entropy (SE), which is a measure of variability of genetic mutations for each amino acid position, calculated on a multiple alignment of spike protein sequences. Decreasing entropy thresholds values of 0.0025, 0.001 and 0.0005 were defined by preliminary analysis of 1,400,000 million spike protein sequences retrieved (as of June 1st 2021) from the daily updated GISAID database.³⁶ Three levels of amino acid (AA) residue conservation were then arbitrarily defined, allowing us to distinguish ‘conserved’, ‘highly conserved’ and ‘hyper-conserved’ spike regions ([Table S6](#)) and to map each epitope to variably constrained portions of the spike protein. Based on this analysis 25 peptides are located within conserved, 8 within highly conserved and 4 within hyper-conserved Spike regions.

HLA-typing analysis

High resolution HLA typing of all samples was performed using different NGS kits. All samples were genotyped for 11 HLA loci, namely HLA-A, -B, -C, -DRB1, -DRB3, -DRB4, and -DRB5, -DQA1, -DQB1, -DPA1, -DPB1. Pooled libraries were loaded on a iSeq 100 Reagent v2 (cartridge and flow cell, Illumina). Paired-end sequencing was performed on the iSeq100 next-generation sequencing (NGS) platform (Illumina), 151 cycles in each direction. HLA typing analyses were made using different HLA typing software packages

(AlloSeq™ Assign®, CareDx; TypeStream Visual, One Lambda; NGSengine®, GenDx) along with the current version of the IPD-IMGT/ HLA database.

SARS-CoV-2 pseudoviruses generation and neutralization assay against the original viral strain and variants

Lentiviral vector-based SARS-CoV-2 S pseudovirus was generated as previously described with minor modifications.⁵⁹ Sub confluent HEK 293T cells were cotransfected with pLV-EF1 α -(turboGFP-Luc2)-WPRE transfer vector, p8.74 packaging vector, pseudotyping vector coding for Spike glycoproteins (Wuhan-Hu-1; B.1 Lineage, China) and pREV with PEI (Polysciences, Inc., Warrington, PA, USA) (1 mg/mL in PBS) (ratio 1:2.5 DNA/PEI). Transfected cells were incubated for 48 h at 37°C and 5% CO₂. The flask was then frozen–thawed at –80°C; transfected cell supernatant (TCS) containing S pseudovirus was clarified via centrifugation, filtered through a 0.45 μ m filter (Millipore, Merck, Darmstadt, Germany), aliquoted, tittered by limited dilution and stored at –80°C. Serum neutralization assay was performed as previously described. S pseudovirus preparation diluted in complete EMEM with 10% FBS was added to each well containing the diluted sera and left to incubate at room temperature for 1.5 h. Next, 104 HEK/ACE2/TMPRSS2/Puro cells, were added to each well and left for 60 h at 37°C and 5% CO₂. Luciferin was added to each well just before the reading of the microplate with the luminometer (Victor, Perkin Elmer). Neutralization titer 50 (NT50/ml) was expressed as the maximal dilution of the sera where the reduction of the signal is $\geq$ 50%. Each serum was tested in triplicate.

Peripheral blood mononuclear cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation and cryopreserved in liquid nitrogen until the day of analysis.

FluoroSPOT assay

IFN- γ /TNF- α /IL-2 three-colour FluoroSPOT assay was performed using a panel of peptides (10-mers overlapping by 9 aa) pooled in 56 mixtures containing most of the VOC and VOI mutations within the spike region (Table S2). The day before the assay, the plates were activated by adding 70% ethanol followed by overnight incubation with IFN- γ /TNF- α /IL-2 capture antibodies. After decanting the plate, 2.4×10^5 PBMCs per well were seeded in duplicate in CTL Medium and Spike-specific T-cell responses were analyzed after overnight incubation with individual peptide mixtures (1 μ M) as IFN- γ /TNF- α /IL-2 production according to the manufacturer's instruction (CTL, Europe, Germany). Spots were counted using an automated reader system (ImmunoSpot Ultimate UV Image analyzer, CTL Europe, Germany). Cytokine-secreting cells were expressed as spot forming cells (SFC) per 1×10^6 cells after subtraction of the background (median background value with 25th–75th percentile range for IFN- γ , TNF- α and IL-2 equal to 12 [3–45], 96 [39–336], 51 [28–82], respectively). Positive controls consisted of PBMCs stimulated with CMV, EBV and influenza peptides. FluoroSPOT was considered positive if the number of spots in the stimulated wells was at least 3 standard deviations above background and the difference between the number of spots in the stimulated and unstimulated wells was above 10.

