Mutations of SARS-CoV-2 variants of concern escaping Spike-specific T cells

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Abstract

The amino acid (AA) mutations that characterise the different variants of concern (VOCs), which replaced the ancestral SARS-CoV-2 Wuhan-Hu-1 isolate worldwide, provide biological advantages such as increased infectivity and partial escape from humoral immunity. Here we analysed the impact of these mutations on vaccination- and infection-induced Spike-specific T cells. We confirmed that, in the majority of infected or vaccinated individuals, different mutations present in a single VOC (Delta) or a combined mosaic of more than 30 AA substitutions and deletions found in Alpha, Beta, Gamma, Delta and Omicron VOCs cause modest alteration in the global Spike-specific T cell response. However, distinct numerically dominant Spike-specific CD4 and CD8 T cells preferentially targeted regions affected by AA mutations and do not recognise the mutated peptides. Importantly, some of these mutations, such as N501Y (present in Alpha, Beta, Gamma, and Omicron) and L452R (present in Delta), known to provide biological advantage to SARS-CoV-2 in terms of infectivity also abolished CD8 T cell recognition.

Taken together, our data show that while global mRNA vaccine- and infection-induced Spike-specific T cells largely tolerate the diverse mutations present in VOCs, single Spike-specific T cells might contribute to the natural selection of SARS-CoV-2 variants.

Living organisms, including viruses, constantly evolve to adapt to their environment. They acquire random mutations during replication and deleterious or neutral mutations are purged. Mutations that aid to their spread and persistence¹ and provide a selective advantage become dominant^{2,3}.

The current SARS-CoV-2 pandemic illustrates this phenomenon: after an initial year of relative evolutionary stasis, variants have emerged to replace the initial SARS-CoV-2 Wuhan-Hu-1 strain^{2,3}. In July 2021 SARS-CoV-2 variants of concern (VOCs) declared by the World Health Organization comprised B.1.1.7-Alpha, B.1.351-Beta, P.1-Gamma, B.1.617.2-Delta³. From end-November 2021, a new VOC called Omicron was declared and has spread rapidly worldwide⁴.

The selection of these variants likely occurs due to the combined effect of immunological pressure and acquisition of advantages in transmissibility and fitness^{1,5}. Amino acid (AA) variations occur throughout the entire genome^{2,3}, however, the most-studied mutations are in the Spike protein^{6–11}. Some mutations increase binding to the ACE-2 receptor^{12–14}, while others reduce antibody neutralization^{6,9,11,15}.

In contrast, the role of SARS-CoV-2-specific T cells in selection pressure has been largely discounted, despite scattered observations that mutations can affect T cell recognition^{9,12,16,17}. The main argument is that T cells recognise various epitopes in vaccinated and convalescent individuals^{7,18,19}. As such, VOC-mutations are unlikely to alter all of them^{7,10}.

This argument is supported by recent reports documenting the ability of Spike-specific T cells to tolerate the high number of mutations present in Omicron^{20–26}, and likely the immunological basis of why vaccinations are still highly effective against VOCs in reducing severe COVID-19²⁷.

However, the role for multi-specific antiviral immunity is not involvement in the selection process of VOCs should not be entirely discounted. Both, humoral and cellular immunity are multi-specific^{28,29} and while most VOCs fully escape recognition of monoclonal antibodies^{6,30}, their ability to escape serum neutralisation from convalescent or vaccinated individuals was less dramatic^{6,15} before the surge of Omicron. In addition, a hierarchy of antiviral efficacy exists within the polyclonal cellular immune response²⁹, and thus some mutations might escape the dominant antiviral T cells, similarly to what has been observed for polyclonal antibodies¹⁵.

Results

Breadth of the Spike-specific T cell response

To understand whether Spike-specific T cells uniformly recognise different Spike regions²⁹, we designed seven pools of 33-39 overlapping 15-mer peptides covering 180-200 AA long regions (Fig S1A, Table S1). Peptide-reactive cells were quantified by IFN- γ ELISpot ex vivo in 35 vaccinated and 31 convalescents individuals. The mean quantity was different (Fig. S1B, S1C), likely reflecting the time of measurement since T cell induction (3 versus 12 months, respectively). Yet, important commonalities were detected. First, as already described³¹, we found significant heterogeneity in the quantity of Spike-specific T cells (Fig. S1B, S1C). Second, most of the individuals exhibited T cells recognising all seven distinct peptide pools (Fig. 1A), in line with the reported T cell multi-specificity^{7,18,19}. However, a dominant T cell response towards a single peptide pool was frequently observed. In 8/35 vaccinated and 9/31 convalescents, with T cells specific for a single pool exceeding 40% of the total Spike-specific T cell response (Fig. 1B). The Spike region 886-1085 was the most immunogenic in both vaccinated (65%) and convalescents (34%) (Fig. 1C). This region is fairly conserved among different VOCs, but includes the D950N, S982A and T1027I mutations in the Delta, Alpha and Gamma VOCs, respectively, and the mutations S954H, S969K, S981F in Omicron. In 16% of vaccinated and 19% of convalescents, a dominant T cell response was observed towards the region 336-510 (Fig. 1C), containing several VOC mutations that affect the receptor binding affinity (N501Y^{13,14}, L452R¹²), antibody (K417³², T478K¹⁵, E484K¹⁵) and T cells (L452R¹²) recognitions.

Impact of VOC mutations on global Spike-specific T cells

Next, we tested the effect of the mutations on Spike-specific T cells. First, we aimed to understand in 100 individuals the impact of Delta mutations on Spike-specific T cells induced by BNT162b2 vaccine. We stimulated whole blood with three peptide pools covering the whole Spike protein (253 peptides) and the regions mutated in Delta (24 peptides) with and without the AA-substitutions/deletions (Fig 2A, Table S2).

We measured the magnitude of the Spike-specific T cell response, its proportion targeting peptides affected by AA changes and calculated the reduction when stimulated with peptides containing Delta-specific mutations (Fig. 2B), which was on average 3.9% (Fig. 2C). The Spike-specific T cell response was not affected in 46%

of the vaccinated individuals; 45% showed less than 10% reduction and only in 3% did we observe more than 20% reduction in Spike-specific T cells (Fig. 2D). Of note, we confirmed in this large population that there is vast heterogeneity of the Spike-specific T cell response in different individuals (Fig. S2).

Second, we defined the combined impact of the mutations that are characteristic of the Alpha, Beta, Gamma and Delta VOCs on Spike-specific T cells in 33 vaccinated and 29 convalescents (Fig. 2E, Table S2). Note, 10 of these mutations are also characteristic of the newly emergent Omicron VOC⁴ (Table S3). The combined effect of 30 AA mutations was tested with IFN- γ ELISpot assays.

This combination of 30 AA mutations reduced the T cell response on average by 11.9% (vaccinated, Fig 2F) and 15.6% (convalescent, Fig 2G), a greater reduction than that detected for the Delta mutations alone (3.9%). Spike-specific T cells were not affected in 7/33 (21%) mRNA vaccinated (Fig. 2H) and 5/29 (17%) convalescent individuals (Fig. 2I). Less than 10% reduction was observed in 39% of vaccinated and 24% of convalescents. Only in 1 individual of both groups did the combined VOC mutations reduce the Spike-specific T cells by 50%.

Definition of single-peptide specificities of dominant and subdominant Spike-specific T cells

Next, we characterized epitope-specificity and CD4/CD8 phenotype of vaccine- and infection-induced T cells. We utilized an unbiased approach, based on PBMC stimulation with a single peptide pool covering whole Spike and expansion of specific T cells. The T cell lines were then used to confirm the single-peptide specificity, define the phenotype of the responsive T cell (CD4/CD8) and, in selected cases, their HLA-Class I restriction (Fig. S3-5). This approach allowed us to define the dominant T cell specificities, irrespective of the HLA-Class I and Class II profile of the tested individuals.

