

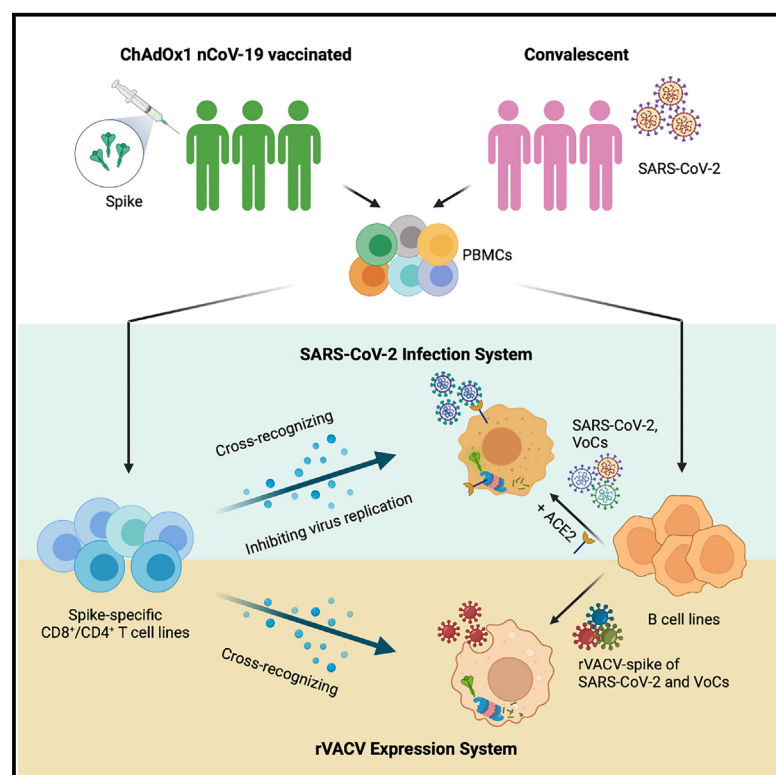


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# Evaluation of T cell responses to naturally processed variant SARS-CoV-2 spike antigens in individuals following infection or vaccination

## Graphical abstract



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## In brief

Yin et al. utilize two informative systems for evaluating overall T cell responses to SARS-CoV-2 and variants, enabling greater understanding of T cell responses to the virus, cross-reactivity to viral variants, and the differences between vaccine- and infection-induced immunity to SARS-CoV-2 and other emerging viruses in the future.

## Highlights

- Systems to evaluate overall T cell responses to naturally processed spike antigens
- Recombinant VACV expression system can be an alternative to SARS-CoV-2 infection
- Infection or vaccination-induced T cells cross-recognize variant SARS-CoV-2 spikes



## Article

# Evaluation of T cell responses to naturally processed variant SARS-CoV-2 spike antigens in individuals following infection or vaccination

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## SUMMARY

Most existing studies characterizing severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-specific T cell responses are peptide based. This does not allow evaluation of whether tested peptides are processed and presented canonically. In this study, we use recombinant vaccinia virus (rVACV)-mediated expression of SARS-CoV-2 spike protein and SARS-CoV-2 infection of angiotensin-converting enzyme (ACE)-2-transduced B cell lines to evaluate overall T cell responses in a small cohort of recovered COVID-19 patients and uninfected donors vaccinated with ChAdOx1 nCoV-19. We show that rVACV expression of SARS-CoV-2 antigen can be used as an alternative to SARS-CoV-2 infection to evaluate T cell responses to naturally processed spike antigens. In addition, the rVACV system can be used to evaluate the cross-reactivity of memory T cells to variants of concern (VOCs) and to identify epitope escape mutants. Finally, our data show that both natural infection and vaccination could induce multi-functional T cell responses with overall T cell responses remaining despite the identification of escape mutations.

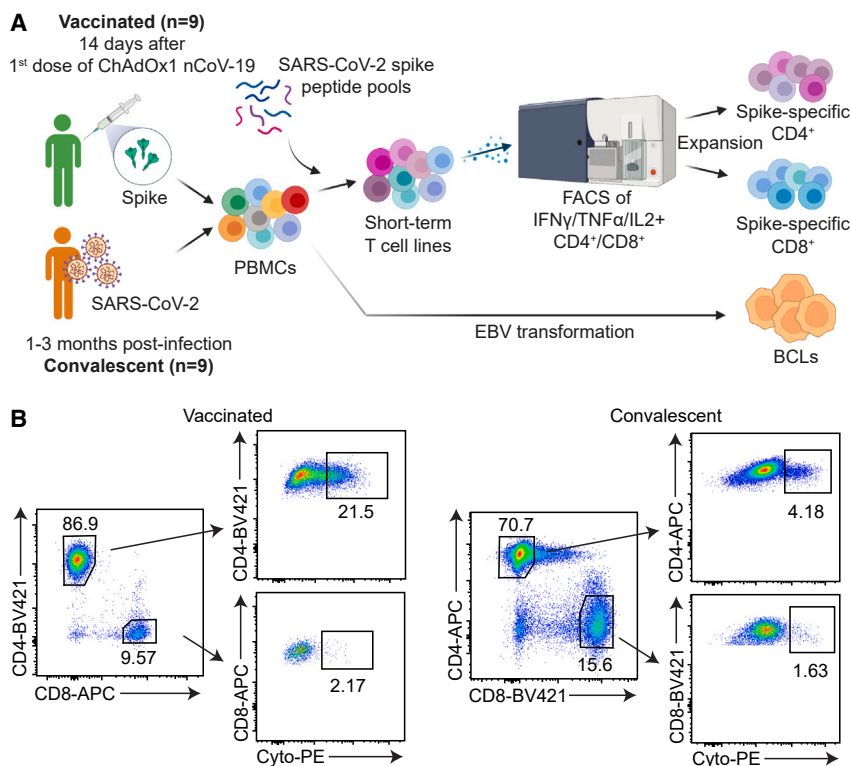
## INTRODUCTION

Understanding of T cell responses, particularly against a novel pathogen, is integral to development of appropriate treatments and vaccines. The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) required rapid study of T cell function and memory in response to infection. Current

studies using peptide pools have revealed that pre-existing T cell responses may contribute to protection from infection as well as reducing disease pathogenesis.<sup>1</sup>

However, the saturating concentration of exogenous pooled peptides typically used in these studies may not fully represent the overall T cell response to virus-infected cells and their antiviral efficacy.<sup>2</sup> This is particularly so if the epitopes presented on the





**Figure 1. Study design and representative FACS of SARS-CoV-2 spike-specific CD4 $^+$  and CD8 $^+$  T cells**

(A) Study design diagram (created with BioRender). (B) Representative FACS plots of IFN $\gamma$  and/or TNF $\alpha$  and/or IL2-secreting T cells. PBMCs were stimulated with SARS-CoV-2 spike peptide pools and cultured for 14 days. Cytokine-secreting CD4 $^+$  or CD8 $^+$  T cells were then sorted after cytokine staining (Cyto-PE) under antigen stimulation. The gating strategy is shown in Figure S2A.

cross-recognition against SARS-CoV-2 VOCs using T cells from both patients recovered from COVID-19 and donors vaccinated with ChAdOx1 nCoV-19. We were also able to identify viral escape at specific T cell epitopes, suggesting that this technique is a robust and efficient tool to study T cell cross-reactivity and identify potential immune escape.

## RESULTS

### Study participants

To assess T cell responses following ChAdOx1 nCoV-19 vaccination or natural

cell surface of infected cells vary due to the different efficiency of antigen processing of antigens and resultant epitope presentation by individual human leukocyte antigen (HLA) molecules.<sup>3</sup> Development of an *in vitro* system that considers the natural processing and presentation of SARS-CoV-2 antigens will be vital for full understanding of T cell responses to SARS-CoV-2.