Flow cytometry analysis

PBMC or expanded T cell lines were stimulated for eighteen hours at 37°C with or without SARS-CoV-2 peptide pools (1 μ M) as mentioned above in the presence of Brefeldin A (GolgiPlug 1 μ g/mL) and Monensin (GolgiStop 0.5 μ g/mL). Cells were washed and surface-stained with Live/Dead fixable dead cell stain kit and anti-CD3, anti-CD4, anti-CD8 (all from BD Biosciences) fluorochrome-conjugated antibodies. PBMC were then fixed, permeabilized using the Fix and Perm kit (Nordic MUBio) and stained with anti-IFN- γ (BD Biosciences) and anti-TNF- α (Miltenyi) conjugated mAbs for the detection of intracellular cytokines, and using an anti-CD107a antibody (BD Biosciences) for the study of the cytotoxic potential. Samples were acquired on a BD LSR Fortessa and analyzed with the FlowJo software (BD Biosciences).

Expanded T cell lines

PBMC were stimulated with selected SARS-CoV-2 peptide pools (1 μ M) at 37°C in RPMI medium supplemented with 8% human serum and cultured for 7–9 days in the presence of 50 UI/ml of recombinant IL-2 (R&D System).

Homology analysis of variant SARS-CoV-2 peptide with unrelated human pathogen sequences

The search for protein sequences of other organisms containing fragments identical or similar to the SARS-CoV-2 variant enhancing peptides was performed as follows. For every peptide used as a query, Blastp analysis (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>) within the non-redundant protein sequences (nr) database, excluding the organism "Severe acute respiratory syndrome coronavirus 2 (taxid:2697049)" and choosing PAM30 matrix plus other default parameters, allowed the isolation of 1000 sequences. Among them, a selection was done using the following criteria: i) sequences still correlated with human respiratory syndrome coronavirus 2 or other animal coronavirus were eliminated; ii) after a multiple alignment of all identified peptides with the corresponding query, using ClustalW program, the peptides with an inserted gap bigger than one amino acid were excluded, as well as all peptides with less than 8 amino acid residues identity; iii) identical peptides derived from the same protein sequences belonging to the same family of micro-organisms were grouped when repeatedly reported in order to consider only a single peptide. The remaining protein sequences were further subjected to selection in order to consider only those corresponding to microorganisms that may have come into contact with, or infected, humans and therefore be capable to trigger an immune response.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed by GraphPad Prism (GraphPad Software, La Jolla, CA). Statistical significance was assessed by the Mann-Whitney U test for non-paired samples and the Wilcoxon signed rank test for paired data. Differences between multiple patient groups were evaluated by the non-parametric one-way ANOVA test corrected for pairwise multiple comparisons. Frequencies were compared by Chi Square with Yates' correction.

To establish relevant differences between T cell responses stimulated by wild type and mutated peptides we applied an *Effect size* calculation.⁶⁰ Fold changes can be assimilated to Risk Ratios (RR) for which their use as "effect size" measures has been suggested in the literature. Effect size reveals how relevant is the relationship between variables or the difference between groups. Measures of effect size are usually divided into "small effect", "medium effect" and "large effect". A large effect size means that a research finding has practical significance, while a small effect size indicates limited practical implications. For our data we chose the "large effect" threshold (RR = 3.0), as the discriminant for the most important values. Taking the logarithm of this value, we defined the corresponding thresholds in the log scale (0.48). Specifically, for each T cell response stimulated by a mutant pool, we calculated the fold-change relative to the ancestral pool (mutated/wild-type). Only T cell responses below or above the "Large Effect size" values were considered significantly decreased or increased (<-0.48 or $>+0.48$).

PRIME 2.0 and IEDB prediction tools were used to predict HLA affinity, MHC binding and immunogenicity of wild type and mutated spike peptides positively modulating CD8 T responses.⁶¹ The PRIME 2.0 immunogenicity predictor tool combines peptide TCR recognition propensity with predicted affinity to HLA-class-I molecules. The lower the percentile rank score PRIME value, the higher the peptide expected ability to elicit CD8 T cell recognition. Prime % score values lower than 0.5% threshold represent good HLA-I binding candidates. The IEDB Class I Immunogenicity tool (<https://www.iedb.org/>) was employed to predict the immunogenicity score and the binding affinity (IC₅₀) of peptides for HLA class I molecules. A higher immunogenicity score indicates a greater probability of eliciting an immune response. The HLA-I binding tool predicted output is IC₅₀nM.