Supplementary Materials for

Post-pandemic T-cell Responses Cross-recognise Animal Sarbecoviruses with Spillover Potential

Ethical approval

The study in Vietnam received approvals from the Institutional Review Board of the HTD in Ho Chi Minh City, Vietnam (CS/BND/21/03) and the Oxford Tropical Research Ethics Committee (513-21). For the study in Oxford, UK, participants were recruited under the GI Biobank Study 16/YH/0247, approved by the research ethics committee (REC) at Yorkshire & The Humber - Sheffield Research Ethics Committee on 29 July 2016, which has been amended for this purpose on 8 June 2020. The study was conducted in compliance with all relevant ethical regulations for work with human participants, and according to the principles of the Declaration of Helsinki (2008) and the International Conference on Harmonization (ICH) Good Clinical Practice (GCP) guidelines. Written informed consent was obtained from all the study participants.

Vaccine evaluation cohorts

For the Vietnam cohort, the study was conducted among members of staff of the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City since March 2021[1]. Two doses of Oxford-AstraZeneca COVID-19 vaccine (ChAdOx1-S) were given as part of the primary series, completed by the first week of May 2021. Pfizer-BioNTech COVID-19 vaccine (BNT162b2) was given the first and the second booster, completed in the third week of December 2021 and the first week of June 2022, respectively. For this analysis, we used available PBMC samples collected before vaccination (March 2021) and at 24 months (May 2024) post second booster dose were used (Table S2). As of March 2021, COVID-19 remained under controlled in Vietnam through the zero COVID-19 policy [2]. As a consequence, at that time, Vietnam reported less than 2500 confirmed COVID-19 cases [3], and HTD staff remained naive to SARS-CoV-2 infection [4].

For the UK cohort, the participants received a primary course of two doses of BNT162b2, or ChAdOx1-S between December 2020 and March 2021. All of participants then received a first booster dose of BNT162b2 in September 2021 to November 2021 and then received a second booster dose of bivalent COMIRNATY® Original/Omicron BA.1 between October 2022 and January 2023, and a third booster of Pfizer monovalent XBB vaccine in October 2023. For this analysis, we used 8 available PBMC samples collected post second or third booster dose (n=4 each, Table S2).

Peptide design

Individual peptides of the spike protein S1 and S2 domains of MERS-CoV and the five sarbecovirus candidates (SARS-CoV-1, SARS-CoV-2, Bat Khosta2, Bat RsYN04, and GX-P4L) were manually designed based on the spike protein sequences retrieved from the GenBank with the corresponding accession numbers of YP_009047204 (MERS-CoV), P59594 (SARS-CoV-1), YP_009724390 (SARS-CoV-2), QVN46569 (Bat Khosta2), QWA14166 (Bat RsYN04), and QIA48614 (GX-P4L). To ensure both CD4 and CD8 T cells can be covered, 15-18-mer peptides overlapping by 10 amino acids were designed with truncated rules applied for the C-terminal amino acid to avoid any of the following G, S, D, E, N, Q, H, P, C, A, and T situated at the C-terminal unless otherwise shorter than 15-mer [5].

Peptide pooling

Lyophilized peptides were dissolved in an appropriate amount of DMSO to yield a final concentration of 100mg/ml. Peptide pools of the spike protein domain S1 or S2 of the corresponding viruses were prepared by pooling individual peptides to the final concentration of 1mg/ml. Then, peptide pools S1 and S2 were combined in an equal volume to create the mega peptide pools with a working solution of 4ug/ml. Prepared mega peptide pools were stored at -80^{o}C until use.

T-cell assays

Two established assays, namely ELISpot and Intracellular Cytokine Stimulation (ICS) assays, were used to assess the T-cell responses to the tested viruses. These two assays were developed by our research team and have been successfully applied to COVID-19 research across the UK, and were carried out as previously described [6].