First, we showed that the expansion procedure preserved the overall hierarchy of Spike-specific T cell recognition detected ex vivo in most of the tested individuals and it was remarkably stable across different time points (Fig. 3SB).

Subsequently, we defined the single peptide specificity and the CD4/CD8 phenotype of T cells in nine vaccinated (Fig. S3C) and 11 convalescents (Fig. S4). Eighteen distinctive Spike-specific CD4 epitopes and 17 different CD8 epitopes were characterized (Fig. S3C, S4; Table1). HLA-Class I restriction of 6 distinct CD8 T cell

lines was identified utilizing EBV-B cell lines with shared/non-shared HLA-Class I molecules (Fig. S5).

Surprisingly, among the defined peptides containing epitopes targeted by Spike-specific T cells, 12/18 CD4 T cells and 10/17 CD8 T cells recognized peptides that contain 30 out of the 55 distinct AA mutations characteristic of the five VOCs (Table 1, Table S2).

Mutations affecting Spike-specific T cells

The impact of the AA mutations affecting the peptide-specific T cells was analysed both directly ex vivo (Fig. 3A) and in T cell lines (Fig. S6). We tested 6 CD8 and 5 CD4 T cell specificities containing AA mutations. PBMCs of the individuals in whom the T cell specificities were defined were stimulated in parallel with peptides containing either the wildtype or the mutated AA sequence.

All AA mutations affected the T cell recognition with the partial exception of mutation T1027I present in S1016-30. This mutation altered T cell recognition in one out of two individuals tested. HLA-restriction (Fig. S5) and visualization with HLA-pentamer on the individual who tolerated T1027I (Fig. S7) showed that the T cells were B40-restricted and recognized the epitope S1016-1024, which lay outside of the mutations. The ability of the other AA mutations to inhibit T cell recognition strongly suggest that they were located within the T cell epitopes. Mutations affected both CD4 (i.e. S76-90, S206-25, S236-50) and CD8 T cell responses (i.e. S411-25, S446-60, S491-510). Of note, peptide S26-40 stimulated a CD4 and a CD8 T cell response in two different convalescents, suggesting this peptide contains two distinct epitopes. P26S strongly inhibited both CD4 and CD8 T cells.

Of particular interest, we demonstrated that HLA-A*02:05-restricted CD8 T cells specific for the peptide 411-25 were completely inhibited by Beta and Omicron mutation K417N and by Gamma mutation K417T. Moreover, we observed that the mutations L452R and N501Y, which have negligible effect on antibody recognition^{33,34} but increase the infectivity of the Delta¹² and Beta/Gamma^{13,14} VOCs respectively, clearly inhibited recognition of CD8 T cells specific for the HLA-A*24:02 epitope GNYNYLYRLF and HLA-B*15:27 epitope FQPTNGVGY.

Impact of L452R and N501Y on Spike-specific T cells

The observation that L452R and N501Y, present in Delta (L452R), Alpha, Beta, Gamma and Omicron (N501Y), respectively, increase infectivity¹² and concomitantly abolish CD8 T cell recognition prompted us to analyse these CD8 T cells in more detail.

The region S446-60 contains the HLA-A*24:02 epitope 448-56 (NYNYLYRLF), has been shown by Motozono et al¹² to induce a dominant CD8 T cell response in convalescents and to be inhibited by L452R. Here we demonstrated that S448-56-specific CD8 T cells are also elicited in A*24:02+ vaccinated individuals and we confirmed the HLA-A*24:02 restriction (Fig. S5).

We then tested the ability of L452R to inhibit T cell activation in comparison with other reported AA mutations that affect the Spike region 446-60, namely L452M, Y453F, L455F and G446V (Fig. 3B,C). The mutation Y453F was characteristic of the SARS-CoV-2 strain infecting minks³⁵, while L452M, Y453F, L455F and G446V, have been occasionally detected³⁶. CD8 T cell lines specific for Spike 446-60 were generated in three different HLA-A*24:02+ individuals and we tested the impact of the 5 distinctive AA substitutions. L452R abolished the CD8 T cell recognition almost completely in all the three individuals, followed by L455F, while the other AA substitutions (G446V, L452M, Y453F) were better tolerated (Fig. 3B,C).

To characterize the HLA-B*15:27 restricted CD8 T cell response to the Spike region containing the mutation N501Y, we engineered T cell receptor (TCR)-redirected T cells. The alpha and beta TCR chains of T cells activated by peptide 491-505 were sequenced and cloned into a pVAX1 vector, which allowed the expression of the introduced TCR in allogenic PBMC (Fig 3D). The TCR-redirected T cells were used to define the short epitope (FQPTNGVGY; Fig. 3E) and we confirmed the ability of N501Y to completely abolish CD8 T cell recognition (Fig. 3F). Titration of the peptide concentration used for T cell activation demonstrated the high affinity of the TCR, a concentration as low as 0.1 ng/ml induced a response which plateaued at 1 µg/ml (Fig. 3G).

Discussion

We designed experiments to address two questions: whether VOCs can escape the global Spike-specific T cell response induced by infection or vaccination and whether T cells can play a role in VOCs selection.

We confirmed the marked multi-specificity of Spike-specific T cells. Although we observed a hierarchy among the T cells recognizing different Spike regions, the dominant Spike-specific T cells rarely occupied more than 40% of the repertoire. Furthermore, the region 886-1085 that is preferentially targeted by T cells contains few mutations present in Alpha (S982A), Gamma (T1027) and Delta (D950N)¹⁵ and also in the newly emerged Omicron VOC (S954H, S969K, S981F)⁴.

The broad multi-specificity translated into the functional ability to largely tolerate AA substitution present in different VOCs. This was first observed in a large population of mRNA vaccinated individuals (n=100). In 91% of them, the effect of mutations present in Delta, inhibited Spike-specific T cells by less than 10%. Second, when we tested the combined effect of 30 distinct AA substitutions/deletions (mutations found in Alpha, Beta, Gamma and Delta VOCs, of which 10 have also been detected in Omicron), we found that convalescent or vaccinated individuals of Asian origin possess Spike-specific T cells that largely tolerate the combined AA substitutions. These data agree with the recent reports of the substantial, although not absolute, preservation of global Spike-specific T cell response against the highly mutated Omicron^{20–26}.

Our data however reveals novel important features of the Spike-specific cellular immunity. In addition to the detection of a broad heterogeneity in the magnitude of the T cell response, we demonstrated that 12 distinct AA substitutions of VOCs alter 11 individual T cell specificities characterized in a relatively small number of vaccinated and convalescent individuals (n=20). VOC mutations affect the activation of CD8 and CD4 T cells and mutations already known to abrogate antibody recognition like K417N³⁴ (present in Beta and Omicron VOC) or K417T and D138Y (present in Gamma VOC)⁸ also inhibit Spike-specific CD8 T cells.

Remarkably, we observed that mutations L452R (present in Delta) and N501Y (present in Alpha, Beta Gamma and Omicron), which have negligible effect on the neutralization ability of polyclonal sera^{33,34}, but increase the binding affinity of Spike protein to the ACE2 receptor^{12–14}, abolish the recognition of CD8 T cells specific for

one HLA-A*24:02 (S447-56) and one HLA-B*15:27 (S497-505)-restricted epitope. This convergence of biological effects is reminiscent of the hypothesis of the causes of influenza hemagglutinin variant selection. In animal models of Influenza, antibodies select viruses with mutations that provide a generalized advantage by increasing receptor avidity⁵.

