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Supplemental information

Immune responses following third COVID-19 vaccination are reduced in patients with hematological malignancies compared to patients with solid cancer

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Supplementary Material

Table S1: Baseline characteristics of post-second vaccine follow-up and booster vaccination cohorts.

	Post second vaccine dose follow-up cohort, n= 353			Third vaccine cohort, n= 199			
	Cohort, All n= 353	NAb to WT n= 316	No NAb WT n= 37	Cohort, All n=199	NAb to Delta n= 97	No NAb Delta, n= 102	p-value
*Age, years (median, range)	59 (18-87)	59 (18-87)	64 (46–78)	60 (19-84)	59 (19-83)	60 (26 – 81)	0.874
Male	197 (56)	180 (57)	17 (46)	113 (57)	53 (55)	60 (59)	0.659
Ethnicity, white	312 (88)	278 (88)	34 (92)	179 (90)	89 (92)	90 (88)	0.172
***Primary COVID-19 vaccine, n (%)**							
ChAdOx1	255 (72)	233 (74)	22 (59)	134 (67)	61 (63)	73 (72)	0.248
BNT162b2	98 (28)	83 (26)	15 (41)	65 (33)	36 (37)	29 (28)	
Third COVID-19 vaccine, n(%)							
ChAdOx1	-	-	-	0	0	0	
BNT162b2	-	-	-	199 (100)	97 (100)	102 (100)	NA
Time to 3rd vaccination, days median (range)	-	-	-	176 (65-274)	175 (97 – 274)	176 (102 – 258)	0.146
***Cancer type**							
Solid	271 (77)	259 (82)	12 (32)	115 (58)	65 (67)	50 (49)	**0.015**
Hematologic	82 (23)	57 (18)	25 (68)	84 (42)	32 (33)	52 (51)	
Solid cancers	**n =271**	**n = 259**	**n = 12**	**n = 115**	**n = 65**	**n = 50**	
Stage I-II	29 (11)	27 (10)	2 (17)	17 (15)	9 (14)	8 (16)	
Stage III	48 (18)	46 (18)	2 (17)	26 (22)	14 (22)	12 (24)	0.877
Stage IV	194 (72)	186 (72)	8 (67)	72 (63)	42 (65)	30 (60)	
Rx at 1st vaccination							
Chemotherapy, <28 days	53 (20)	30 (12)	3 (25)	26 (23)	15 (23)	11 (22)	1
Targeted therapy, <28 days	87 (32)	73 (28)	3 (25)	39 (34)	21 (32)	18 (36)	0.829
Anti-PD(L)1 ± anti-CTLA4, <183 days	75 (28)	54 (21)	4 (33)	28 (24)	16 (25)	12 (24)	1
No recent SACT	82 (30)	48 (19)	4 (33)	36 (31)	28 (43)	13 (26)	0.958
Rx at 3rd vaccine dose	-	-	-				
Chemotherapy, <28 days	-	-	-	21(18)	12 (18)	9 (18)	1
Targeted therapy, <28 days	-	-	-	41 (36)	21 (32)	20 (40)	0.510
Anti-PD(L)1 ± anti-CTLA4, <183 days	-	-	-	26 (23)	11 (17)	13 (26)	0.591