In brief, for the IFN-γ ELISpot assay 96-well Multiscreen-I plates (Millipore, UK) were coated with 10μg/ml of catcher antibody (IFN-γ human Elispot antibody clone 1-D1K - Mabtech) for 3 hours at room temperature. PBMC samples were added to each well at a density of 200,000 cells, and were then stimulated with 50μl of the prepared peptide pools of the correspnding viruses (2μg/ml per peptide). The experiments were conducted in duplicates. A medium containing 0.4% DMSO was used as negative control and as positive controls, CEFX peptide pool (2μg/ml, Proimmune) and Concanavalin A (5μg/ml final concentration) were used. The reaction plates were then incubated at 37 °C for 16-18 hours under a 5% CO_2 and 95% humidity

condition in a tissue culture incubator for IFN-γ secretion. After incubation, cells were washed off and the plates were incubated with 1 μg/ml anti-IFN-γ biotinylated mAb (7-B6-1-biotin, Mabtech) for 2–3 hours, followed by 1 μg/ml streptavidin alkaline phosphatase for 1–2 hours. Finally, BCIP/NBT was added to the reaction wells which acts as substrate for alkaline phosphatase. The plates were finally incubated at room temperature for 5 min to allow for color development. Dried ELISpot plates were scanned and counted using a CTL ImmunoSpot® S6 Ultimate platform. Results were reported as spot-forming units (SFU) per million PBMC. Negative control sample shoud gave a result of less than 50 SFU/10^6 PBMC. ELISpot assays were considered positive if the number of SFU/million PBMC was greater than the mean +2 SD of all the background values after background subtraction.

For the ICS assay, PBMCs were rested in R10 media for 4-6 hours after thawing. PBMCs were then plated at 1 million cells/well in a 96-well U-bottom plate and were stimulated with the prepared peptide pools of the corresponding viruses (2μg/ml final concentration per peptide pool) and co-stimulatory molecules CD28/CD49d (1 μg/ml final concentration). DMSO (equivalent concentration to the peptides) and phorbol-12-myristate 13-acetate (PMA, 81 μM final concentration, Biolegend)/ionomycin (1.3 μM final concentration, Biolegend) were used as negative and positive controls, respectively. After one hour of incubation with peptide pools at 37°C, in 5% CO_2 and 95% humidity, Brefeldin A (Biolegend) was added at 5 μg/ml final concentration to the reaction wells to block the cytokine secretion. The cells were then incubated for further 15 hours at 37°C, in 5% CO_2 and 95% humidity. After incubation, the cells were washed with a cell staining buffer (Biolegend), and were then stained with a live/dead and human specific antibodies against CD4 and CD8 (Live/Dead NiR 1:1000 dilution (Invitrogen); CD4 APC 1:200 dilution (clone RPA-T4, Biolegend); CD8 BV510 1:600 dilution (clone RPA-T8, Biolegned); CD14 APC-Fire750 1:200 dilution (clone M5E2, Biolegend); CD154 V421 1:100 dilution (clone24-31, Biolegend) and human Fc blocking reagent (Miltenyi Biotec) for 20 min at 4 °C. Next, the cells were fixed with a fixation/permeabilization solution (BD Biosciences), and incubated with lineage and functional markers CD3 PerCP (clone UCHT1, Biolegend); IFN-γ PE (clone 4S.B3, Biolegend) and human Fc blocking reagent (Miltenyi Biotec) for 20 min at 4 °C followed by washing step using Perm/Wash buffer (BD Biosciences). Finally, the cells were resuspended in a staining buffer and stored at 4 °C in the dark until data

acquisition. Data acquisition was carried out on a MACSquant analyser X (Miltenyi Biotec), and visualised using FlowJo Version 10.7.1 (BD Biosciences).

Spike based phylogenetic tree

Thirty unique RBD sequences of sarbecoviruses exhibiting human ACE2 binding properties as previously suggested [7], were used for phylogenetic analysis. Multiple sequence alignment was performed using MAFFT (v7.520) [8]. The maximum likelihood phylogenetic tree was inferred using GTR+F+G4 model, as suggested by IQ tree (v2.2.6). Support for individual nodes was assessed using a bootstrap analysis with 1000 replicates. The Hibecovirus sequence Hp-BetaCoV_Zhejiang_2013 (Accession number: KF636752) was employed as an outgroup to root the tree. The re-constructed tree was visualized and annotated using Figtree (v1.4.2, http://tree.bio.ed.ac.uk/software/figtree/).