In general, immune escape mutations affecting T cell epitopes are selected in individuals with chronic viral infections (i.e. HIV, HBV, HCV, HDV, HCMV, EBV)³⁷ but are unlikely to uniformly affect the wider human population. Since the biology of T cell recognition makes the T cell repertoire largely unique for each individual²⁹, it is unlikely that a virus variant with a single set of immune escape mutations will affect the whole human population identically and successfully spread globally. However, if mutations permit escape from a specific T cell specificity in parallel with increasing the receptor binding affinity of Spike, as in the case of L452R and N501Y, such advantage will no longer be solely restricted to a selected population. Interestingly, recent mathematical models of SARS-CoV-2 variant spread suggested that mutations able to concurrently increase infectivity and immune escape are likely to rapidly propagate in the population³⁸.

Our data do not provide the demonstration that this chain of events took place during this pandemic. Nevertheless, they indicate an alternative possibility to the prevalent theory that postulates that VOCs emerged exclusively under the pressure of neutralizing antibodies.

We made the unexpected observation that a large number of T cells (20 out of 35) selected in vitro by PBMC stimulation with whole Spike peptide pool recognized peptides carrying the VOC mutated regions of Spike. This is at odds with ex-vivo results obtained by us and others, since T cells specific for variant regions represent a minority of the global Spike T cell repertoire in the ex vivo analysis^{7,18–26}. One might speculate that the in vitro expansion mimics the immunological events occurring after SARS-CoV-2 infection and, as such, the analysis of T cells after in vitro expansion selects for the dominant effector T cell response present during the acute phase of response. These dominant T cells might thus exert higher selective pressure. Future studies will be necessary to verify the robustness of the observation and its real mechanisms. However, by determining HLA-restrictions of a number of these T cell

epitopes targeting mutated regions (at least for HLA-Class I), we provide the possibility to test whether, for example, breakthrough infection might occur more frequently in individuals with such HLA-Class I profiles. Finally, the demonstration that AA mutations escaping specific CD8 T cells and concomitantly offer the virus a biological advantage in term of increased infectivity¹⁴, provides the theoretical possibility that T cell pressure might contribute to the selection of VOCs.

There are some limitations to this study. We tested the impact of AA substitutions on Spike-specific T cells utilizing peptide-pulsed target cells and not infected cells. This method might overestimate the quantity of T cells specific for SARS-CoV-2 because low affinity T cells might have been quantified and be more sensitive to AA mutations. On the other hand, peptide-pulsed target cells cannot be used to evaluate the impact that AA substitutions might have on the processing of T cell epitopes³⁷. AA outside the T cell epitopes can alter their generation³⁹ and as such we might have underestimate the impact of the mutations on the Spike-specific cellular immunity.

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Table 1: Details of identified Spike-specific T cell specificities

T cell	Sample	Peptides	Sequence	VOC Mutations	Dominant	HLA
CD8	Vaccine recipients	81-95	NPVLPFNDGVYFAST	T95I	yes	B*35:01
		131-145	CEPQFCNDPFLGVY	D138Y, G142D Δ144, Δ143-5		
		266-280	YVGYLQPRFTLLKYN		yes	A*02:01
		346-360	RFASVYAWNRRKRISN			
		360-375	KRISNCVADYSVLYN	S371L		
		375-390	SASFSTFKCYGVSP			
		411-425	APGQTGKIADYNYKL	K417T, K417N	yes	A*02:05
		446-460	GGNYNYLYRLFRKSN	L452R	yes	A*24:02
		491-505	PLQSYGFGPTNGVGY	Q493R, G496S, Q498R, N501Y, Y505H	yes	B*15:27
		641-655	NVFQTRAGCLIGAEH	H655Y		
	Convalescents	681-695	PRRARSVASQSIIAY	P681H, P681R		
		1016-1030	AEIRASANLAATKMS	T1027I	yes	B*40:01
		1051-1065	SFPQSAPHGVVFLHV			B*54:01
		26-40	PAYTNSFTRGVYYP	P26S	yes	
		281-295	ENGITDAVDCALDP		yes	
		421-435	YNYKLDDFTGVIA		yes	
		446-460	GGNYNYLYRLFRKSN	L452R	yes	A*24:02
		1001-1015	LQSLQTYVTQQLIRA			
		1051-1065	SFPQSAPHGVVFLHV		yes	B*54:01
		211-230	NLVRDLPPQGFSALEPLVDLP	N211I, Δ221, EPE214, D215G	yes	
CD4	Vaccine recipients	236-250	TRFQTLALHRSYLT	Δ241-3		
		416-440	GKIADYNYKLDDFTGCVIWNNSN	K417N, K417T		
		446-465	GGNYNYLYRLFRKSNLKPFE	L452R		
		556-570	NKKFLPFQFGRDIA	A570D	yes	
		896-920	IPFAMQMAYRFNGIGVTQNVLYENQ		yes	
		931-950	IGKIQDSLSTASALGKLQD	D950N		
		976-1000	VLNDILSRDLKVEAEVQIDRLITGR	L981F, S982A		
		1006-1020	LQSLQTYVTQQLIRAAEIRA			
		1051-1075	SFPQSAPHGVVFLHVTYVPAQEKNF			
		1095-1120	VSNQTHWFVTQRNFYEPQIITDNT	D1118H	yes	
	Convalescents	26-40	PAYTNSFTRGVYYP	P26S		
		76-90	TKRFDNPVLPFNDGV	D80A	yes	
		201-220	FKIYSKHTPI NLVRDLPPQG	N211I, Δ221, EPE214, D215G		
		311-325	GIYQTSNFRVQPTES		yes	
		411-430	APGQTGKIADYNYKLDDFT	K417N, K417T		
		446-460	GGNYNYLYRLFRKSN	L452R		
		556-570	NKKFLPFQFGRDIA	A570D		
		921-940	KLIANQFNSAIGKIQDSLSS		yes	
		931-945	IGKIQDSLSTASAL			
		956-970	AQALNTLVKQLSSNF			
		1016-1030	AEIRASANLAATKMS	T1027I		

Methods:

Ethics statement: All donors provided written consent. The study was conducted in accordance with the Declaration of Helsinki and approved by the NUS institutional review board (H-20-006) and the SingHealth Centralised Institutional Review Board (reference CIRB/F/2018/2387).

Human samples: Donors were recruited based on their clinical history of SARS-CoV-2 infection and their vaccination status. Blood samples of recovered COVID-19 patients (n=35) were obtained 6-12 months post PCR negativity. Blood samples of two vaccinated cohorts were taken, 35 donors were recruited at multiple timepoints until 3 months post second dose BNT162b, and additional 100 donors at 9 months post second dose BNT162b. Vaccinated individuals were all healthy adults, 20-65 years old, of Asian origin.

PBMC isolation: Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation using Ficoll-Paque. Isolated PBMC were either studied directly or cryopreserved and stored in liquid nitrogen until used in the assays.

Peptide pools: 15-mer peptides overlapping by 10 amino acids spanning the entire protein sequence of SARS-CoV-2 Spike were synthesized (GenScript; see Table S1). To stimulate whole blood or PBMC, the peptides were divided into 7 pools of about 40 peptides. For single peptide identification, peptides were organized in a matrix of 16 numeric and 16 alphabetic pools. Peptides with and without VOC mutations were mixed into two separate pools (Table S2).

Cytokine release assay (CRA) from whole peripheral blood: 320 µl of whole blood drawn on the same day were mixed with 80 µl RPMI and stimulated with the indicated SARS-CoV-2 Spike peptide pools at 2 µg/ml or with DMSO as a control. After 16 hours of culture, the culture supernatant (plasma) was collected and stored at -80°C. Cytokine concentrations in the plasma were quantified using an Ella machine with microfluidic multiplex cartridges measuring IFN-γ and IL-2 following the manufacturer's instructions (ProteinSimple). The level of cytokines present in the plasma of DMSO controls was subtracted from the corresponding peptide pool stimulated samples. The positivity threshold was set at 10x times the lower limit of

quantification of each cytokine (IFN- γ = 1.7pg/ml; IL-2 = 5.4pg/ml) after DMSO background subtraction.