No recent SACT	-	-	-	45 (39)	28 (43)	13 (26)	0.089
***Hematologic malignancies**	**n = 82**	**n = 57**	**n = 25**	**n = 84**	**n = 32**	**n = 52**	
Diagnosis							
Lymphoma	26 (32)	16 (28)	10 (40)	25 (30)	6 (19)	19 (37)	
Myeloma	29 (35)	27 (47)	2 (8)	29 (35)	11 (34)	18 (35)	
CLL	10 (12)	3 (5)	7 (28)	17 (20)	7 (22)	10 (19)	0.1052
Acute Leukaemia	14 (17)	10 (18)	4 (16)	10 (12)	5 (16)	5 (10)	
Myelodysplastic syndrome	3 (4)	1 (2)	2 (8)	3 (4)	3 (9)	0 (0)	
Rx at first vaccination							
Chemotherapy	5 (6)	3 (5)	2 (8)	7 (8)	2 (6)	5 (10)	0.892
Targeted therapy, <28 days	40 (49)	26 (46)	14 (56)	26 (31)	8 (25)	18 (35)	0.795
Anti-CD20 mAb, <12 mths	8 (10)	0 (0)	8 (32)	7 (8)	0 (0)	7 (13)	0.078
BTKi therapy, <28 days	5 (6)	3 (5)	2 (8)	5 (6)	1 (3)	4 (8)	0.701
No recent SACT	38 (46)	30 (53)	8 (32)	50 (60)	22 (69)	28 (54)	0.261
HSCT, ever	39 (48)	33 (58)	6 (24)	35 (42)	18 (56)	17 (33)	0.057
*Autograft, ever	19 (23)	16 (28)	3 (12)	22 (26)	9 (28)	13 (25)	0.751
Allograft, ever	19 (23)	16 (28)	3 (12)	13 (15)	9 (28)	4 (8)	**0.027**
HSCT, <6 months	3 (4)	2 (4)	1 (4)	7 (8)	4 (13)	2 (4)	0.136
*CAR-T, <6 months	2 (2)	0 (0)	2 (8)	3 (4)	0 (0)	3 (6)	0.436
Rx at 3rd vaccine dose							
*Chemotherapy, <28 days	-	-	-	13 (15)	5 (16)	8 (15)	1
*Targeted therapy, <28 days	-	-	-	31 (37)	11 (34)	20 (38)	1
*Anti-CD20 mAb, <12 mths	-	-	-	10 (13)	0 (0)	10 (19)	**0.022**
*BTKi therapy, <28 days	-	-	-	5 (6)	1 (3)	4 (8)	0.701
*No recent SACT	-	-	-	42 (50)	18 (56)	22 (42)	0.308

Post-second vaccine follow-up cohort: All patients with cancer received 2 doses of COVID-19 vaccination (n=353) and were followed up longitudinally, split by presence or absence of NAb to WT SARS-CoV-2 at 14-28 days post vaccination. **Third vaccination cohort**: patients that received a third COVID-19 vaccination (n=199), with the cohort split by presence or absence of detectable NAb to Delta variant of concern *prior* to third vaccination.

Values are numbers and percentages n(%) unless otherwise stated. Comparison of baseline characteristics was performed using either chi-squared test for categorical variables or Mann-Whitney U test for continuous variables. Variables utilised in multivariate analysis are denoted with *.

BTK-I, Bruton's tyrosine kinase inhibitor; CAR-T, chimeric antigen receptor T cell; CLL, chronic lymphocytic leukaemia; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; HSCT, hematopoietic stem cell transplant; mAB, monoclonal antibody; NR, non-responders; PD-1, programmed death ligand-1; Rx, treatment; SACT, systemic anti-cancer therapy; WT, wildtype.