Statistical analysis

Comparisons between pre and post vaccination groups and data of the UK and VN cohorts were carried out using Wilcoxon rank sum test and adjusted p values displayed. The line in the middle of each boxplot indicate the median and the box edges show the 25th and 75th percentiles. The correlation of sequence homology and T-cell response to sarbecovirus was calculated using Pearson's Correlation Test. A threshold of p values less than 0.05 was used to define a significant result. Statistical analyses were carried out in h R, version 4.4.1, or GraphPad Prism, version 10.2.3, where appropriate.

Legends to Supplementary Figures

Figure S1: Maximum likelihood phylogenetic tree depicting the relatedness between sarbecoviruses that exhibit hACE2 binding properties.
hACE2-dependent sarbecoviruses (SARS-CoV-1&2, pangolin coronavirus GX-P4L, and bat coronaviruses RsYN04 and Khosta2) selected for T-cell response analysis are marked by the stars. MERS-CoV belongs to merbecoviruses and employs dipeptidyl peptidase-4 (DDP4) as an entry receptor. It was thus not included for phylogenetic analysis. Information about lineage assignment of bat coronavirus RsYN04 is currently not available.

Figure S2: Schematic illustration showing the sampling time

Figure S3: The proportion of PBMC samples collected post-vaccination with detectable T-cell responses against the tested viruses.

Figure S4: Comparison of the levels of T-cell responses obtained from post-pandemic samples, A) between SARS-CoV-2 and the remaining tested viruses using combined data of the UK and Vietnam cohorts, B) between SARS-CoV-2 and the remaining tested viruses using data of the UK cohort, and C) between SARS-CoV-2 and the remaining tested viruses using data of the Vietnam cohort
Note to Figure S4: Shown p values were the results of comparing T-cell responses to SARS-CoV-2 and the corresponding viruses.

Figure S5: Correlations of post vaccine IFN- γ intracellular cytokine responses and IFN-γ ELISpot responses to SARS-CoV-2 (WT), GX-P4L (GXP), Bat Khosta2 (BatKh) and Bat RsYN04 (BatRs) S1 and S2 pools. Spearman's correlation coefficient (colour coded and values in white text) are shown in case of significant values ($p < 0.05$). Adjustment for multiple testing were not performed.

References

1. Chau, N.V.V., et al., *Kinetics of Neutralizing Antibodies against Omicron Variant in Vietnamese Healthcare Workers after Primary Immunization with ChAdOx1-S and Booster Immunization with BNT162b2.* Am J Trop Med Hyg, 2023. **108**(1): p. 137-144.
2. Van Tan, L., *COVID-19 control in Vietnam.* Nat Immunol, 2021. **22**(3): p. 261.
3. https://ourworldindata.org/coronavirus/country/vietnam.
4. Chau, N.V.V., et al., *Absence of SARS-CoV-2 antibodies in health care workers of a tertiary referral hospital for COVID-19 in southern Vietnam.* J Infect, 2021. **82**(1): p. e36-e37.
5. Draenert, R., et al., *Impact of intrapeptide epitope location on CD8 T cell recognition: implications for design of overlapping peptide panels.* AIDS, 2004. **18**(6): p. 871-6.
6. Ogbe, A., et al., *T cell assays differentiate clinical and subclinical SARS-CoV-2 infections from cross-reactive antiviral responses.* Nat Commun, 2021. **12**(1): p. 2055.
7. Starr, T.N., et al., *ACE2 binding is an ancestral and evolvable trait of sarbecoviruses.* Nature, 2022. **603**(7903): p. 913-918.

8. Nguyen, L.T., et al., *IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies.* Mol Biol Evol, 2015. **32**(1): p. 268-74.