ELISpot assay: ELISpot plates (Millipore) were coated with human IFN- γ antibody (1-D1K, Mabtech; 5 μ g/ml) overnight at 4°C. 400,000 PBMC were seeded per well and stimulated for 18h with pools of SARS-CoV-1/2 peptides (2 μ g/ml). For stimulation with peptide matrix pools or single peptides, a concentration of 5 μ g/ml was used. Subsequently, the plates were developed with human biotinylated IFN- γ detection antibody (7-B6-1, Mabtech; 1:2000), followed by incubation with Streptavidin-AP (Mabtech) and KPL BCIP/NBT Phosphatase Substrate (SeraCare). Spot forming units (SFU) were quantified with ImmunoSpot. To quantify positive peptide-specific responses, 2x mean spots of the unstimulated wells were subtracted from the peptide-stimulated wells, and the results expressed as SFU/10⁶ PBMC. We excluded the results if negative control wells had >30 SFU/10⁶ PBMC or positive control wells (PMA/Ionomycin) were negative.

Flow Cytometry: PBMC or expanded T cell lines were stimulated for 5h at 37°C with or without SARS-CoV-2 peptides (2 μ g/ml) in the presence of 10 μ g/ml brefeldin A (Sigma-Aldrich). Cells were stained with the yellow LIVE/DEAD fixable dead cell stain kit (Invitrogen) and anti-CD3 (clone SK7; 3:50), anti-CD4 (clone SK3; 3:50), and anti-CD8 (clone SK1; 3:50) antibodies. Cells were subsequently fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences-Pharmingen) and stained with anti-IFN- γ (clone 25723, R&D Systems; 1:25) and anti-TNF- α (clone MAb11; 1:25) antibodies and analyzed on a BD-LSR II FACS Scan. Data were analyzed by FlowJo (Tree Star Inc.). Antibodies were purchased from BD Biosciences-Pharmingen unless otherwise stated.

Expanded T cell lines: T cell lines were generated as follows: 20% of PBMC were pulsed with 10 μ g/ml of the overlapping SARS-CoV-2 peptides (all pools combined) or single peptides for 1 hour at 37°C, subsequently washed, and cocultured with the remaining cells in AIM-V medium (Gibco; Thermo Fisher Scientific) supplemented with 2% AB human serum (Gibco; Thermo Fisher Scientific). T cell lines were cultured for 10 days in the presence of 20 U/ml of recombinant IL-2 (R&D Systems).

HLA-restriction assay: The HLA-haplotype (4 digit HLA-typing) of individuals was determined and different EBV transformed B cells lines with one common allele each were selected for presentation of the indicated peptides. B cells were pulsed with 10 µg/ml of the peptide for 1 hour at 37°C, washed three times, and cocultured with the expanded T cell line at a ratio of 1:1 in the presence of 10 µg/ml brefeldin A (Sigma-Aldrich). Non-pulsed B cell lines served as a negative control detecting potential allogeneic responses and autologous peptide-pulsed cells served as a positive control.

TCR-redirected SARS-CoV-2-specific CD8⁺ T cells: Spike-specific T cell line was stimulated for 5 hours with peptide S491-505 and the activated antigen-specific T cells were identified through the expression of CD107a. The CD107a⁺ T cells were sorted and single cell TCR sequencing was performed and analyzed using the 10x Genomics human T cell V(D)J amplification kit (10x Genomics) according to the manufacturer's recommendations.

Spike 491-510-specific TCR α and β chain genes were subcloned into T7 expression vector (p-VAX1), the SARS-CoV-2-TCR mRNA was transcribed in vitro using the mMESSAGE mMACHINE™ T7 ULTRA Transcription Kit (ThermoFisher Scientific) following the manufacturer's protocols. To introduce the TCR expression in non-memory T cells, PBMCs from healthy individuals were isolated and expanded in vitro for 7 days in the presence of 50 ng/ml of OKT-3 (Miltenyi) and 600 IU/ml IL-2 (R&D Systems) in AIM-V (Gibco) medium supplemented with 2% human AB serum (Gibco). The concentration of IL-2 was increased to 1000 IU/ml on day 7 and the expanded T cells were electroporated with the indicated mRNA at a concentration of 2 pg mRNA/cell on day 8 using 4D Nucleofector™ System (Lonza) according to the manufacturer's instructions. Electroporated T cells were rested for 5 mins before been maintained in AIM-V media supplemented with 10% human AB serum and 100 IU/ml IL-2 overnight. The expression of the introduced TCR were examined using Live/Dead Fixable Yellow Dead Cell Stain Kit (ThermoFisher Scientific), anti-human TCR V β 1 (Beckman Coulter), anti-human CD3 and CD8 antibodies (BD Bioscience).

Peptide-pulse experiment: The EBV-transformed lymphoblastoid B (EBV-B) cell lines with identified HLA-B*15:27 phenotype were used as antigen-presenting cells and cocultured with the indicated concentrations of peptides for 1 hour at 37 °C. The

peptide-pulsed EBV-B cells were washed twice with HBSS (Gibco) before cocultured with the TCR-redirected T cells at a 1:1 ratio in the presence of brefeldin A (2 µg/ml) overnight. The cells were stained with Live/Dead in 1 × PBS for 10 min at room temperature and then stained with anti-human CD3 and CD8 antibodies for 30 min at 4°C. The cells were fixed and permeabilized using the Cytofix/Cytoperm fixation/permeabilization (BD Biosciences) buffer following the manufacturer's protocols. Intracellular cytokine staining was performed with anti-human IFN γ and TNF α (BD Biosciences) antibodies for 30 min at room temperature, followed by washing and analysis by flow cytometry.

Figure 1: Breath of the Spike-specific T cell response in BNT162b mRNA vaccinated donors and SARS-CoV-2 convalescents.

A, Bar graphs show the percentage of donors (vaccinated n=35; convalescent n=31) reacting to the number of Spike-peptide pools tested (total 7 distinct peptide pools). **B**, Heatmap is indicating the percentage of the response towards a single peptide pool in proportion to the total Spike-specific response in each of the tested individuals. **C**, Percentage of tested individuals with a dominant response to one of the 7 peptide pools is shown.

Figure 2: Impact of VOC mutations on the global Spike-specific T cell response

A, Schematic of Spike protein with the amino acid (AA) substitutions/deletions characteristic for the Delta VOC. Red lines indicate the locations of the 15-mer peptides covering the mutated regions, which were tested as separate pools with and without the AA substitutions/deletions. **B**, Whole blood of n=100 BNT162b2 vaccinated donors was stimulated with 3 separate peptide pools covering 1) the whole Spike protein, 2) the Delta mutations without and 3) with the AA substitutions/deletions. IFN- γ release was quantified after overnight stimulation in the plasma. Bars indicate the percentage of the Spike-peptide response targeting the conserved regions (black), the mutated regions but not affected (grey) and the percentage inhibited by the mutations (red). **C**, Mean reduction of the response to Delta AA substitutions/deletions in proportion to the total Spike-response. **D**, Proportion of donors whose response was reduced by the indicated percentages. **E**, Schematic of Spike protein with 30 tested AA substitutions/deletions characteristic for the VOCs (Alpha, Beta, Gama, Delta, Omicron). Red lines indicate the locations of the 15-mer peptides covering the mutated regions, which were tested as separate pools with and without the AA substitutions/deletions. PBMCs of 33 BNT162b2 vaccinated donors (**F**) and 29 SARS-CoV-2 convalescents (**G**) was stimulated with 3 separate peptide pools covering 1) the whole Spike protein, 2) the VOC mutations without and 3) with the AA substitutions/deletions. Bars indicate the percentage of the Spike-peptide response targeting the conserved regions (black), the mutated regions but not affected (grey) and the percentage inhibited by the mutations (red). Pie charts are indicating the mean reduction of the response to 30 tested VOC AA substitutions/deletions in proportion to the total Spike-response. Proportion of vaccinated donors (**H**) and convalescents (**I**) whose response was reduced by the indicated percentages.