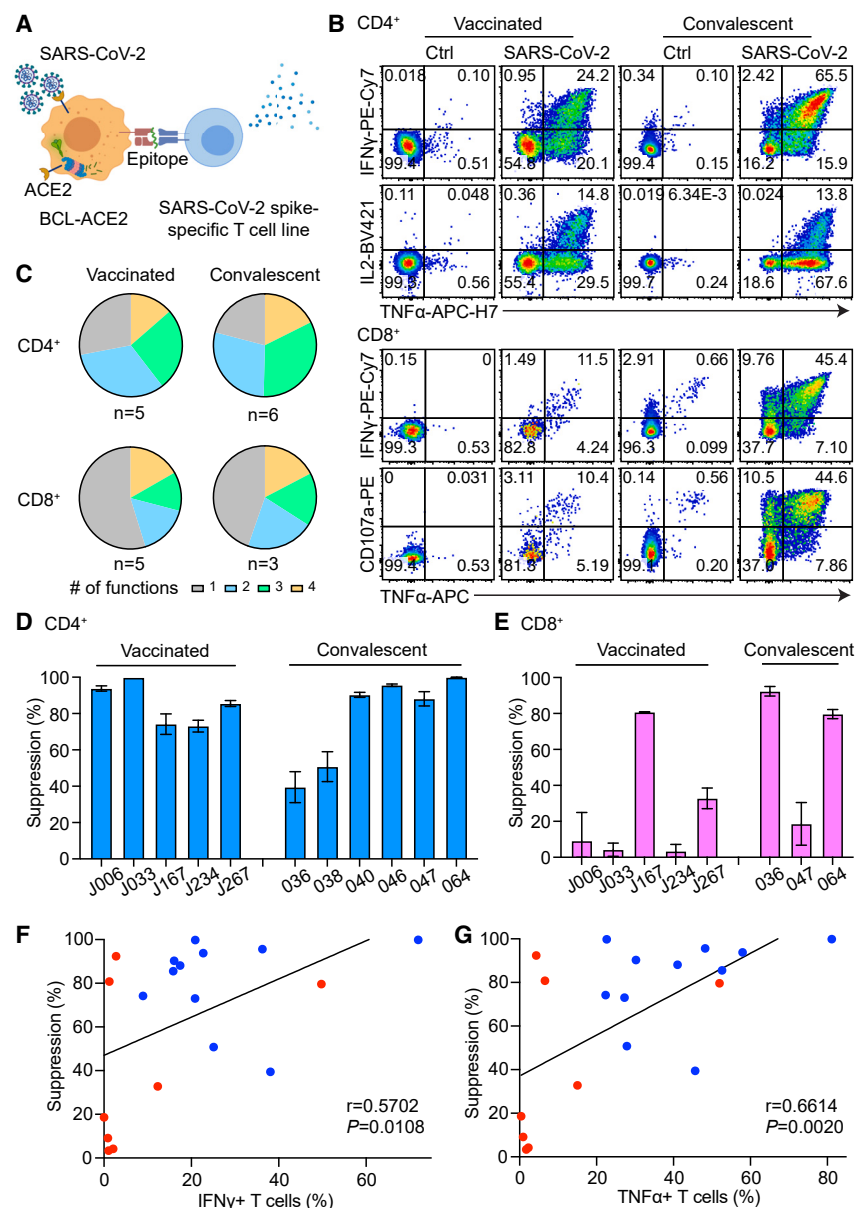
Current licensed vaccines against SARS-CoV-2 have primarily focused on the spike protein as neutralizing antibody responses would block infection and subsequent disease. Since SARS-CoV-2 was first identified in 2019, many mutations have been detected in the spike protein. This has led to the emergence of SARS-CoV-2 variants of concern (VOCs) in multiple parts of the world, including the Alpha, Beta, Gamma,<sup>4</sup> and highly transmissible Delta and Omicron strains.<sup>5</sup> The mutations in the spike protein result in reduced neutralization of these strains by antibodies induced by past infection with an earlier variant or vaccines based on non-homologous strains.<sup>6</sup> Using exogenous peptides spanning the spike protein from the ancestral strain and VOCs, many studies have demonstrated that SARS-CoV-2 mutations are unlikely to alter the overall T cell responses, with scattered observations of mutations that affect T cell recognition.<sup>2,3,7–12</sup> To the best of our knowledge, no study into the effect of these mutations on antigen processing, presentation, and subsequent T cell responses has been published.

Here, we established two systems to evaluate overall T cell responses to naturally processed and presented SARS-CoV-2 spike antigen using recombinant vaccinia virus (rVACV)-mediated expression of SARS-CoV-2 spike protein<sup>13,14</sup> and SARS-CoV-2 infection of angiotensin-converting enzyme (ACE) 2-transduced B cell lines, respectively. We observed strong

infection, we isolated peripheral blood mononuclear cells (PBMCs) from nine vaccinated participants 14 days after the first dose of ChAdOx1 nCoV-19 and nine convalescent patients 1–3 months post infection. The vaccinated participants were naive to SARS-CoV-2 before vaccination, while the convalescent donors were not vaccinated when sampling. PBMCs were stimulated with SARS-CoV-2 spike peptide pools and cultured for 14 days. Spike-specific CD4 $^+$  and CD8 $^+$  T cell lines were then isolated and expanded by fluorescence-activated cell sorting (FACS) of interferon gamma (IFN $\gamma$ ) and/or tumor necrosis factor alpha (TNF $\alpha$ ) and/or interleukin (IL) 2-secreting T cells after stimulation with spike peptides (Figures 1B and S2A). Meanwhile, autologous B cell lines (BCLs) were established from PBMCs by Epstein-Barr virus (EBV) transformation. The study design is shown in Figure 1A. Participant characteristics are summarized in Table S1 and their HLA allele coverage is shown in Figure S1.

### SARS-CoV-2 infection to evaluate antiviral T cell immune responses

To evaluate antiviral activity of T cell immune responses induced by vaccination or infection, we established a SARS-CoV-2 infection system by co-culturing T cells with virus-infected target cells. To set up this system, we first transduced autologous BCLs isolated from our donors with ACE2, which accommodates SARS-CoV-2 entry into BCLs (Figure S3). We then infected these ACE2-transduced BCLs (BCLs-ACE2) with the original SARS-CoV-2 strain and evaluated the function of spike-specific CD4 $^+$  and CD8 $^+$  T cells in both vaccinated and convalescent cohorts (Figure 2A).



**Figure 2. Evaluation of spike-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses to BCLs-ACE2 infected with SARS-CoV-2 ancestral strain**

(A) Diagram of the SARS-CoV-2 infection system (created with BioRender).

(B) Representative cytokine responses of CD4<sup>+</sup> and CD8<sup>+</sup> T cells of a vaccinated participant and a convalescent patient to SARS-CoV-2-infected BCLs-ACE2.

(C) Proportion of T cells expressing one, two, three, or all four of the following functional markers: CD4<sup>+</sup> T cells are defined by the different combination of IFN $\gamma$ , TNF $\alpha$ , IL2, and CD107a; CD8<sup>+</sup> are defined by IFN $\gamma$ , TNF $\alpha$ , MIP1 $\beta$ , and CD107a.

(D) SARS-CoV-2 suppression by spike-specific CD4<sup>+</sup> T cells.

(E) SARS-CoV-2 suppression by spike-specific CD8<sup>+</sup> T cells. Data in (C) and (D) are represented as mean  $\pm$  SEM, n = 3.

(F) Correlation of SARS-CoV-2 suppression with proportion of CD4<sup>+</sup> (blue dots, n = 11) or CD8<sup>+</sup> (red dots, n = 8) T cells producing IFN $\gamma$ .

(G) Correlation of SARS-CoV-2 suppression with proportion of CD4<sup>+</sup> (blue dots, n = 11) or CD8<sup>+</sup> (red dots, n = 8) T cells producing TNF $\alpha$ . Correlation analysis was performed using Spearman's rank correlation coefficient and the two-tailed p value was calculated. p < 0.05 statistically significant.

molecules, including multiple cytokines and degranulation markers of CD107a (Figures 2B, 2C, and S2B). No differences in CD4<sup>+</sup> or CD8<sup>+</sup> T cell functionality was observed between convalescent and vaccinated cohorts. A strong suppression of SARS-CoV-2 replication was observed in the presence of CD4<sup>+</sup> T cells generated from both vaccinated donors and convalescent individuals (Figure 2D), while suppression by CD8<sup>+</sup> T cells (Figure 2E) showed great variation. However, the suppression of virus replication by both spike-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells strongly correlated with the proportion of IFN $\gamma$ - (Figure 2F) and

TNF $\alpha$ -producing (Figure 2G) cells when encountering with virus-infected targets.