Table S1: Spike-protein sequence similarities

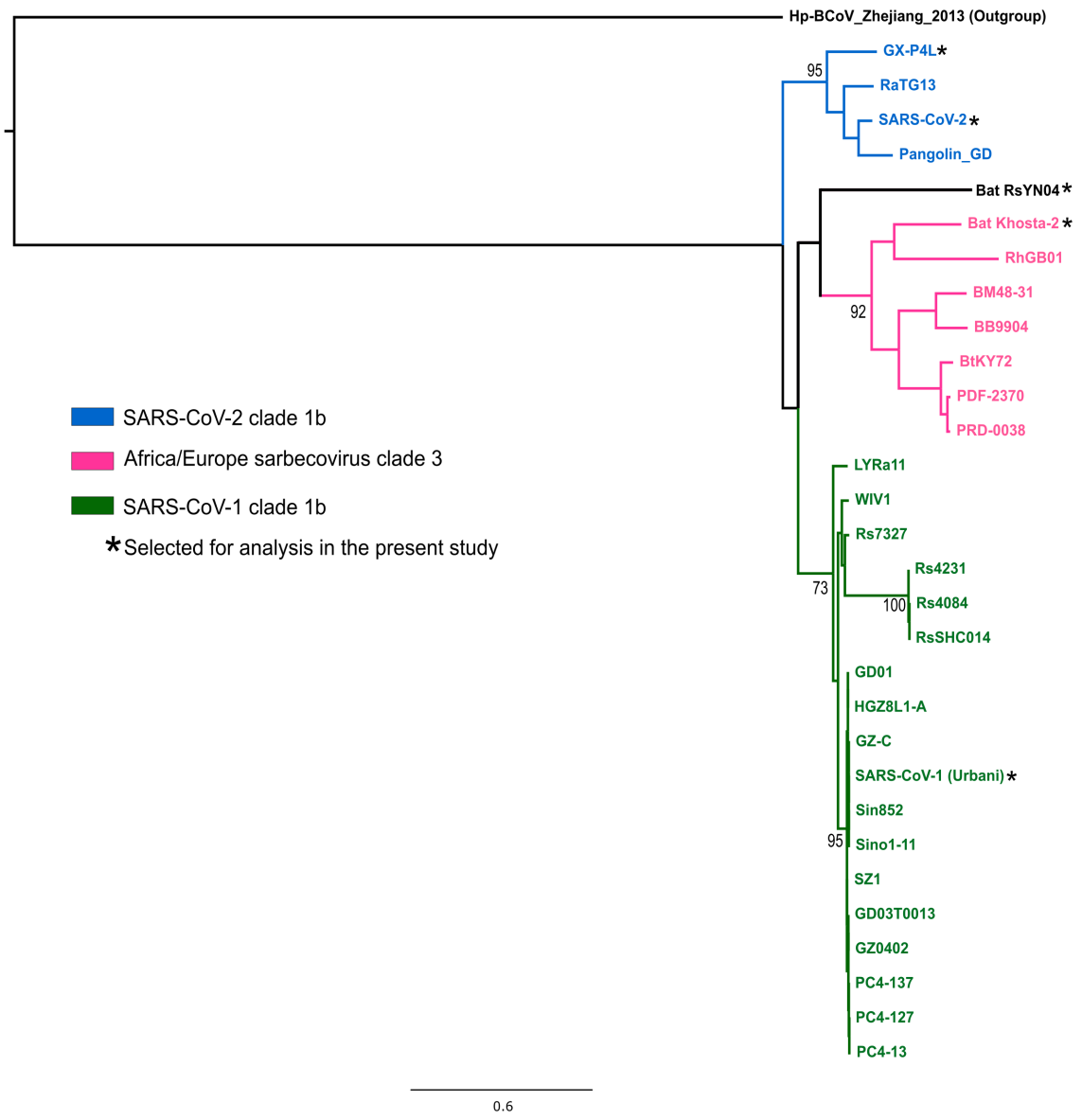
	SARS-CoV-2 *	Pangolin_GD	RaTG13	Pangolin_GX *	Urbani *	WIV1	Rs7327	LYRa11	Rs4231	BM48-31	BB9904	BtKY72	PDF-2370	PRD-0038	Khosta-2 *	RhGB01	RsYN04 *
SARS-CoV-2 *		86.7	85.6	79.5	73.1	74.1	73	72.3	72.6	64.4	64.9	69.5	69.5	69.1	63.2	66.7	62.1
Pangolin_GD	86.7		81.6	78.3	72.8	73.1	73.5	72.9	72.6	66.9	66.1	67.8	68.1	67.7	64.5	65.3	61.3
RaTG13	85.6	81.6		79.3	73.6	73.1	72.8	72.1	70.6	64.7	64.4	69.3	68.6	68.6	63.5	64.4	63.2
Pangolin_GX *	79.5	78.3	79.3		72.5	73.8	72.7	72.7	72	64.7	63.9	68.2	67.9	67.9	66.9	63.9	61.9
Urbani *	73.1	72.8	73.6	72.5		93.3	93.2	90.7	81.8	66	65.2	68.6	67.5	68.2	68.5	67.8	64.1
WIV1	74.1	73.1	73.1	73.8	93.3		95.2	91.2	82.5	66.1	65.3	68.5	68.8	68.8	68.5	67	64.3
Rs7327	73	73.5	72.8	72.7	93.2	95.2		90.8	83.3	66.6	66.3	68.3	68.2	68.5	68.2	67.5	64