A

CD8 epitopes convalescents CD8 epitopes vaccinated CD4 epitopes convalescents CD4 epitopes vaccinated

B **C** **D** **E** **F** **G**

Figure 3: Impact of VOC AA mutations on Spike-specific T cells.

A, PBMCs of the individuals in whom the T cell specificities were defined (and cells were available) were stimulated ex vivo in parallel with peptides containing either the wildtype or the mutated sequence. Peptide reactivity was analysed by IFN-γ ELISpot assay. Frequency of spot forming cells (SFC)/10⁶ PBMC are shown. **B**, PBMCs of two vaccinated and one convalescent HLA-A*24:02+ donor were stimulated with the 446-460 peptide for 10 days and subsequently tested in parallel with peptides containing either the wildtype or the five indicated mutated sequences. Peptide response was measured by IFN-γ ELISpot assay. **C**, The 446-460 short-term T cell line of a vaccinated HLA-A*24:02+ donor was stimulated in parallel with peptides containing either the wildtype sequence (446-460), or the G446V and L452R mutations. Peptide response was measured by intracellular cytokine staining. **D**, TCR Vβ1 antibody was used to detect the introduced TCR. Representative FACS histogram plots showing TCR Vβ1 expression in allogenic T cells 24 hours after mRNA electroporation. **E**, TCR-redirected T cells stimulated in parallel with the 15-mer peptide 491-505 (PLQSYGFQPTNGVG), 9-mer peptides 497-505 (FQPTNGVG) and 496-504 (GFQPTNGVG) analysed by intracellular cytokine staining. **F**, TCR-redirected T cells stimulated in parallel with peptide 497-505 with and without the N501Y mutation. Peptide reactivity was analysed by intracellular cytokine staining. **G**, TCR-redirected T cells stimulated with a 10-fold dilution series of the 497-505 (FQPTNGVG) peptide and analysed by intracellular cytokine staining.

Figure S1: Breath of the Spike-specific T cell response in BNT162b mRNA vaccinated donors and SARS-CoV-2 convalescents.

A, Schematic of the 7 Spike-specific peptide pools containing 15-mer overlapping peptides spanning the entire Spike protein. **B**, Frequency of peptide-reactive cells in 35 individuals 3 months post 2-dose BNT162b2 vaccination and **C**, frequency of peptide-reactive cells in 31 individuals 12 months post infection with SARS-CoV-2. Black bar graphs in **B** and **C** show the total of IFN- γ spot forming cells (SFC)/ 10^6 PBMC to all 7 peptide pools combined. The pie charts show the mean proportion of the response to the 7 distinct Spike-peptide pools. Coloured bar graphs below show the frequency of SFC to the 7 distinct Spike-peptide pools in the 35 different vaccinated individuals (**B**) and in the 31 different convalescents (**C**).

Figure S2: Heterogeneity of the Spike-specific T cell response in BNT162b2 vaccinated donors.
A, Whole blood of n=100 healthy BNT162b2 vaccinated donors was stimulated with one peptide pool covering the whole Spike protein. IFN- γ release was quantified after overnight stimulation in the plasma. **B**, Donors were divided based on peptide pool reactivity in high, medium, low and non-responder.

Figure S4: Definition of single-peptide specificities of dominant and subdominant Spike-specific T cells in SARS-CoV-2 convalescent donors.

Heatmap of the response of the short-term T cell lines of the 11 convalescent donors to the numerical pools of the peptide matrix covering 16 distinct regions of the Spike-protein (left). Single-peptide responses were confirmed by ICS (right).

HLA-Class I haplotype

A1	A2	B1	B2	C1	C2
02:05	24:XX	35:03	50:01	06:02	12:03

Peptide: 411-425

A*02:05	+	-	-	-	-	-	-
A*24:02	-	+	-	-	-	-	-
B*35:03	-	-	+	-	-	-	-
C*06:02	-	-	-	+	-	-	-
C*12:03	-	-	-	-	+	-	-

B cell lines

HLA-Class I haplotype

A1	A2	B1	B2	C1	C2
02:01	11:01	35:01	35:01	04:01	04:01

Peptide: 81-95

A*02:01	+	-	-	-	-	-	-
A*11:01	-	+	-	-	-	-	-
B*35:01	-	-	+	-	-	-	-
C*04:01	-	-	-	+	-	-	-

B cell lines

HLA-Class I haplotype

A1	A2	B1	B2	C1	C2
11:01	24:02	40:01	40:01	03:04	04:82

Peptide: 1016-1030

A*11:01	+	-	-	-	-	-	-
A*24:02	-	+	-	-	-	-	-
B*40:01	-	-	+	-	-	-	-
C*03:04	-	-	-	+	-	-	-

B cell lines

HLA-Class I haplotype

A1	A2	B1	B2	C1	C2
02:03	11:02	40:01	54:01	01:02	12:02

Peptide: 1051-1065

A*02:03	+	-	-	-	-	-	-
A*11:02	-	+	-	-	-	-	-
B*40:01	-	-	+	-	-	-	-
C*54:01	-	-	-	+	-	-	-
C*01:02	-	-	-	-	+	-	-

B cell lines

Figure S5: HLA-Class-I restriction of single-peptide specificities of 6 dominant CD8+ Spike-specific T cells
The HLA-class I haplotypes of vaccinated donors 1, 2, 3, 4 are 6 are shown in the tables. HLA-restriction of the indicated peptide-specific T cells from the donors was deduced by co-culturing the T cells with peptide-pulsed EBV-transformed B cell lines that shared the indicated HLA-Class I molecule (+). Activation of the peptide-specific T cells by autologous cells was achieved by the direct addition of the peptide and used as a positive control.

Figure S6: The impact of VOC AA mutations on Spike-specific T cells after in vitro expansion

Expanded T cell lines of the individuals in whom the T cell specificities were defined (and cells were available) were stimulated in parallel with peptides containing either the wildtype or the mutated sequence. Peptide reactivity was analysed by IFN-γ ELISpot assay. Frequency of spot forming cells (SFC)/10⁵ PBMC are shown.

Figure S7: Definition of a CD8 T cell epitope in peptide S1016-30-specific T cells

S1016-30 expanded T cell lines of a HLA-B*40:01+ and a HLA-B*40:01- vaccine recipient were stained with HLA-B*40:01 tetramer containing peptide 1016-24. Dot plots show tetramer staining on CD3+ cells.