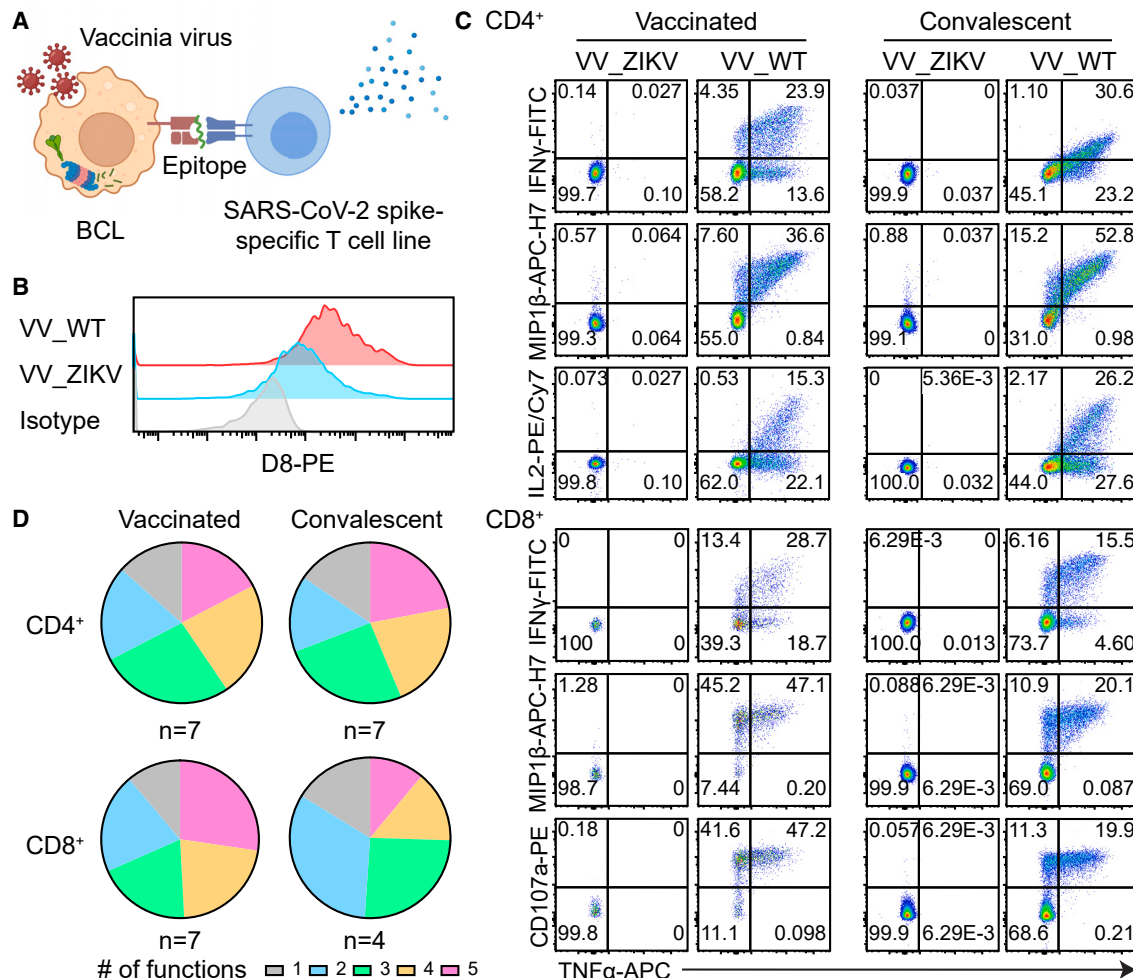
### Use of rVACV expression system to evaluate antiviral T cell immune responses

As described above, the SARS-CoV-2 infection system is able to evaluate antiviral efficacy of SARS-CoV-2 spike-specific T cell responses. However, ACE2 transduction into BCLs is challenging with a low efficiency of success. Moreover, the antigen load of viral CD8<sup>+</sup> epitopes on infected BCLs is generally low and this makes identification of variance in T cell responses difficult. To overcome these obstacles, we established an alternative system that also takes into account the natural processing of viral antigens by infecting target cells with rVACV expressing

We first assessed the effector functions, including cytokine production and degranulation (CD107a expression) of the T cells by intracellular cytokine staining (ICS); CD4<sup>+</sup> T cell responses were measured using IFN $\gamma$ , TNF $\alpha$ , IL2, and CD107a, while CD8<sup>+</sup> responses were measured using IFN $\gamma$ , TNF $\alpha$ , MIP1 $\beta$ , and CD107a. In parallel, suppression of virus replication by each T cell line was evaluated by quantification of the copies of N1 regions from SARS-CoV-2 nucleoprotein. In total, antiviral activity of CD4<sup>+</sup> T cell responses from 11 subjects (n = 6 convalescent, n = 5 vaccinated) and CD8<sup>+</sup> T cell responses from eight subjects (n = 3 convalescent, n = 5 vaccinated) were evaluated in this study.

All the CD4<sup>+</sup> and CD8<sup>+</sup> T cell lines showed strong responses against SARS-CoV-2 with expression of multiple effector



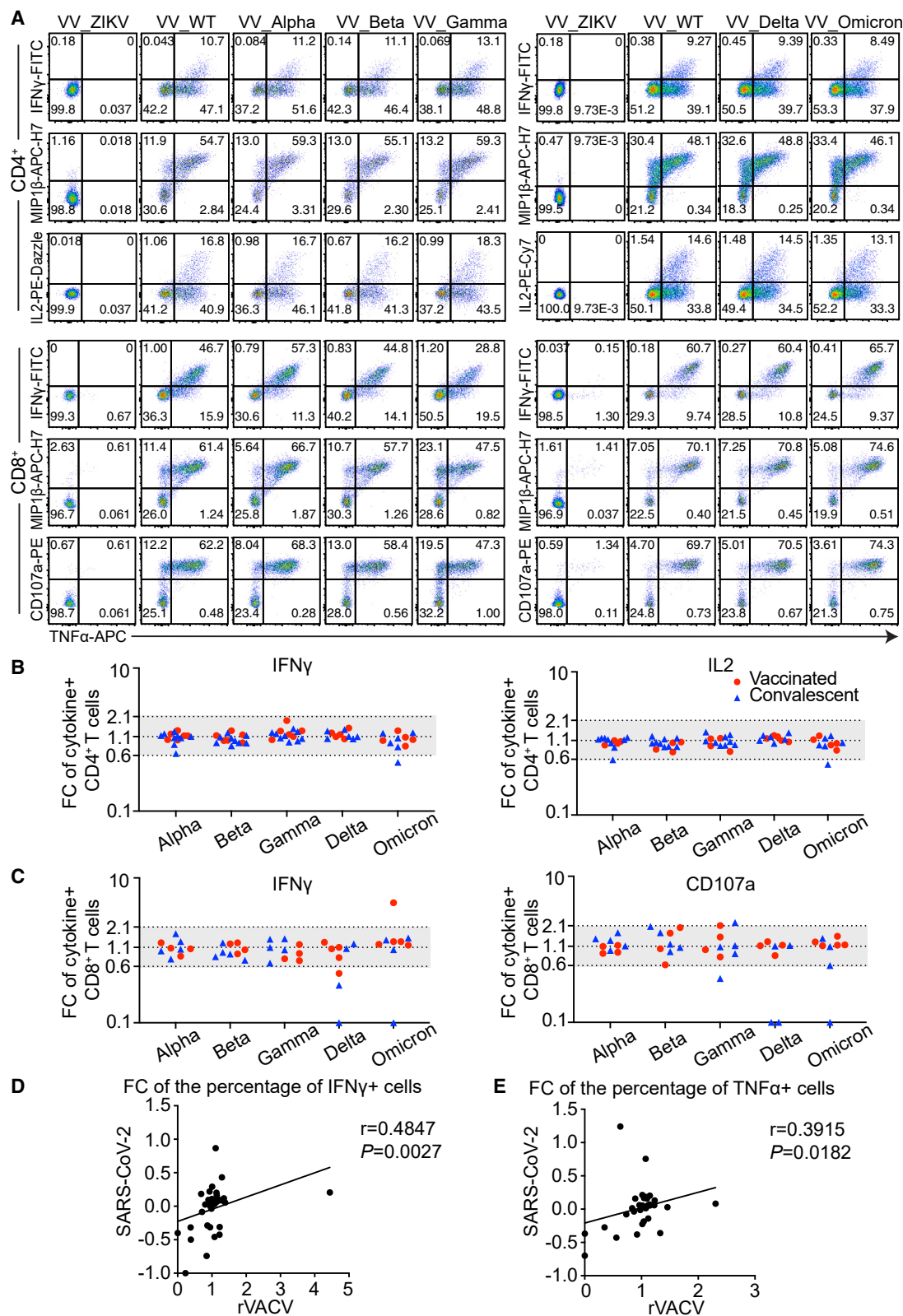


**Figure 3. Evaluation of spike-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses to BCLs infected with rVACV expressing WT SARS-CoV-2 spike**  
(A) Diagram of the rVACV system (created with BioRender).  
(B) Expression of antigens by rVACV after infection of BCLs. The infection rate was assessed by intracellular staining of the VACV D8 protein. Isotype: unstained control.  
(C) Representative cytokine responses of CD4<sup>+</sup> and CD8<sup>+</sup> T cells of a vaccinated participant and a convalescent patient to BCLs infected with VV\_WT or VV\_ZIKV.  
(D) Proportion of T cells expressing one, two, three, four, or all five of the following functional markers: IFN $\gamma$ , TNF $\alpha$ , IL2, MIP1 $\beta$ , and CD107a.