LYRa11	72.3	72.9	72.1	72.7	90.7	91.2	90.8		80.3	66.8	65	68	68	68.5	68.8	66.5	64.8
Rs4231	72.6	72.6	70.6	72	81.8	82.5	83.3	80.3		66.9	64.2	67.8	66.7	67.3	64.2	65.2	61.1
BM48-31	64.4	66.9	64.7	64.7	66	66.1	66.6	66.8	66.9		83.5	75	75.8	76.5	69.5	69.7	59.5
BB9904	64.9	66.1	64.4	63.9	65.2	65.3	66.3	65	64.2	83.5		76.8	76.8	76.8	69.7	68	59.6
BtKY72	69.5	67.8	69.3	68.2	68.6	68.5	68.3	68	67.8	75	76.8		92.7	93.2	72.7	67.7	61.2
PDF-2370	69.5	68.1	68.6	67.9	67.5	68.8	68.2	68	66.7	75.8	76.8	92.7		97.5	72.5	67.8	59.9
PRD-0038	69.1	67.7	68.6	67.9	68.2	68.8	68.5	68.5	67.3	76.5	76.8	93.2	97.5		72.8	68.3	60.4
Khosta-2 *	63.2	64.5	63.5	66.9	68.5	68.5	68.2	68.8	64.2	69.5	69.7	72.7	72.5	72.8		70	61.4
RhGB01	66.7	65.3	64.4	63.9	67.8	67	67.5	66.5	65.2	69.7	68	67.7	67.8	68.3	70		57.8
RsYN04 *	62.1	61.3	63.2	61.9	64.1	64.3	64	64.8	61.1	59.5	59.6	61.2	59.9	60.4	61.4	57.8	