Table S1: Seven pools of 15-mer overlapping peptides covering the SARS-CoV-2 Spike protein

S-1 peptide pool		S-2 peptide pool		S-3 peptide pool		S-4 peptide pool		S-5 peptide pool		S-6 peptide pool		S-7 peptide pool	
aa	Sequence	aa	Sequence	aa	Sequence	aa	Sequence	aa	Sequence	aa	Sequence	aa	Sequence
1-15	MFVFLVLLPLVSSQC	171-185	VSQPFLMDLEGKQGN	336-350	CPFGEVFNATRFASV	501-515	NGVGYPYRVVVLSF	696-710	TMSLGAENSVAYSNN	886-900	WTFGAGAAALQIPFAM	1076-1090	TTAPAICHDGKAHPF
6-20	VLLPLVSSQCQVNLTT	176-190	LMDLEGKQGNFKNLNR	341-355	VFNATRFASVYAWNR	506-520	QPYRVVLSFELLHA	701-715	AENSVAYSNNNSIAIP	891-905	GAALQIPFAMQMAYR	1081-1095	ICHDGKAHPFPREGVF
11-25	VSSQCQVNLTTTRTQLP	181-195	GKQGNFKNLREFVFK	346-360	RFASVYAWNRKRISN	511-525	VVLSFELLHAPATVC	706-720	AYSNNNSIAIPTNFTTI	896-910	IPFAMQMAYRFNGIG	1086-1100	KAHPFPREGVFSVNGT
16-30	VNLTTTRTQLPPPAYTN	186-200	FKNLREFVFKNIDGY	351-365	YAWNRKRISNCVADY	516-530	ELLHAPATVCGPKKS	711-725	SIAIPTNFTTISVTTE	901-915	QMAYRFNGIGVTQNV	1091-1105	REGVFSVNGTHWFVT
21-35	RTQLPPPAYTNSFTRG	191-205	EFVFKNIDGYFKIYS	356-370	KRISNCVADYSVLYN	521-535	PATVCGPKKSTNLVK	716-730	TNFTISVTTEILPVS	906-920	FNGIGVTQNVLYENQ	1096-1110	VSNGTHWFVTQRNFY
26-40	PAYTNSFTRGVVYPD	196-210	NIDGYFKIYSKHTPI	361-375	CVADYSVLYNSASF	526-540	GPKKSTNLVKNKCVN	721-735	SVTTEILPVSMTKTS	911-925	VTQNVLYENQKLIAN	1101-1115	HWFVTQRNFYEPQII
31-45	SFTRGVVYPDKVFRS	201-215	FKIYSKHTPINLVRD	366-380	SVLYNSASFSTFKCY	531-545	TNLVKNKCVNFNFNG	726-740	ILPVSMTKTSVDCTM	916-930	LYENQKLIANQFN	1106-1120	QRNFYEPQIITDNT
36-50	VYYPDKVFRSSVLHS	206-220	KHTPINLVRDLPPQGF	371-385	SASFSTFKCYGVSP	536-550	NKCVNFNFNGLTGTG	731-745	MTKTSVDCTMYICGD	921-935	KLIANQFN	1111-1125	EPQIITDNTFVSGN
41-55	KVFRSSVLHSTQDLF	211-225	NLVRDLPPQGS	376-390	TFKCYGVSP	541-555	FNFNGLTGTGVL	736-750	VDCTMYICGDSTEC	926-940	QFN	1116-1130	TTDNTFVSGNCDVVI
46-60	SVLHSTQDLFLPFFS	216-230	LPQGS	381-395	GVSPTK	546-560	LTGTGVL	741-755	YICGDSTEC	931-945	IGKIQDSL	1121-1135	FVSGNCDVVIGIVNN
51-65	TQDLFLPFFSNVTWF	221-235	SALEPLVDLPIGINI	386-400	KLNDLCFTNVYADSF	551-565	VLTESNKKFLPFQQF	746-760	STEC	936-950	DSL	1126-1140	CDVVIGIVNNTVYDP
56-70	LPFFSNVTWFHAIHV	226-240	LVDLPIGINITRFQT	391-405	CFTNVYADSFVIRGD	556-570	NKKFLPFQQFGRDIA	751-765	NLLQYGSFCTQLNR	941-955	TASALGKLQDVVNQN	1131-1145	GIVNNTVYDPLQPEL
61-75	NVTWFHAIHVSGTNG	231-245	IGINITRFQTL	396-410	YADSFVIRGDEV	561-575	PFQQFGRDIADTTDA	756-770	YGSFCTQLNRALTGI	946-960	GKLQDVVNQNAQALN	1136-1150	TVYDPLQPELDSFKE
66-80	HAIHVSGTNGTKRFD	236-250	TRFQTL	401-415	VIRGDEV	566-580	GRDIADTTDAVRDPQ	761-775	TQLNRALTGIAVEQD	951-965	VVNQNAQALNTLVKQ	1141-1155	LQPELDSFKEELDKY
71-85	SGTNGTKRFDNPVLP	241-255	LLALHRSYLT	406-420	EV	571-585	DTTDAVRDPQ	766-780	ALTGIAVEQDKNTQE	956-970	AQALNTLVKQLSSNF	1146-1160	DSFKEELDKYFKNHT
76-90	TKRFDNPVLPFNDGV	246-260	RSYLT	411-425	APGQT	576-590	VRDPQ	771-785	AVEQDKNTQEVFAQV	961-975	TLVKQLSSNFGAISS	1151-1165	ELDKYFKNHTSPDVD
81-95	NPVLPFNDGVYFAST	251-265	PGDSSSGWTAGAAAY	416-430	GKIADYNYKL	581-595	TLEILDITPCSF	776-790	KNTQEVFAQVKQIYK	966-980	LSSNFGAISSVLNDI	1156-1170	FKNHTSPDVDLGDIS
86-100	FNDGVYFAST	256-270	SGWTAGAAAYYVGYL	421-435	YNYKL	586-600	DITPCSF	781-795	VFAQVKQIYKTPPIK	971-985	GAISSVLNDILSRLD	1161-1175	SPDVDLGDISGINAS
91-105	YFAST	261-275	GAAAYYVGYLQ	426-440	PDDFTGCVIAWNSNN	591-605	SFGGVSVITPGTNTS	786-800	KQIYKTPPIKDFGGF	976-990	VLNDILSRLDKVEAE	1166-1180	LGDISGINASVVNIQ
96-110	EKSNIIRGWIFGTTL	266-280	YVGYLQ	431-445	GCVIAWNSNNLDSKV	596-610	SVITPGTNTS	791-805	TPPIKDFGGFNFSQI	981-995	LSRLDKVEAEVQIDR	1171-1185	GINASVVNIQKEIDR
101-115	IRGWIFGTTLDSKTQ	271-285	QPRTFLLKYN	436-450	WNSNNLDSKVGGNYN	601-615	GTNTSNQVAVLYQDV	796-810	DFGGFNFSQILPDPS	986-1000	KVEAEVQIDRLITGR	1176-1190	VVNIQKEIDRLNEVA
106-120	FGTTLDSKTQSL	276-290	LLKYN	441-455	LDSKVGGNYNYLRL	606-620	NQVAVLYQDVNCTEV	801-815	NFSQILPDPSKPSKR	991-1005	VQIDRLITGR	1181-1195	KEIDRLNEVAKNLNE
111-125	DSKTQSL	281-295	ENG	446-460	GGNYNYLRLFRKSN	611-625	LYQDVNCTEVPVAIH	806-820	LPDPSKPSKRSFIED	996-1010	LITGR	1186-1200	LNEVAKNLNESLIDL
116-130	SL	286-300	TDAVDCALDPLSETK	451-465	YLYRLFRKSNLKPFE	616-630	NCTEVPVAIHADQLT	811-825	KPSKRSFIEDLLFNK	1001-1015	LQSLQTYVTYQQLIRA	1191-1205	KNLNESLIDLQELGK
121-135	NNATNVV	291-305	CALDPLSETKCTLKS	456-470	FRKSNLKPFERDIST	621-635	PVAIHADQLTPTWRV	816-830	SFIEDLLFNKVTLAD	1006-1020	TYVTYQQLIRAAEIRA	1196-1210	SLIDLQELGKYEQYI
126-140	VV	296-310	LSETKCTLKSFTVEK	461-475	LKPFERDISTEIQ	626-640	ADQLTPTWRVYSTGS	821-835	LLFNKVTLADAGFIK	1011-1025	QLIRAAEIRASANLA	1201-1215	QELGKYEQYIKWPWY
131-145	CE	301-315	CTLKSFTVEKGIYQT	466-480	RDISTEIQAGSTPC	631-645	PTWRVYSTGSNVFQT	826-840	VTLADAGFIKQYGDC	1016-1030	AEIRASANLAATKMS	1206-1220	YEQYIKWPWYIWLGF
136-150	CNDPFLGVYYHKNK	306-320	FTVEKGIYQTSNFRV	471-485	EIQAGSTPCNGVEG	636-650	YSTGSNVFQTRAGCL	831-845	AGFIKQYGDCLGDIA	1021-1035	SANLAATKMSECVLG	1211-1225	KWPWYIWLGF
141-155	LG	311-325	GIYQTSNFRVQPTES	476-490	GSTPCNGVEGFNCYF	641-655	NVFQTRAGCLIGAEH	836-850	QYGDCLGDIAARDLI	1026-1040	ATKMSECVLGQSKRV	1216-1230	IWLGF
146-160	HKNKSWMESEFRVY	316-330	SNFRVQPTESIVRFP	481-495	NGVEGFNCYFP	646-660	RAGCLIGAEHVNNSY	841-855	LGDIARDLICAQKF	1031-1045	ECVLGQSKRVDFCGK	1221-1235	IAGLIAIVMVTIMLC
151-165	SWMESEFRVYSSANN	321-335	QPTESIVRFPNITNL	486-500	FNCYFP	651-665	IGAEHVNNSYEC	846-860	ARDLICAQKFNGLTV	1036-1050	QSKRVDFCGKGYHLM	1226-1240	AIVMVTIMLCCMTSC
156-170	EFRVYSSANNCTFEY	326-340	IVRFPNITNLCPFGE	491-505	PLQSYGFQPTNGVGY	656-670	VNNSYEC	851-865	CAQKFNGLTVLPPLL	1041-1055	DFCGKGYHLSFPQS	1231-1245	TIMLCCMTSCCCLK
161-175	SSANNCTFEYVSQPF	331-345	NITNLCPFGEVFNAT	496-510	GFQPTNGVGYQPYRV	661-675	ECDIP	856-870	NGLTVLPPLLTDEMI	1046-1060	GYHLSFPQSAPHGV	1236-1250	CMTSCCCLKGCCSC
166-180	CTFEYVSQPFLLMDLE					666-680	IGAGICASYQTQTNS	861-875	LPPLLTDEMIAQYTS	1051-1065	SFPQSAPHGVVFLHV	1241-1255	CCLKGCCSCGSCCK
						671-685	CASYQTQTNSPRRAR	866-880	TDEMIAQYTSALLAG	1056-1070	APHGVVFLHVTVVPA	1246-1260	GCCSCGSCCKFDEDD
						676-690	TQTNSPRRARSVASQ	871-885	AQYTSALLAGTITSG	1061-1075	VFLHVTVVPAQEKNF	1251-1265	GSCCKFDEDDSEPV
						681-695	PRRARSVASQSIIAY	876-890	ALLAGTITSGWTFGA	1066-1080	TVVPAQEKNFTTAPA	1256-1270	FDEDDSEPV
						686-700	SVASQSIIAYTMSLG	881-895	TITSGWTFGAGAA	1071-1085	QEKNFTTAPAICHDG	1261-1273	SEPV
						691-705	SIIAYTMSLGAENSV						