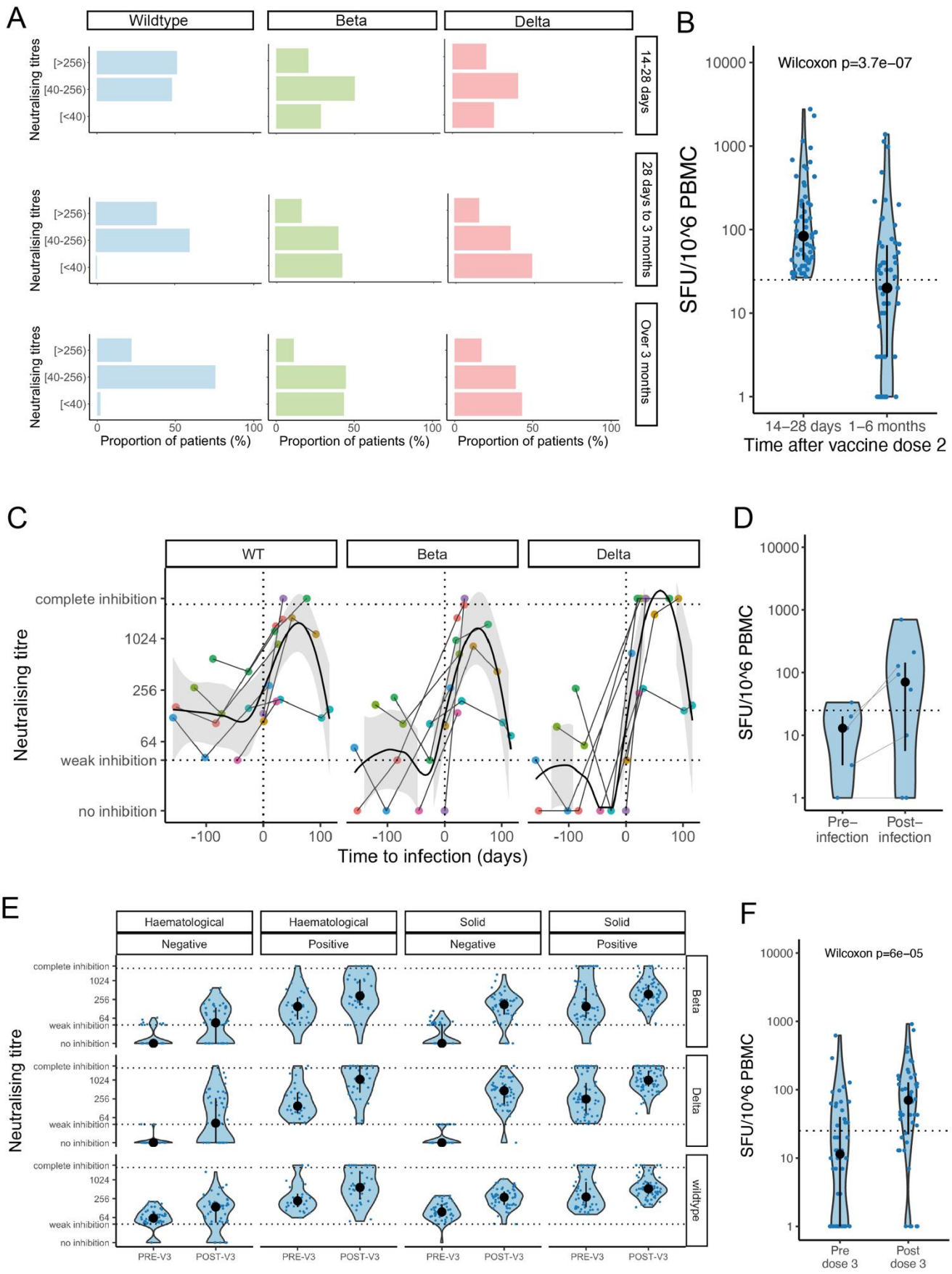
A
Wildtype
Beta
Delta
Neutralising titres
[>256)
[40-256)
[<40)
14-28 days
28 days to 3 months
Over 3 months
0
50
100
Proportion of patients (%)
B
Wilcoxon p=3.7e−07
SFU/10^6 PBMC
10000
1000
100
10
1
14–28 days
1–6 months
Time after vaccine dose 2
C
WT
Beta
Delta
Neutralising titre
complete inhibition
1024
256
64
weak inhibition
no inhibition
-100
0
100
Time to infection (days)
D
SFU/10^6 PBMC
Pre–infection
Post–infection
E
Haematological
Negative
Haematological
Positive
Solid
Negative
Solid
Positive
Beta
Delta
wildtype
PRE-V3
POST-V3
F
Wilcoxon p=6e−05
SFU/10^6 PBMC
Pre dose 3
Post dose 3