SARS-CoV-2 spike proteins (Figure 3A). The spike sequence of ancestral SARS-CoV-2 (wild-type [WT] spike) linked to an early/late vaccinia virus (VACV) promoter was inserted into the thymidine kinase locus of VACV strain Lister. Additional rVACVs expressing spike proteins from SARS-CoV-2 VOCs Alpha, Beta, Gamma, Delta, and Omicron were constructed. As a specificity control, an rVACV expressing the Zika virus (ZIKV) pre-membrane protein (prM)<sup>15</sup> was constructed and analyzed in parallel. The expression of each foreign antigen was confirmed by immunoblotting extracts of HEK293T cells infected by these rVACVs using antibody to a FLAG epitope added to the N terminus of each protein (Figure S4). It is unlikely that any of the donors from our cohorts would have been exposed to ZIKV, due to their county of residence being the UK, and therefore T cells tested in this study would not respond to the rVACV carrying this viral antigen.

We infected autologous BCLs with rVACV expressing either WT spike (VV\_WT) or ZIKV prM (VV\_ZIKV) and evaluated the function of spike-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells in both vaccinated and convalescent cohorts. Both VV\_WT and VV\_ZIKV showed a similar infection rate in the target BCLs (Figure 3B). Similar to SARS-CoV-2 infection, cytokine secretion of SARS-CoV-2 spike-specific CD4<sup>+</sup> or CD8<sup>+</sup> T cell lines was assessed by ICS after co-culturing with rVACV-infected BCLs. As shown in Figures 3C and 3D, strong CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses against WT spike were observed, with both CD4<sup>+</sup> and CD8<sup>+</sup> T cells displaying poly-functionality by expressing at least two functional effector molecules, including IFN $\gamma$ , TNF $\alpha$ , IL2, MIP1 $\beta$ , and CD107a.

Consistent with SARS-CoV-2 infection results in BCLs-ACE2 cells, no differences in functionality were observed between CD4<sup>+</sup> or CD8<sup>+</sup> T cells elicited by vaccination and infection. Taken



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together, these data suggest that rVACV can be used as an alternative to SARS-CoV-2 for rapid evaluation of T cell responses to naturally processed spike antigens.

### Evaluation of cross-reactivity of spike-specific T cells against SARS-CoV-2 variants using rVACV system

We next used the rVACV system to investigate the impact of SARS-CoV-2 viral mutations on T cell recognition. In order to do this, rVACVs expressing spike protein from Alpha, Beta, Gamma, Delta and Omicron strains (named VV\_Alpha, VV\_Beta, VV\_Gamma, VV\_Delta and VV\_Omicron, respectively) were used.

As before, T cells were cocultured with rVACV-infected autologous BCLs and their responses to the spike antigen were assessed subsequently by ICS. Strong CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses against rVACV carrying WT or mutant spikes were observed (Figure 4A). For each donor, we calculated the fold change (FC) in both CD4<sup>+</sup> T cell response (IFN $\gamma$ - or IL2-producing cells; Figure 4B) and CD8<sup>+</sup> T cell response (IFN $\gamma$ - or CD107a-expressing cells; Figure 4C) against spike protein of VOCs compared with that seen to the WT spike. We found that most of donors showed minimal variance in T cell responses to each VOC spike protein with an FC threshold of 2 (in a range between 0.6 and 2.1 as indicated by the dotted lines). This suggests that most of the memory CD4<sup>+</sup> and CD8<sup>+</sup> spike T cell responses elicited by vaccination and natural infection are cross-reactive to SARS-CoV-2 VOCs.

In parallel with the rVACV system, we also assessed the T cell recognition of BCLs-ACE2 infected with SARS-CoV-2 variants including Delta and Omicron, as well as the ancestral strain, by co-culturing the T cells with infected BCLs. Comparable CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses were observed against each SARS-CoV-2 strain (Figure S5), showing that these spike-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells can cross-recognize VOCs. The pattern of T cell cross-recognition to the VOCs tested here is similar to that observed in the rVACV system. As shown in Figures 4D and 4E, a strong correlation of the FC in IFN $\gamma$ -producing cells and TNF $\alpha$ -producing cells between these two systems was observed. This indicates that rVACVs can be used as an alternative to SARS-CoV-2 for rapid evaluation of cross-reactivity of T cells to emerging viral variants.

### Identification of T cell escape mutants using the rVACV infection system

While the majority of responses fell within the threshold of FC we set to show variance in the T cell response, several outliers were also observed (Figures 4B and 4C). These outliers showed

reduced or absent responses to spike variants, indicating potential T cell escape. As summarized in Figure 5A, we identified one potential CD4<sup>+</sup> T cell escape in response to Alpha and Omicron spike protein, three CD8<sup>+</sup> T cell escapes in response to Delta and Omicron, and one CD8<sup>+</sup> immune escape by Omicron (three convalescent, one vaccinated). These potential T cell immune escapes were confirmed by the same pattern of compromised T cell responses in the SARS-CoV-2 infection system (Figure 5B).

### Confirmation of immune escape at dominant T cell epitopes

To identify the epitopes responsible for the escape seen above, dominant T cell epitopes in the individuals with compromised T cell responses to SARS-CoV-2 variants were mapped and the T cell response to the epitopes was then evaluated. In brief, the dominant epitope for each individual was determined using long spike peptides.<sup>16</sup> Peptide-specific T cells were isolated and expanded prior to assessment by ICS of their response against SARS-CoV-2 variants.

As shown in (Figures 4B and 5A), compromised recognition of Delta spike by CD8<sup>+</sup> T cells was detected in a vaccinated individual, J033. Using exogenous pulsing of WT spike peptide pools<sup>16</sup> and analysis via ELISpot assay, we determined that this donor responded most dominantly to pool 6 and pool 16 (Figure 6A). Within each of these pools, we had already identified a dominant epitope response against the long peptides S90 (<sub>446</sub>GGNYNYLYRLFRKSN<sub>460</sub>) and S242 (<sub>1206</sub>YEQYIKWPWYIWLGF<sub>1220</sub>), respectively. The sequence of S242 is conserved among all SARS-CoV-2 spike variants tested in this study, while S90 has an L452R substitution in the Delta variant (Table S2), suggesting that this mutation may be responsible for the escape seen here.