Table S2: Overlapping 15-mer peptides covering the region of the VOC mutations with and without the amino acid substitutions and deletions.

Alpha				Beta				Gamma				Delta			
mutation	aa	Wuhan-Hu-1	B.1.1.7	mutation	aa	Wuhan-Hu-1	B.1.351	mutation	aa	Wuhan-Hu-1	P.1	mutation	aa	Wuhan-Hu-1	B.1.617.2
del HV 69-70	56-70	LPFFSNVTWFHAIHV	LPFFSNVTWFHAI--	L18F	6-20	VLLPLVSSQCVNLTT	VLLPLVSSQCVNFTT	L18F T20N	6-20	VLLPLVSSQCVNLTT	VLLPLVSSQCVNFTN	T19R	6-20	VLLPLVSSQCVNLTT	VLLPLVSSQCVNLRT
	61-75	NVTWFHAIHVSGTNG	NVTWFHAI--SGTNG		11-25	VSSQCVNLTTTRTQLP	VSSQCVNFTTTRTQLP		11-25	VSSQCVNLTTTRTQLP	VSSQCVNFTNRTQLP		11-25	VSSQCVNLTTTRTQLP	VSSQCVNLRTTRTQLP
	66-80	HAIHVSGTNGTKRFD	HAI--SGTNGTKRFD		16-30	VNLTTTRTQLPPAYTN	VNFTTTRTQLPPAYTN		16-30	VNLTTTRTQLPPAYTN	VNFTNRTQLPPAYTN		16-30	VNLTTTRTQLPPAYTN	VNLRTTRTQLPPAYTN
del Y 144	131-145	CEFQFCNDPFLGVYY	CEFQFCNDPFLGV-Y	D80A	66-80	HAIHVSGTNGTKRFD	HAIHVSGTNGTKRFA	P26S	16-30	VNLTTTRTQLPPAYTN	VNLTNRTQLPSAYTN	G142D	131-145	CEFQFCNDPFLGVYY	CEFQFCNDPFLDVYY
	136-150	CNDPFLGVYYHKNNK	CNDPFLGV-YHKNNK		71-85	SGTNGTKRFDNPVLP	SGTNGTKRFPANVLP		21-35	RTQLPPAYTNSFTRG	RTQLPSAYTNSFTRG		136-150	CNDPFLGVYYHKNNK	CNDPFLDVYYHKNNK
	141-155	LGVYYHKNNKSWMES	LGV-YHKNNKSWMES		76-90	TKRFDNPVLPFNDGV	TKRFANPVLPFNDGV		26-40	PAYTNSFTRGVVYPD	SAYTNSFTRGVVYPD		141-155	LGVYYHKNNKSWMES	LDVYYHKNNKSWMES
N501Y	491-505	PLQSYGFQPTNGVGY	PLQSYGFQPTYGVGY	D215G	201-215	FKIYSKHTPINLVRD	FKIYSKHTPINLVRG	D138Y	126-140	VVIKVCCEFQFCNDPF	VVIKVCCEFQCNYPF	EFR156-8G	146-160	HKNNKSWMES	HKNNKSWMESG--VY
	496-510	GFQPTNGVGYPYRV	GFQPTYGVGYQPYRV		206-220	KHTPINLVRLDPQGF	KHTPINLVRLGPQGF		131-145	CEFQFCNDPFLGVYY	CEFQFCNYPFLGVYY		151-165	SWMES	SWMESG--VYSSANN
	501-515	NGVGYPYRVVLSF	YGVGYQPYRVVLSF		211-225	NLVRDLPGQFSALEP	NLVRGLPGQFSALEP		136-150	CNDPFLGVYYHKNNK	CNYPFLGVYYHKNNK		156-170	EFVRVYSSANNCTFEY	G--VYSSANNCTFEY
A570D	556-570	NKKFLPFQQFGRDIA	NKKFLPFQQFGRDID	del LLA 241-3	231-245	IGINITRFQTLALH	IGINITRFQT---LH	R190S	176-190	LMDLEGKQGNFKNLS	LMDLEGKQGNFNKLS	L452R	441-455	LDSKVGGNYNYLYRL	LDSKVGGNYNYRYRL
	561-575	PFQQFGRDIADTTDA	PFQQFGRDIDDTTDA		236-250	TRFQTLALHRSYLT	TRFQT---LHRSYLT		181-195	GKQGNFKNLREFVFK	GKQGNFKNLSEFVFK		446-460	GGNYNYLYRLFRKSN	GGNYNYRYRLFRKSN
	566-580	GRDIADTTDAVRDPQ	GRDIDTTDAVRDPQ		241-255	LLALHRSYLTGPDSS	---LHRSYLTGPDSS		186-200	FKNLREFVFKNIDGY	FKNLSEFVFKNIDGY		451-465	YLYRLFRKSNLKPFE	YRYRLFRKSNLKPFE
D614G	601-615	GTNTSNQVAVLYQDV	GTNTSNQVAVLYQGV	K417N	406-420	EVQRQIAPGQTGKIAD	EVQRQIAPGQTGNIAD	K417T	406-420	EVQRQIAPGQTGKIAD	EVQRQIAPGQTGTIAD	T478K	466-480	RDISTEIQAGSTPC	RDISTEIQAGSKPC
	606-620	NQVAVLYQDVNCTEV	NQVAVLYQGVNCTEV		411-425	APGQTGKIADYNYKL	APGQTGNIADYNYKL		411-425	APGQTGKIADYNYKL	APGQTGTIADYNYKL		471-485	EIQAGSTPCNGVEG	EIQAGSKPCNGVEG
	611-625	LYQDVNCTEVPVAIH	LYQGVNCTEVPVAIH		416-430	GKIADYNYKLDDFT	GNIADYNYKLDDFT		416-430	GKIADYNYKLDDFT	GTIADYNYKLDDFT		476-490	GSTPCNGVEGFNCYF	GSKPCNGVEGFNCYF
P681H	671-685	CASYQTQTNSPRRAR	CASYQTQTNSHRRAR	E484K	471-485	EIQAGSTPCNGVEG	EIQAGSTPCNGVKG	E484K	471-485	EIQAGSTPCNGVEG	EIQAGSTPCNGVKG	D614G	601-615	GTNTSNQVAVLYQDV	GTNTSNQVAVLYQGV