Figure S1: Immune responses of patients with cancer after two and three doses of COVID-19 vaccine A) NAbT against WT, Beta, and Delta were measured 14-28 days after first dose and at least 37 days after second vaccine dose. NAbT were grouped into high (IC_{50} titres >256), medium (IC50 titres >40 and ≤ 256) and undetectable (IC50 titres <40). Data are shown as proportion of patients within the indicated timespan. B) Duration of T cell responses in patients with initial positive response after two doses. Responses were measured 14-28 days after second dose or at least one months after second dose and are defined as IFN-y spot-forming units (SFU) per 10^6 PBMC. Dotted line denotes the limit of positivity. Significance was tested by 2-sided Wilcoxon signed rank test. Violin plots denote density of data, Pointrange denotes the median and the 25^{th} and 75^{th} percentile. Patients are indicated as individuals datapoints. C) NAbT against WT, Beta, and Delta in patients with breakthrough infections. Time of infection is denoted with a vertical line; upper and lower limit of detection is denoted with horizontal lines. Values from individual patients are indicated by different colours and are connected. Regression line and 95% CI was fitted using LOESS regression. D) T cell responses in patients with breakthrough infection. Responses are defined as IFN-y spot-forming units (SFU) per 10^6 PBMC. Dotted line denotes the limit of positivity. Violin plots denote density of data, Pointrange denotes the median and the 25^{th} and 75^{th} percentile. Patients are indicated as individuals datapoints when values for both timepoints were available. E) NAbT against Wt, Beta, and Delta after three vaccine doses (POST-V3) in patients with undetectable (Negative) or detectable (Positive) NAbT against Delta after two vaccine doses (PRE-V3). Haematological: patients with haematological malignancies; Solid: patients with solid cancer. Horizontal lines denote upper and lower limit of detection. Violin plots denote density of data, Pointrange denotes the median and the 25^{th} and 75^{th} percentile. Patients are indicated as individuals datapoints F) T cell responses in patients before and after third vaccine dose. Responses are defined as IFN-y spot-forming units (SFU) per 10^6 PBMC. Dotted line denotes the limit of positivity. Significance was tested by 2-sided Wilcoxon signed rank test. Violin plots denote density of data, Pointrange denotes the median and the 25^{th} and 75^{th} percentile. Patients are indicated as individuals datapoints.

Methods

Study design

CAPTURE (NCT03226886) is a prospective, longitudinal cohort study that commenced recruitment in May 2020, and continues to enrol patients at the Royal Marsden NHS Foundation Trust. The study design has been previously published (Au et al., 2020). In brief, adult patients with current or history of invasive cancer are eligible for enrolment. Inclusion criteria are intentionally broad, and patients were recruited irrespective of cancer type, stage, or treatment. Patients recruited to the CAPTURE study who have received at least one dose of COVID-19 vaccine will be included in an analysis to explore vaccine immunogenicity in cancer patients. Patients were included in the analysis regardless of prior SARS-CoV-2 infection status. The primary outcome for this analysis was be the seroconversion rate in cancer patients at 14-28 days following the second dose of vaccine. At establishment of the study protocol, there was no prior published data of seroconversion in cancer patients in this setting and thus sample size was exploratory. The most precise estimate of seroconversion in cancer patients would therefore be achieved through recruitment of as many patients as possible in the time period.

CAPTURE was approved as a substudy of TRACERx Renal (NCT03226886). TRACERx Renal was initially approved by the NRES Committee London, Fulham, on January 17, 2012. The TRACERx Renal sub-study CAPTURE was submitted as part of Substantial Amendment 9 and approved by the Health Research Authority on April 30, 2020 and the NRES Committee London - Fulham on May 1, 2020. CAPTURE was being conducted in accordance with the ethical principles of the Declaration of Helsinki, Good Clinical Practice and applicable regulatory requirements. All patients provided written, informed consent to participate.

Study schedule and follow-up

Clinical data and sample collection for participating cancer patients was performed at baseline (pre-first dose vaccine or within 14 days of first dose vaccine), at timepoints follow-up 1 (FU1; 2-4 weeks post-first dose vaccine); FU2 (within 14 days prior to second vaccine); FU3 (2-4 weeks post-second dose vaccine).