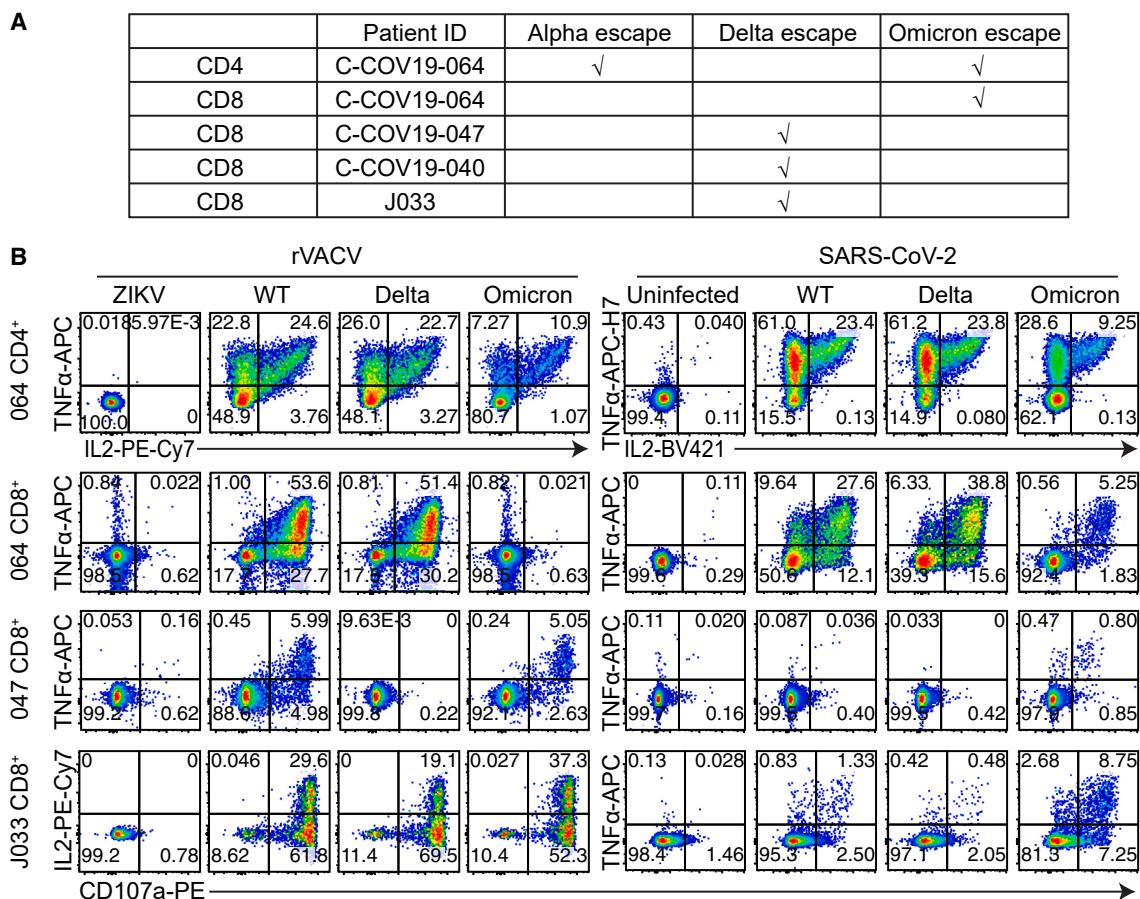
Within the S90 peptide, a CD8 epitope spike<sub>448-456</sub> (NYNYLYRLF), restricted by HLA-A\*24:02, was reported.<sup>17,18</sup> In accordance with this, S90-specific CD8<sup>+</sup> T cells were isolated by cytokine production and their HLA restriction was confirmed by ELISpot with partially HLA-matched BCLs (Figure 6B). The HLA restriction of HLA-A\*24:02 and epitope sequence spike<sub>448-456</sub> (NYNYLYRLF) of this CD8 response were further confirmed by a positive staining with peptide-MHC class I tetramer (Figure 6C).

Next, we tested the recognition of WT spike<sub>448-456</sub> (NYNYLYRLF) and spike<sub>448-456</sub> L452R (<sub>448</sub>NYNYRYRLF<sub>456</sub>) by S90-specific CD8<sup>+</sup> T cells. Strong recognition of WT spike<sub>448-456</sub> was observed while responses to spike<sub>448-456</sub> L452R were completely abolished (Figure 6D), confirming the T cell immune escape due to L452R mutation that has previously been

**Figure 4. Evaluation of spike-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses to SARS-CoV-2 VOCs using rVACV and SARS-CoV-2 infection systems**

(A) Representative cytokine responses of CD4<sup>+</sup> and CD8<sup>+</sup> T cells of a participant to BCLs following rVACV infection.  
(B) FCs of the percentage of IFN $\gamma$  or IL2+ CD4<sup>+</sup> T cells in response to spike variants were calculated relative to that in response to WT spike. For Alpha, Beta, and Gamma, n = 13 (four vaccinated in red and nine convalescent in blue); for Delta and Omicron, n = 11 (five vaccinated in red and six convalescent in blue).  
(C) FCs of the percentage of IFN $\gamma$  or CD107a+ CD8<sup>+</sup> T cells in response to spike variants were calculated relative to that in response to WT spike. For Alpha, Beta, and Gamma, n = 9 (four vaccinated in red and five convalescent in blue); for Delta and Omicron, n = 9 (five vaccinated in red and four convalescent in blue). Pseudocounts 0.1 was added to each FC value (B and C).  
(D and E) Correlation of FC of the percentage of IFN $\gamma$ + (D) or TNF $\alpha$ + (E) CD4<sup>+</sup> or CD8<sup>+</sup> T cells evaluated using SARS-CoV-2 and rVACV. Correlation analysis was performed using Spearman's rank correlation coefficient and the two-tailed p value was calculated. The final y values plotted on graph (D and E) were derived with the equation  $y = \log_{10}(FC + 0.1)$ . n = 36 were 18 tested lines responding to Delta and Omicron.





**Figure 5. Reduced T cell responses against SARS-CoV-2 variants**

(A) Potential T cell immune escape observed by rVACV infection system.

(B) Flow cytometry plots showing one reduced CD4<sup>+</sup> cytokine response against Omicron and three reduced CD8<sup>+</sup> cytokine responses against Delta.

reported.<sup>17</sup> Reduced responses against rVACV expressing Delta spike and SARS-CoV-2 Delta were also observed, confirming T cell escape via the L452R mutation (Figure 6E).

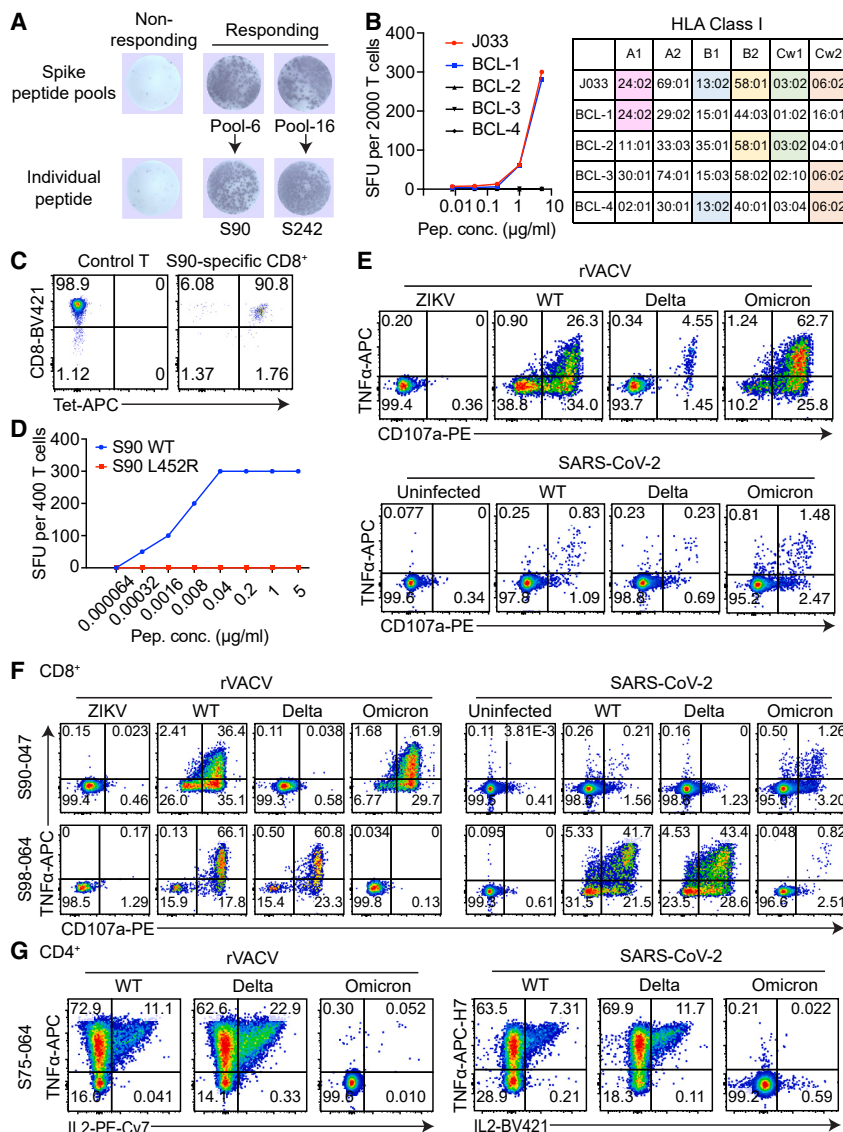
The additional potential escape variants as shown in Table S1 were mapped as above and confirmed through both rVACV and SARS-CoV-2 infection using epitope-specific T cells (Figures 6F and 6G). We identified escape of the S90 CD8<sup>+</sup> epitope in another convalescent donor (C-COV19-047) as well as additional escape within the Omicron variant through the CD8<sup>+</sup> epitope S98<sub>(489YFPLQSYGF<sub>497</sub>)</sub> in convalescent donor C-COV19-064. In addition, this donor C-COV19-064 displayed escape in CD4<sup>+</sup> cells through the epitope S75<sub>(371SASFSTFKCYGVSP<sub>385</sub>)</sub>. Summary data for escape variants identified can be found in Table 1. Alignment of epitope sequence is shown in Table S2.