Note to Table S1: Viruses are colour coded according to Figure 1A. Viruses selected for T-cell response analysis are marked by the stars. Viruses of clade 1 (in green) sharing >=98% sequence homology with SARS-CoV-2 were not included in the Table.

Table S2: Cohort characteristics, COVID-19 vaccination history and sample timing

Parameters		Vietnam cohort		UK cohort
		Pre-vaccination[&], n=11	**Post-vaccination*, n=25**	**Post-vaccination**, n=8**
Age median in years, (range)		38 (30-59)	41 (31-60)	52 (34-58)
Gender, female, n (%)		10 (90.9)	18 (72)	8 (100)
Occupation, n (%)				
Nurse		5 (45.5)	14 (56)	5 (62.5)
Clinician		1 (9.1)	5 (20)	0
Pharmacist		3 (27.3)	1 (4)	0
Cleaner		0	3 (12)	0
Others		2 (18.2)[$]	2 (8)[$$]	3[$$$]
Comorbidity, n/N (%)		2/4 (50)[#]	5 (20)[##]	2 (25)[###]
COVID-19 vaccination				
Dose 1	Vaccine type	N/A	ChAdOx1-S	BNT162b2 (n=6), and ChAdOx1-S (n=2)
	Time period	N/A	8-15/Mar/2021	10/Dec/2020 - 3/Mar/2021
	n/N, (%)	N/A	25/25 (100)	8/8 (100)
Dose 2	Vaccine type	N/A	ChAdOx1-S	BNT162b2 (n=6), and ChAdOx1-S (n=2)
	Time period	N/A	20/Apr-4/May/2021	2/Jan – 22/Apr/2021
	n/N, (%)	N/A	25/25 (100)	8/8 (100)
Dose 3	Vaccine type	N/A	BNT162b2	BNT162b2
	Time period	N/A	16-21/Dec/2021)	26/Sep – 4/Nov/2021
	n/N, (%)	N/A	25/25 (100)	8/8 (100)
Dose 4	Vaccine type	N/A	BNT162b2	BNT162b2 bivalent (original/BA.1)
	Time period	N/A	30/May-7/Jun/2022)	1/Oct – 2/Nov/2022
	n/N, (%)	N/A	18/25 (72)	4/4 (100)
Dose 5	Vaccine type	N/A	NA	COMIRNATY (Omicron XBB.1.5)
	Time period	N/A	NA	12-23/Oct/2023
	n/N, (%)	N/A	NA	4/4 (100)
Time interval in day (range)[@]		N/A	729 (714-736)	86 (69-93)

Note to table S2: [$]administrator and retirement (n=1 each), [$$]accountant and engineer (n=1 each), [$$$]biomedical engineer, consultant and manager (n=1 each). [#]obese (n=1) and thyroid goiter (n=1). [##]diabetes (n=1), hypertension (n=3), and obese (n=1). [###]cancer (n=1), and urticaria and migraines (n=1). [&]SARS-CoV-2 infection naive individuals, inferred from the results of testing for antibodies against anti-nucleocapsid protein (n=1) or neutralising antibodies (n=6, data not shown). *post vaccination: after dose 4. **post vaccination: after dose 5, [@]from last vaccination to bleeding. NA = not applicable


Hp-BCoV_Zhejiang_2013 (Outgroup)
GX-P4L*
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RaTG13
SARS-CoV-2*
Pangolin_GD
Bat RsYN04*
Bat Khosta-2*
RhGB01
92
BM48-31
BB9904
BtKY72
PDF-2370
PRD-0038
LYRa11
WIV1
Rs7327
Rs4231
73
100
Rs4084
RsSHC014
GD01
HGZ8L1-A
GZ-C
SARS-CoV-1 (Urbani)*
Sin852
95
Sino1-11
SZ1
GD03T0013
GZ0402
PC4-137
PC4-127
PC4-13
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Africa/Europe sarbecovirus clade 3
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*Selected for analysis in the present study
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Figure S1