Patient data and sample Sources

Demographic, epidemiological and clinical data (e.g. cancer type, cancer stage, treatment history) were collected from the internal electronic patient record and pseudonymised data was entered into in a cloud-based electronic database (Ninox Software, Berlin, Germany). Regarding systemic-anticancer therapy (SACT), we deemed chemotherapy, targeted therapy (small molecule inhibitors or monoclonal antibodies) or endocrine therapy to be current if given within 28 days of vaccination. CPI given within six months was considered significant given the prolonged receptor occupancy with these agents(Fessas et al., 2017). Concomitant medications were recorded for corticosteroids (considered significant if >10mg prednisolone equivalent given for at least 7 days); GCSF when given within 48 hours of vaccination or five days if pegylated preparation; other immunosuppressive drugs taken within 48 hours of vaccination.

Patients were grouped by cancer diagnosis (solid vs hematological malignancy) for downstream analysis. Where two independent diagnoses of cancer were identified in the same patient, the case was reviewed by two clinicians (STCS & AMS) and the highest stage and/or cancer receiving active

treatment was used for classification. Patients with haematological malignancies were grouped by conventional subtypes.

Detailed sampling schedule and methodology has been previously described(Au et al., 2020). Study biospecimens included per-protocol blood samples, oropharyngeal swabs and cryostored serum from routine clinical investigations. Collected data and study samples were de-identified and stored with only the study-specific study identification number.

Definition of breakthrough SARS-CoV-2 infection

Most patients underwent RT-PCR screening as part of routine clinical care. We considered patients to have had a breakthrough SARS-CoV-2 infection if they had SARS-CoV-2 positive RT-PCR at least 7 days following the second COVID-19 vaccine dose.

WHO classification of severity of COVID-19

We classified severity of COVID-19 according to the WHO clinical progression scale(Reynolds et al., 2020). Uninfected: uninfected, no viral RNA detected – 0; Asymptomatic: viral RNA and/or S1-reactive IgG detected – 1; mild (ambulatory): symptomatic, independent – 2; symptomatic, assistance needed - 3; moderate (hospitalised): no oxygen therapy (if hospitalised for isolation only, record status as for ambulatory patient) – 4; oxygen by mask or nasal prongs - 5; severe (hospitalised): oxygen by non-invasive ventilation or high flow – 6; intubation and mechanical ventilation, $pO_2/FiO_2 \geq 150$ or $SpO_2/FiO_2 \geq 200$ – 7; mechanical ventilation, $pO_2/FiO_2 < 150$ ($SpO_2/FiO_2 < 200$) or vasopressors – 8; mechanical ventilation, $pO_2/FiO_2 < 150$ and vasopressors, dialysis, or extracorporeal membrane oxygenation - 9; Dead - 10.

Handling of whole blood samples

All blood samples and isolated products were handled in a CL2 laboratory inside a biosafety cabinet using appropriate personal protective equipment and safety measures, which were in accordance with a risk assessment and standard operating procedure approved by the safety, health and sustainability committee of the Francis Crick Institute. For indicated experiments, serum or plasma samples were heat-inactivated at 56ºC for 30 minutes prior to use after which they were used in a CL1 laboratory.

Plasma and PBMC isolation

Whole blood was collected in EDTA tubes (VWR) and stored at 4ºC until processing. All samples were processed within 24 hours. Time of blood draw, processing, and freezing was recorded for each sample. Prior to processing, tubes were brought to room temperature (RT). PBMC and plasma were isolated by density-gradient centrifugation using pre-filled centrifugation tubes (pluriSelect). Up to 30 ml of undiluted blood was added on top of the sponge and centrifuged for 30 minutes at 1000g at RT. Plasma was carefully removed then centrifuged for 10 minutes at 4000g to remove debris, aliquoted and stored at -80ºC. The cell layer was then collected and washed twice in PBS by centrifugation for 10 minutes at 300g at RT. PBMC were resuspended in Recovery cell culture freezing medium (Fisher Scientific) containing 10% DMSO, placed overnight in CoolCell freezing containers (Corning) at -80ºC and then stored at -80ºC.