## DISCUSSION

Peptide-based and predictive assays have been highly informative for T cell responses since the start of the SARS-CoV-2 pandemic; however, while invaluable for the rapid collection of information and determination of T cell responses, these assays

do not take into account the processing of the antigens that occurs naturally. Here we have established both a SARS-CoV-2 infection system and an rVACV system to evaluate T cell responses to naturally processed spike antigens. Using these two systems, we evaluated SARS-CoV-2 spike-specific T cell responses from individuals after the first dose of ChAdOx1 nCoV-19 vaccination as well as patients who recovered from COVID-19. Our data demonstrate that SARS-CoV-2 spike-specific T cells can cross-recognize SARS-CoV-2 variants in the majority of individuals studied in both cohorts. However, T cell immune escape to both CD4<sup>+</sup> and CD8<sup>+</sup> overall responses were observed in one out of nine donors within the vaccine cohort and three out of nine in the convalescent cohort, providing further evidence of SARS-CoV-2 escape from dominant T cell responses during long-term chronic infection.<sup>17,19–22</sup> Mutations in several dominant epitope responses responsible for observed escape were subsequently identified.

The systems established in this study allowed us to evaluate overall T cell responses and the level of cross-reactivity to VOCs by memory T cell responses induced by both vaccinated and naturally infected individuals. We are able to assess the direct impact of epitope mutations on T cell receptor



**Figure 6. Identification and confirmation of single escape mutations in Delta and Omicron**

(A) Example of CD8<sup>+</sup> epitope mapping by ELISpot using autologous BCLs loaded with spike peptide pools and single peptides.

(B) Identification of HLA restriction. T cell responses to peptide-loaded BCLs expressing HLA molecules partially overlapped with autologous BCLs were assessed by IFN $\gamma$  ELISpot.

(C) S90-HLA-A\*24:02 tetramer staining of S90-specific CD8<sup>+</sup> T cells.

(D) IFN $\gamma$  ELISpot assay showed response of S90-specific CD8<sup>+</sup> T cells to BCLs loaded with WT peptide and peptide variant of S90 L452R.

(E) ICS showed response of S90-specific CD8<sup>+</sup> T cells to BCLs infected with rVACV expressing WT spike or spike variants and BCLs infected with live WT SARS-CoV-2 or variants.

(F) ICS showed the recognition of T cells to CD8<sup>+</sup> epitopes containing escape mutations.

(G) ICS showed the recognition of T cells to one CD4<sup>+</sup> epitope containing escape mutation. The recognition of T cells to WT epitope and epitope variants was assessed by rVACV expressing WT spike or spike variants and BCLs infected with WT SARS-CoV-2 or variants.

vides a very high bar for immune escape, which is very relevant in this context.

In this study, we were able to assess the differences in vaccine- and infection-induced immunity. In our small cohort, which was limited by the proportion of BCLs transduced with ACE2, it was encouraging that we saw relatively few differences in the activation or functionality of the memory T cells induced by natural infection or vaccination.

Our study has demonstrated two informative systems for the analysis of T cell responses to SARS-CoV-2 and VOCs. The

use of these systems will enable greater understanding of T cell responses to the virus, cross-reactivity to viral variants, and the differences between vaccine- and infection-induced immunity to SARS-CoV-2 and other emerging viruses in the future.

### Limitations of the study

There are some limitations of our study. The system requires short-term culture and expansion of T cells *in vitro*. While this method may mimic the recall memory response of T cells to

engagement.<sup>20</sup> The systems also allow us to evaluate the overall contribution to T cell escape through the entire antigen processing and presentation pathway with newly emerging viral variants.<sup>23</sup> Using the rVACV system in particular, we are able to assess T cell responses to new variants in a rapid manner. We acknowledge that rVACV expresses different levels of recombinant proteins, although the use of autologous human BCLs provides a far more relevant model of naturally occurring antigen levels, and supraphysiological antigen expression by rVACV pro-

**Table 1. T cell epitopes that contain immune escape mutations**

Epitope ID	Epitope sequence	CD4/CD8	HLA restriction	Escaped variant	Escape mutations	Donors
S90	NYNYLYRLF	CD8	A*24:02	Delta	L452R	J033, C-COV19-047
S98	YFPLQSYGF	CD8	A*29:02	Omicron	G496S	C-COV19-064
S75	SASFSTFKCYGVSP	CD4	HLA-DRB1 15:01	Omicron	Not confirmed	C-COV19-064

expand rapidly upon viral infection *in vivo*, it may also bias toward rapidly expanded T cell clonal types with high antigen sensitivity *in vitro*. Furthermore, for this study, we focused on the evaluation of effector function of the T cells such as cytokine production and cytotoxicity to quantify CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses. Although such methods would ensure the specificity of the T cell detected, it might not be as sensitive as other commonly used methods for antigen-specific T cell detection and quantification, such as activation-induced marker (AIM) assay.<sup>24</sup> We also acknowledge that rVACV-expressed antigens can sometimes completely bypass normal requirements for the transporter associated with antigen processing (TAP) and expresses different levels of recombinant proteins. In addition, entry of antigen into processing for HLA class II presentation can be limited by intracellular expression systems. Finally, our cohorts have small sample size and limited genotypic diversity in the UK population.

## 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.celrep.2023.112470>.

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## AUTHOR CONTRIBUTIONS

T.D., G.L.S., Y.P., T.L., S.G., and A.P. conceptualized the project. Y.P. and T.D. designed and supervised T cell experiments. T.D. coordinated the study. G.L.S. supervised the rVACV work. T.L. supervised work from the vaccination donor cohort. Y.P. generated T cell lines from COVID-19-recovered donors. L.G. generated T cell lines from vaccinated donors. Z.Y., J.-L.C., B.W., G.L., and X.Y. performed all T cell experiments. Y.L. and Q.Z. made the rVACVs. P.H. and R.B. made the ACE2 constructs and lentivirus. T.R. performed HLA typing. J.C.K. and A.J.M. established the convalescent cohort and collected clinical samples and data. T.L., S.G., K.W., and A.P. established the vaccinated cohort and collected clinical samples and data. C.W., S.-A.C., K.C., P.S., P.A.C.W., W.D., P.S., C.L., J.M., and G.R.S. provided technical assistance and critical reagents. Z.Y., J.-L.C., B.W., and Y.L. analyzed the data. Y.P. and T.D. supervised data analysis. T.D., Y.P., Z.Y., J.-L.C., and D.W. wrote the original draft. All authors reviewed and edited the manuscript.

## DECLARATION OF INTERESTS

A.J.P. is chair of the DHSC Joint Committee on Vaccination and Immunisation (JCVI) but does not take part in the JCVI COVID-19 committee. Oxford University has entered into a partnership with AstraZeneca for development of a COVID-19 vaccine. A.J.P. has provided advice to Shionogi & Co., Ltd., on the development of a COVID-19 vaccine.