	676-690	TQTNSPRRARSVASQ	TQTNSHRRARSVASQ		476-490	GSTPCNGVEGFNCYF	GSTPCNGVKGFCNCYF		476-490	GSTPCNGVEGFNCYF	GSTPCNGVKGFCNCYF		606-620	NQVAVLYQDVNCTEV	NQVAVLYQGVNCTEV
	681-695	PRRARSVASQSI IAY	HRRARSVASQSI IAY		481-495	NGVEGFNCYFP LQSY	NGVKGFCNCYFP LQSY		481-495	NGVEGFNCYFP LQSY	NGVKGFCNCYFP LQSY		611-625	LYQDVNCTEVPVAIH	LYQGVNCTEVPVAIH
T716I	706-720	AYSNNIAIPTNFTI	AYSNNIAIPINFTI	N501Y	491-505	PLQSYGFQPTNGVGY	PLQSYGFQPTYGVGY	N501Y	491-505	PLQSYGFQPTNGVGY	PLQSYGFQPTYGVGY	P681R	671-685	CASYQTQTNSPRRAR	CASYQTQTNSRRRAR
	711-725	SIAIPTNFTISVTTE	SIAIPINFTISVTTE		496-510	GFQPTNGVGYPYRV	GFQPTYGVGYQPYRV		496-510	GFQPTNGVGYPYRV	GFQPTYGVGYQPYRV		676-690	TQTNSPRRARSVASQ	TQTNSRRRARSVASQ
	716-730	TNFTISVTTEILPVS	INFTISVTTEILPVS		501-515	NGVGYPYRVVLSF	YGVGYQPYRVVLSF		501-515	NGVGYPYRVVLSF	YGVGYQPYRVVLSF		681-695	PRRARSVASQSI IAY	RRRARSVASQSI IAY
S982A	971-985	GAISSVLNDILSRDL	GAISSVLNDILARLD	D614G	601-615	GTNTSNQVAVLYQDV	GTNTSNQVAVLYQGV	D614G	601-615	GTNTSNQVAVLYQDV	GTNTSNQVAVLYQGV	D950N	936-950	DSLSTASALGKLQD	DSLSTASALGKLND
	976-990	VLNDILSRDLKVEAE	VLNDILARLDKVEAE		606-620	NQVAVLYQDVNCTEV	NQVAVLYQGVNCTEV		606-620	NQVAVLYQDVNCTEV	NQVAVLYQGVNCTEV		941-955	TASALGKLQDVVNQN	TASALGKLQNVVNQN
	981-995	LSRLDKVEAEVQIDR	LARLDKVEAEVQIDR		611-625	LYQDVNCTEVPVAIH	LYQGVNCTEVPVAIH		611-625	LYQDVNCTEVPVAIH	LYQGVNCTEVPVAIH		946-960	GKLQDVVNQNAQALN	GKLQNVVNQNAQALN
D1118H	1106-1120	QRNFYEPQIITDNT	QRNFYEPQIITHTNT	A701V	691-705	SIIAYTMSLGAENSV	SIIAYTMSLGVENSV	H655Y	641-655	NVFQTRAGCLIGAEH	NVFQTRAGCLIGAEY	V1176F	1166-1180	LGDISGINASVFNQI	LGDISGINASFVNIQ
	1111-1125	EPQIITDNTFVSGN	EPQIITHTNTFVSGN		696-710	TMSLGAENSVAYSNN	TMSLGVENSVAYSNN		646-660	RAGCLIGAEHVNNNSY	RAGCLIGAEYVNNNSY		1171-1185	GINASVFNQIKEIDR	GINASFVNIQIKEIDR
	1116-1130	TDNTFVSGNCDVVI	THNTFVSGNCDVVI		701-715	AENSVAYSNNIAIP	VENSVAYSNNIAIP		651-665	IGAETHVNNSEYCDIP	IGAETHVNNSEYCDIP		1176-1190	VVNIQKEIDRLNEVA	FVNIQKEIDRLNEVA
								T1027I	1016-1030	AEIRASANLAATKMS	AEIRASANLAAIKMS				
									1021-1035	SANLAATKMSECVLG	SANLAAIKMSECVLG				
									1026-1040	ATKMSECVLGQSKRV	AIKMSECVLGQSKRV				
									1166-1180	LGDISGINASVFNQI	LGDISGINASFVNIQ				
									1171-1185	GINASVFNQIKEIDR	GINASFVNIQIKEIDR				
									1176-1190	VVNIQKEIDRLNEVA	FVNIQKEIDRLNEVA				

Table S3: Spike mutations in VOCs vs Wuhan-Hu1. Grey highlighted mutations were tested in this manuscript.

	Alpha	Beta	Gamma	Delta	Omicron
	B.1.1.7	B.1.351	P.1	B.1.617.2	B.1.1.529
L18F		(F)	F		
T19R				R	
T20N			N		
P26S			S		
A67V					V
del 69-70	del				del
D80A		A			
T95I				(I)	I
D138Y			Y		
G142D				(D)	D
del 143-5					del
del 144	del				
E156G				G	
del 157-8				del	
R190S			S		
N211I					I
del 212					del
ins EPE 214					ins
D215G		G			
del 241-3		del			
G339D					D
S371L					L
S373P					P
S375F					F
K417N/T		N	T		(N)
L452R				R	
S477N					N
T478K				K	K
E484A/K		K	K		A
Q493R					R
G496S					S
Q498R					R
N501Y	Y	Y	Y		Y
Y505H					H
T547K					K
A570D	D				
D614G	G	G	G	G	G
H655Y			Y		Y
N679K					K
P681R/H	H			R	H
A701V		V			(V)
T716I	I				
D796Y					Y
N856K					K
D950N				N	
Q954H					H
N969K					K
L981F					F
S982A	A				
T1027I			I		
D1118H	H				
V1176F			F		