Serum isolation

Whole blood was collected in serum coagulation tubes (Vacuette CAT tubes, Greiner) for serum isolation and stored at 4ºC until processing. All samples were processed within 24 hrs. Time of blood draw, processing, and freezing was recorded for each sample. Tubes were centrifuged for 10 minutes at 2000g at 4ºC. Serum was separated from the clotted portion, aliquoted and stored at -80ºC.

Virus variants & culture

The SARS-CoV-2 reference isolate (referred to as WT) was hCoV19/England/02/2020, obtained from the Respiratory Virus Unit, Public Health England, UK, (GISAID EpiCov accession EPI_ISL_407073). The B.1.1 strain ("D614G") was isolated from a swab from an infected healthcare worker at UCLH, obtained through the SAFER study,2 and carries only the D614G mutation in its spike. The B.1.1.7 isolate ("B.1.1.7") was the hCoV19/England/204690005/2020, which carries the D614G, Δ69-70, Δ144, N501Y, A570D, P681H, T716I, S982A and D1118H mutations,3 obtained from Public Health England (PHE), UK, through Prof. Wendy Barclay, Imperial College London, London, UK through the Genotype-to-Phenotype National Virology Consortium (G2P-UK). The B.1.351 virus isolate was the 501Y.V2.HV001, which carries the D614G, L18F, D80A, D215G, Δ242-244, K417N, E484K, N501Y, A701V mutations, and was kindly provided by Prof. Alex Sigal and Prof. Tulio de Oliveira; 4 sequencing of viral isolates received identified the Q677H and R682W mutations at the furin cleavage site in approximately 50% of the genomes, which was maintained upon passage in cell culture. The B.1.617.2 isolate was MS066352H (GISAID accession number EPI_ISL_1731019), which carries the T19R, K77R, G142D, Δ156-157/R158G, A222V, L452R, T478K, D614G, P681R, D950N, and was kindly provided by Prof. Wendy Barclay, Imperial College London, London, UK through the Genotype-to-Phenotype National Virology Consortium (G2P-UK). All viral isolates were propagated in Vero V1 cells. Briefly, 50% confluent monolayers of Vero E6 cells were infected with the given SARS CoV-2 strains at an MOI of approx. 0.001. Cells were washed once with DMEM (Sigma; D6429), then 5 ml virus inoculum made up in DMEM was added to each T175 flask and incubated at room temperature for 30 minutes. DMEM + 1% FCS (Biosera; FB-1001/500) was added to each flask. Cells were incubated at 37° C, 5% CO^2 for 4 days until extensive cytopathogenic effect was observed. Supernatant was harvested and clarified by centrifugation at 2000 rpm for 10 minutes in a benchtop centrifuge. Supernatant was aliquoted and frozen at -80°C.

Virus PCR and sequencing

All virus stocks generated for use in neutralisation assays were sequence-validated prior to use. To confirm the identity of cultured VoC samples, 8ul of viral RNA was prepared for sequencing by the ARTIC method (https://www.protocols.io/view/ncov-2019-sequencingprotocol-v3-locost-bh42j8ye) and sequenced on the ONT GridION platform to >30k reads / sample. The data was demultiplexed and processed using the viralrecon pipeline (https://github.com/nf-core/viralrecon).

High-throughput live virus microneutralisation assay

High-throughput live virus microneutralisation assays were performed as described previously(Faulkner et al., 2021). Briefly, Vero E6 cells (Institut Pasteur) or Vero E6 cells expressing ACE2 and TMPRSS2 (VAT-1) (Centre for Virus Research)(Rihn et al., 2021) at 90-100% confluency in 384-well format were first titrated with varying MOI of each SARS-CoV-2 variant and varying concentrations of a control monoclonal nanobody in order to normalise for possible replicative differences between variants and select conditions equivalent to wild-type virus. Following this calibration, cells were infected in the presence of serial dilutions of patient serum samples. After