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## STAR★METHODS

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Antibodies</b>		
BV421-anti-human IL-2	BioLegend	Cat# 500328; RRID:AB_10962947
APC/Cy7-anti-human TNF- $\alpha$	BioLegend	Cat# 502944; RRID:AB_2562870
APC-anti-human CD4	Invitrogen	Cat# 17-0047-42;RRID:AB_1548692
APC-H7-anti-human MIP-1 $\beta$	BD Biosciences	Cat# 17-7321-82; RRID:AB_469508
BV421-anti-human CD8	BioLegend	Cat# 344748; RRID:AB_2629584
PE/Cy7-anti-human IFN $\gamma$	BD Biosciences	Cat#560924; RRID:AB_396760
APC-anti-human TNF- $\alpha$	Invitrogen	Cat# 17-7321-82; RRID:AB_469508
PE-anti-human CD107a	BD Biosciences	Cat#560948; RRID: AB_396135
<b>Bacterial and Virus Strains</b>		
SARS-CoV-2 Victoria strain	Public Health England	N/A
SARS-CoV-2 Delta strain	Gavin Screaton *Dejnirattisai, et al. 2022	N/A
SARS-CoV-2 Omicron strain	Gavin Screaton *Liu C,et al. 2021 <sup>25</sup>	N/A
<b>Biological Samples</b>		
Human blood	COVID-19 patients were recruited from the John Radcliffe Hospital (Oxford University Hospitals NHS Trust) in Oxford, UK. The vaccinated cohort were participants in COV002; a phase 2/3 study to determine the efficacy, safety and immunogenicity of the candidate Coronavirus Disease (COVID-19) vaccine ChAdOx1 nCoV-19. Sponsor: University of Oxford. Chief Investigator: Professor Andrew Pollard. REC Number: 20/SC/0179 EudraCT Number: 2020-001228-32 IRAS Reference: 281904.	N/A
<b>Chemicals, Peptides, and Recombinant Proteins</b>		
SARS-CoV-2 spike peptides	GenScript	N/A
<b>Critical Commercial Assays</b>		
ELISpot assay	Mabtech AB	Cat#3420-2APT-2
<b>Experimental Models: Cell Lines</b>		
SARS-CoV-2 spike-specific CD4 <sup>+</sup> and CD8 <sup>+</sup> T cell lines	this paper	N/A
EBV-transformed B cell lines	this paper	N/A
<b>Software and Algorithms</b>		
GraphPad Prism 9	Dotmatics	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, Yanchun Peng ([yanchun.peng@imm.ox.ac.uk](mailto:yanchun.peng@imm.ox.ac.uk)).

#### Materials availability

Materials and reagents generated in this study are available from the [lead contact](#) upon request.

#### 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 SUBJECT DETAILS

### Study participants and clinical definitions

COVID-19 patients were recruited from the John Radcliffe Hospital (Oxford University Hospitals NHS Trust) in Oxford, UK, between March 2020 and September 2021 by identification of patients hospitalized during the SARS-CoV-2 pandemic and recruited into the Sepsis Immunomics study and International Severe Acute Respiratory and Emerging Infection Consortium World Health Organization Clinical Characterisation Protocol UK (IRAS260007 and IRAS126600). Patients were sampled at least 28 days after symptom onset. Written informed consent was obtained from all patients. Ethical approvals were given by the South Central-Oxford C Research Ethics Committee in England (reference: 19/SC/0296 and 13/SC/0149). Clinical definitions, recruitment, sampling and phenotyping were defined as previously described.<sup>16</sup> The vaccinated cohort were participants in COV002<sup>26</sup>; a phase 2/3 study to determine the efficacy, safety and immunogenicity of the candidate Coronavirus Disease (COVID-19) vaccine ChAdOx1 nCoV-19. Sponsor: University of Oxford. Chief Investigator: Professor Andrew Pollard. REC Number: 20/SC/0179 EudraCT Number: 2020-001228-32 IRAS Reference: 281904. Clinical characteristics, age and sex of participants in both cohorts are summarized in Table S1.

### SARS-CoV-2 spike-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell lines

Short-term SARS-CoV-2-specific T cell lines were established as described.<sup>27</sup> Briefly,  $3 \times 10^6$  to  $5 \times 10^6$  PBMCs were pulsed for 1 h at 37°C with 10  $\mu$ M SARS-CoV-2 spike peptide pools and cultured in R10 (RPMI 1640 medium with 10% foetal calf serum, 2 mM glutamine and 100 mg ml<sup>-1</sup> of penicillin-streptomycin) at  $2 \times 10^6$  cells per well in a 24-well Costar plate. IL-2 was added to a final concentration of 100 U ml<sup>-1</sup> on day 3 and cultured for a further 10–14 d. Spike-specific CD4<sup>+</sup> and CD8<sup>+</sup> T cell lines were then isolated by FACS of IFN- $\gamma$ , TNF $\alpha$  or IL-2-secreting T cells after stimulation with spike peptides and expanded as described.<sup>28</sup> S98 T cell clones were generated by FACS of S<sub>486-500</sub>-A\*29:02 CD8<sup>+</sup> T cells at a single-cell level from short-term cell lines.

### ACE2-transduced EBV-transformed B cell lines

EBV-transformed BCLs were generated as described.<sup>29</sup> The cDNA for the human ACE2 mRNA (ENSG00000130234) was cloned into a lentiviral vector that allows coexpression of enhanced green fluorescent protein and a puromycin resistance marker (Addgene, plasmid no. 17488). The plasmids were cotransfected with packaging plasmids pMD2.G and psPAX2 into HEK293-TLA using PEIpro (Polyplus). Lentiviral supernatant was collected 48 h and 72 h post-transfection and concentrated by ultracentrifugation. EBV-transformed BCLs were infected by ACE2-expressing lentivirus at a multiplicity of infection (MOI) of 0.1 with 8  $\mu$ g ml<sup>-1</sup> of polybrene (Sigma-Aldrich) overnight, and then washed and cultured for 3–5 d. ACE2-expressing B cells were stained using primary goat anti-human ACE2 antibody (R&D, 1:20) and donkey anti-goat AF647 secondary antibody (Abcam, 1:1,000), followed by cell sorting via flow cytometry. B cells with stable expression of ACE2 were maintained with 0.5  $\mu$ g ml<sup>-1</sup> of puromycin (Thermo Fisher Scientific). Mycoplasma testing was carried out every 4 weeks with all cell lines using MycoAlert detection kit (Lonza).

### SARS-CoV-2 strains

SARS-CoV-2 Victoria 01/20 strain (BVIC01) was originally from Public Health England and provided by J. McKeating (University of Oxford).<sup>30</sup> The Delta (B.1.617.2) variant was originally from W. Barclay and T. De Silva (G2P-UK) and provided by G. Screaton (University of Oxford).<sup>25</sup> The Omicron (B.1.1.529) variant was provided by G. Screaton.<sup>31</sup>

## METHOD DETAILS

### Generation of rVACV-expressing spike proteins and Zika pre-membrane protein (prM)

A plasmid containing a codon-optimised SARS-CoV-2 spike gene from the Wuhan 2019 SARS-CoV-2 strain with a C-terminal HA tag was a kind gift from James Edgar and Sean Munro (University of Cambridge, UK). This plasmid was modified to, first, replace the HA tag with the FLAG epitope by overlapping polymerase chain reaction (PCR), and, second, to remove a cryptic poxvirus early transcription termination signal (TTTTTCT),<sup>32</sup> at nucleotides 172 to 178. This was mutated to TTCTTCT by overlap extension PCR, without changing the amino acid sequence. Given that amino acid mutations N501Y and D614G substitution were present in Alpha, Beta and Gamma variants, the spike gene was further modified to introduce these mutations. The spike sequence modified with D614G was prepared for Delta the variant. Other amino acid substitutions of Alpha, Beta, Gamma and Delta were introduced by site-directed mutagenesis (NEB, catalog no. E0554S) following the manufacturer's instruction. Lastly, a codon-optimized spike gene from SARS-CoV-2 variants Omicron BA.1 and BA.2 were synthesized by GenScript, again with removal of any cryptic poxvirus early termination signal. The plasmids containing these different SARS-CoV-2 spike genes were digested with *KpnI* and *SacII* and the resulting fragment was cloned into a modified VACV expression vector, pSC11,<sup>33</sup> downstream of the VACV p7.5 early/late promoter. To facilitate this cloning, pSC11 was first modified by insertion of the sequence GGATACCGCGGCCCGCCGCGG to introduce unique *KpnI* and *SacII* restriction sites downstream of the VACV promoter. The ZIKV prM sequence was amplified by PCR from a ZIKV infectious cDNA (icDNA) clone (pCCI-SP6-PE243 ZIKV), a kind gift from Nerea Irigoyen, (Department of Pathology, University of Cambridge, UK). An FLAG tag was added to the C-terminus of the ZIKV prM gene by PCR amplification. The resulting PCR fragment was digested with *KpnI* and *SacII* and inserted into the pSC11 vector as described above.