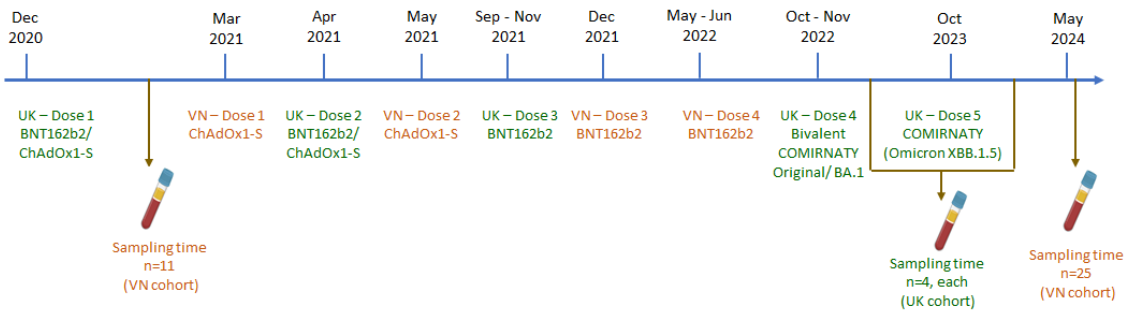

Dec 2020
Mar 2021
Apr 2021
May 2021
Sep - Nov 2021
Dec 2021
May - Jun 2022
Oct - Nov 2022
Oct 2023
May 2024
UK – Dose 1 BNT162b2/ ChAdOx1-S
VN – Dose 1 ChAdOx1-S
UK – Dose 2 BNT162b2/ ChAdOx1-S
VN – Dose 2 ChAdOx1-S
UK – Dose 3 BNT162b2
VN – Dose 3 BNT162b2
VN – Dose 4 BNT162b2
UK – Dose 4 Bivalent COMIRNATY Original/ BA.1
UK – Dose 5 COMIRNATY (Omicron XBB.1.5)
Sampling time n=11 (VN cohort)
Sampling time n=4, each (UK cohort)
Sampling time n=25 (VN cohort)

Figure S2

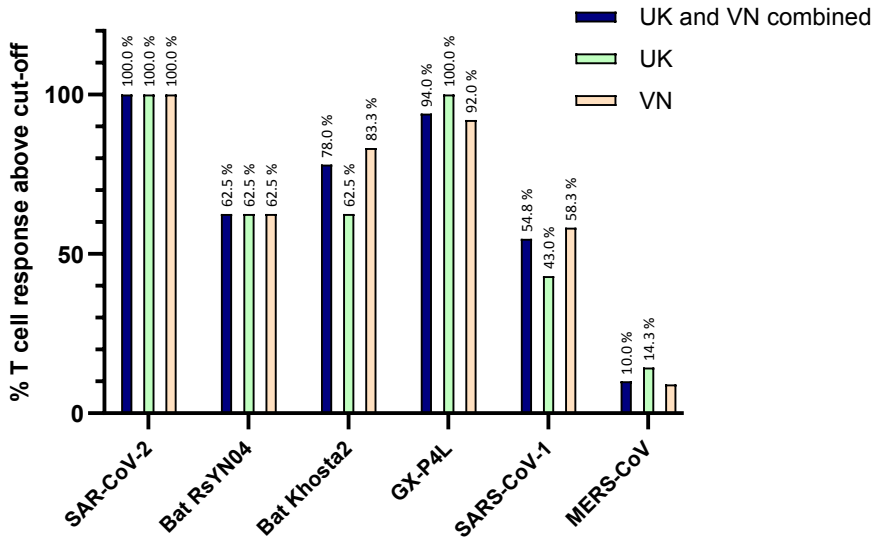
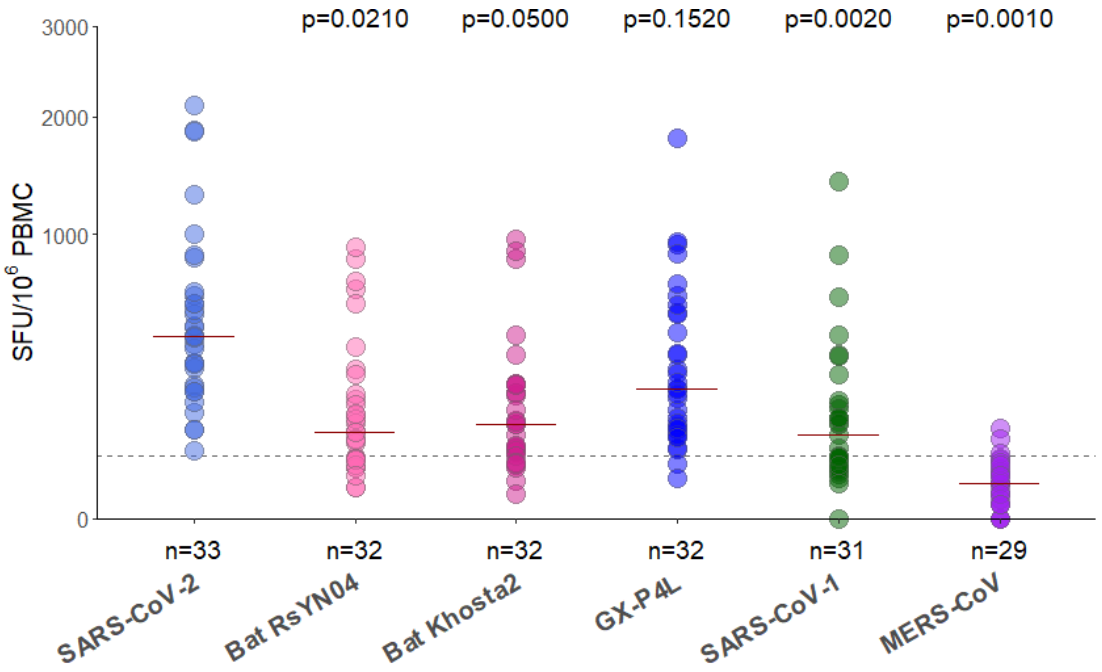