infection (24 hrs Vero E6 Pasteur, 16hrs VAT-1), cells were fixed with 4% final Formaldehyde, permeabilised with 0.2% TritonX-100, 3% BSA in PBS (v/v), and stained for SARS-CoV-2 N protein using Alexa488-labelled-CR3009 antibody produced in-house and cellular DNA using DAPI(Brink et al., 2005). Whole-well imaging at 5x was carried out using an Opera Phenix (Perkin Elmer) and fluorescent areas and intensity calculated using the Phenix-associated software Harmony 9 (Perkin Elmer). Inhibition was estimated from the measured area of infected cells/total area occupied by all cells. The inhibitory profile of each serum sample was estimated by fitting a 4-parameter dose response curve executed in SciPy. Neutralising antibody titres are reported as the fold-dilution of serum required to inhibit 50% of viral replication (IC_{50}), and are further annotated if they lie above the quantitative (complete inhibition) range, below the quantitative range but still within the qualitative range (i.e. partial inhibition is observed but a dose- response curve cannot be fit because it does not sufficiently span the IC_{50}), or if they show no inhibition at all. IC_{50} values above the quantitative limit of detection of the assay (>2560) were recoded as 3000; IC_{50} values below the quantitative limit of the assay (< 40) but within the qualitative range were recoded as 39 and data below the qualitative range (i.e. no response observed) were recoded as 10.

ELISpot assay

IFN-γ Precoated ELISpot (Mabtech, UK) plates were blocked with complete medium (RPMI, 5% human AB serum) before 300,000 PBMC were seeded per well and stimulated for 18 h. Synthetic SARS-CoV-2 PepTivator peptides (Miltenyi Biotec, Surrey, UK), consisting of 15-mer sequences with 11 amino acid overlap were used at a final concentration of 1 µg/ml/peptide, as follows: (1) PepTivator SARS-CoV-2 Prot_S1 (amino acids 1-692); (2) PepTivator SARS-CoV-2 Prot_S (covering the sequences 304-338, 421-475, 492-519, 683-707, 741-770, 785-802 and 885-1273) and PepTivator SARS-CoV-2 Prot_S+ (amino acids 689-895) combined into a single pool broadly representing S2; (3) PepTivator SARS-CoV-2 Prot_M (covering the complete membrane glycoprotein); (4) PepTivator SARS-CoV-2 Prot_N (covering the complete nucleocapsid phosphoprotein), (5) PepTivator SARS-CoV-2 Prot_S B.1.1.7 Mutation Pool (34 peptides covering the mutated regions in spike of the Alpha VOC); (6) The PepTivator SARS-CoV-2 Prot_S B.1.617.2 Mutation Pool covers selectively the mutated regions (32 peptides covering the mutated regions in spike of the Delta).

Plates were developed with human biotinylated IFN-γ detection antibody (7-B6-1-ALP, 1:200), followed by incubation BCIP/NBT Phosphatase Substrate (SeraCare). Spot forming units (Mabtech) were quantified with ImmunoSpot. To quantify positive peptide-specific responses, spots of the unstimulated wells were subtracted from the peptide-stimulated wells, and the results expressed as SFU per million. Samples where positive controls were <10 SFU/10^6 spots per well were excluded, as were samples with negative control >50 SFU/10^6. The cut-off threshold for a positive result was the mean of the negative control well plus 2 times the standard deviation (24 SFU/10^6)(Adriana et al., 2021). The magnitude of the response (i.e. SFU/10^6) could not be compared between WT and VOC due to the reduced number of peptides in the VOC pools.

Quantification and statistical analysis

Data and statistical analysis were done in R v3.6.1 in R studio v1.2.1335. Gaussian distribution was tested by Kolmogorov-Smirnov test. Wilcoxon signed rank test, Chi2, Fisher's exact test were performed for statistical significance. A p-value <0.05 was considered significant. All tests were performed two-sided. Statistical details for each experiment are provided in the figure legends. The ggplot2 package in R was used for data visualization and illustrative figures were created with

BioRender.com. Data are usually plotted as single data points and violin or box plots on a logarithmic scale. PointRange in violin plots denotes median and upper and lower quartiles. Multivariable binary logistic regression analysis was performed using the glm function with the stats package in R.

Supplemental References

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The authors declare no competing interests.

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