### Construction and titration of rVACVs

A set of rVACVs expressing the different SARS-CoV-2 spike proteins was produced as described.<sup>33,34</sup> In brief, HEK293T cells (American Type Culture Collection (ATCC), catalog no. CRL-11268) were transfected with 3  $\mu$ g of each pSC11-derived plasmid containing spike with poly(ethylenimine). At 2 h post-transfection, cells were infected with VACV strain Lister at an MOI of 1 for 48 h. Infected cells were harvested and rVACVs were identified and plaque purified using TK<sup>-</sup>143B cells (ATCC, catalog no. CRL-8303) in the presence of 25  $\mu$ g mL<sup>-1</sup> bromodeoxyuridine (BUdR). The rVACVs were identified by addition of 25  $\mu$ g mL<sup>-1</sup> of X-gal in the agarose overlay so that cells expressing  $\beta$ -galactosidase turned blue, thereby enabling visual identification of rVACV plaques. After 3 rounds of plaque purification on TK<sup>-</sup>143B cells in the presence of BUdR, master stocks of rVACV were prepared by infection of rabbit kidney 13 (RK13) cells (ATCC, catalog no. CCL37) and these stocks were titrated by plaque assay on African green monkey BS-C-1 cells (ATCC, catalog no. CCL26) cells. DNA from rVACVs was prepared with proteinase K digestion at 56°C for 30 min, followed by heat-inactivation at 95°C for 10 min. The TK locus of each rVACV was amplified by PCR and sequenced to confirm the insertion of indicated sequences.

### Infection of BCLs with rVACV-expressing spike or Zika-M

EBV transformed BCLs were infected with rVACVs expressing SARS-CoV-2 spike variants or ZIKA prM at a MOI of 3 for 2 h at 37°C. Cells were then washed three times to remove unbound virus and incubated 16–18 h in R10 at 37°C. Cells were then counted and co-cultured with autologous CD8<sup>+</sup> or CD4<sup>+</sup> T cells at an E:T ratio of 1:1. Degranulation (CD107a expression) and cytokine production of T cells were then evaluated by intracellular cytokine staining (ICS) as described below.

### Quantitation of rVACV infection of B cells

To check for equal infection of B cells by rVACVs, expression of VACV structural protein D8 was analysed at 24 h p.i. Dead cells were labelled using Live/Dead Fixable Aqua dye (Invitrogen). Live cells were then washed and fixed with intracellular fixation buffer (Invitrogen) at 4°C for 30 mins, washed twice with permeabilization buffer (Invitrogen), and then stained with the mouse monoclonal antibody AB.1.1 specific for VACV protein D8,<sup>35,36</sup> or isotype control. Cells were then incubated with PE-conjugated goat anti-mouse IgG (Poly4053, Biolegend) prepared in permeabilization buffer for 30 mins at 4°C before being washed once with permeabilization buffer and followed by FBS buffer (10% FBS with PBS). Finally, cells were analysed by an Attune NxT flow cytometer (Invitrogen, software v.3.2.1) with compensation applied from single-stained beads, and analysed using FlowJo v.10 software (FlowJo LLC).

### SARS-CoV-2 propagation and titration

In brief, Victoria 01/20, Delta and Omicron variants were propagated in Vero E6/TMPRSS2 cells (provided by A.R.M. Townsend, University of Oxford). Naive Vero E6/TMPRSS2 cells were plated overnight and infected with SARS-CoV-2 at an MOI of 0.003. Cultures were harvested when visible cytopathic effect was observed 48–72 h later. Virus-containing supernatant was collected, aliquoted and stored at –80°C. The viral titre (pfu/mL) was determined by plaque assay on Vero E6/TMPRSS2 cells.

### IFN $\gamma$ ELISpot with T cell lines

IFN- $\gamma$  ELISpot assays were performed using antigen-specific T cell lines as described.<sup>16</sup> In brief, 20,000 autologous EBV-transformed BCLs were first loaded with peptides at five-fold titrated concentrations for 1 h and subsequently cocultured with 2,000 T cells at an effector: target (E:T) ratio of 1:10 for 16–18 h. Assays were performed in standard human IFN $\gamma$  ELISPOT assays according to suppliers' instruction (MABTECH). To quantify antigen-specific responses, data were collected with AID ELISpot 7.0, mean spots of the control wells were subtracted from the positive wells and the results were expressed as spot-forming units (s.f.u.) per 2,000 cells. Responses were considered positive if results were at least three times the mean of the negative control wells and >1 s.f.u. per 1,000 cells.

### Intracellular cytokine staining (ICS) for SARS-CoV-2 infection

Intracellular cytokine staining (ICS) was performed as described.<sup>16</sup> Briefly, T cells were cocultured with SARS-CoV-2 strains Victoria 01/20, Delta or Omicron-infected BCLs-ACE2 at an E:T ratio of 1:2 for 6 h together with GolgiPlug and GolgiStop. The cells were then surface stained with PE-anti-CD107a (BD Biosciences, 1:20). Dead cells were labelled using Live/Dead Fixable Aqua dye (Invitrogen, 1:1000). After fixated with Cytofix/Cytoperm (BD Biosciences), CD8<sup>+</sup> T cells were stained with BV421-anti-CD8 (Biolegend, 1:33), PE-Cy7-anti-IFN $\gamma$  (BD Biosciences, 1:33), APC-anti-TNF $\alpha$  (eBioscience, 1:500) and APC-H7-anti-MIP1 $\beta$  (BD Biosciences, 1:33); CD4<sup>+</sup> T cells were stained with APC-anti-CD4 (ThermoFisher, 1:33), PE-Cy7-anti-IFN $\gamma$  (BD Biosciences, 1:33), APC-H7-anti-TNF $\alpha$  (Biolegend, 1:33) and BV421-anti-IL2 (Biolegend, 1:33). Negative controls without virus infection were run for each sample. All samples were acquired on Attune NxT Flow Cytometer (software v.3.2.1) and analyzed using FlowJo v.10 software (FlowJo LLC).

### Peptide-MHC class I tetramer staining

A total of  $1 \times 10^5$  live cells from S90 specific T cell lines or control T cell lines were labelled with tetramer-APC:HLA-A\*24:02 complexed with S<sub>451–459</sub> peptide NYNYLYRLF, produced in-house using standard methods,<sup>29</sup> and incubated for 15 min at 37°C. Cells were then incubated with BV421-anti-CD8 (1:40, BD, Oxford, UK) and propidium iodide (eBioscience). All samples were acquired on Attune NxT Flow Cytometer (software v.3.2.1) and analyzed using FlowJo v.10 software (FlowJo LLC).

### **Evaluation of T cell response to rVACV-spike antigens**

EBV transformed BCLs were infected with rVACVs expressing spike variants or ZIKA prM at a MOI of 3 for 2 h at 37°C. Cells were then washed three times to remove any unbound virus and incubated for 16–18 h in R10 at 37°C. Cells were then counted and co-cultured with autologous CD8<sup>+</sup> or CD4<sup>+</sup> T cells at an E:T ratio of 1:1. Degranulation (CD107a expression) and cytokine production of T cells were then evaluated by ICS as described above.

### **Evaluation of T cell response to SARS-CoV-2 infection**

EBV-transformed BCLs expressing ACE2 were infected with SARS-CoV-2 strains at an MOI of 1 for 2 h at 37°C. Cells were washed and incubated in R10 at 37°C. After 24 h, cells were cocultured with T cells at an E:T ratio of 1:2. Degranulation (CD107a expression) and cytokine production of T cells were evaluated by ICS as described above.

### **SARS-CoV-2 suppression assay**

EBV-transformed BCLs expressing ACE2 were infected with SARS-CoV-2 strains at an MOI of 0.1 for 2 h at 37°C. Cells were then washed and cocultured with T cells at an E:T ratio of 4:1. Control wells containing SARS-CoV-2-infected targets with T cells unspecific to SARS-CoV-2 were also included. After 48 h incubation, cells were washed with PBS and lysed with buffer RLT (QIAGEN). RNA was extracted using RNeasy 96 kit (QIAGEN). Virus genome copies were quantified with Takyon Dry one-step RT-qPCR (Eurogentec) using SARS-CoV-2 (2019-nCoV) CDC qPCR Probe Assay (IDT, ISO 13485:2016) and human  $\beta$ 2-microglobulin as an endogenous control (Applied Biosystems). The suppression rate was calculated by the percentage reduction of virus replication by T cells.

### **QUANTIFICATION AND STATISTICAL ANALYSIS**

Statistical analysis was performed with GraphPad Prism 9 and the figures were made with GraphPad Prism 9. All statistical details of experiments were included in the figure legends. Correlations were performed via Spearman's rank correlation. Statistical significance was set at \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  and \*\*\*\* $p < 0.0001$ . All of the tests were two tailed.