UK and VN combined
UK
VN
% T cell response above cut-off
100
50
0
100.0 %
100.0 %
100.0 %
62.5 %
62.5 %
62.5 %
78.0 %
62.5 %
83.3 %
94.0 %
100.0 %
92.0 %
54.8 %
43.0 %
58.3 %
10.0 %
14.3 %
SAR-CoV-2
Bat RsYN04
Bat Khosta2
GX-P4L
SARS-CoV-1
MERS-CoV

Figure S3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1000
0
SFU/10^6 PBMC
n=33
n=32
n=32
n=32
n=31
n=29
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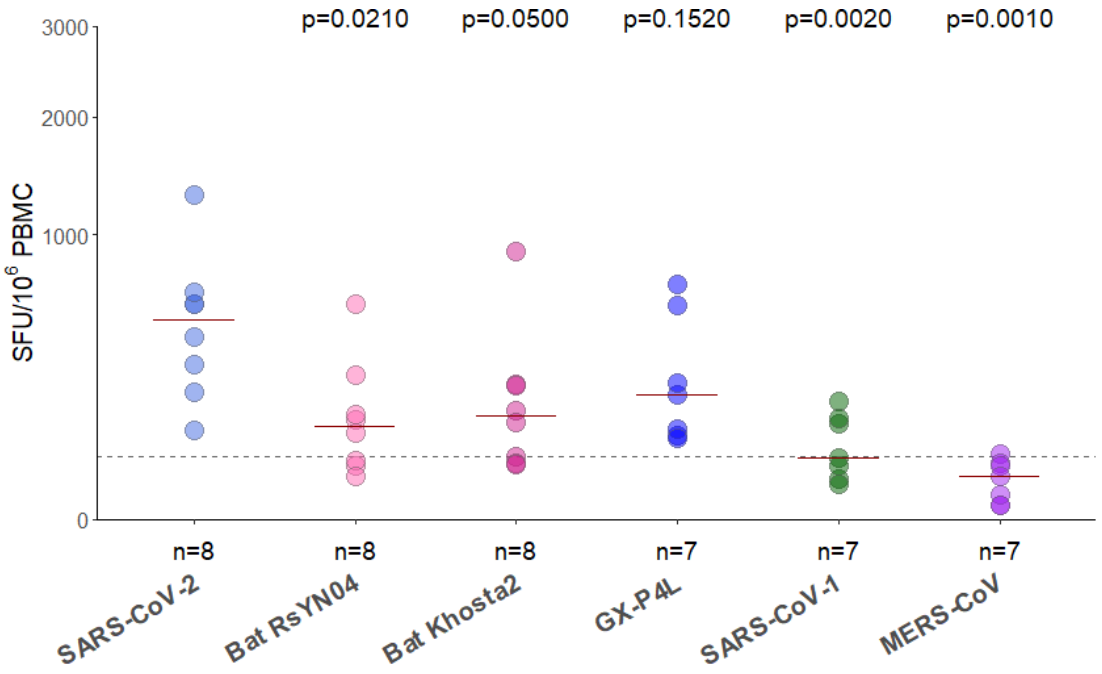
Bat RsYN04
Bat Khosta2
GX-P4L
SARS-CoV-1
MERS-CoV

Figure S4A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2000
1000
0
SFU/10^6 PBMC
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n=8
n=8
n=7
n=7
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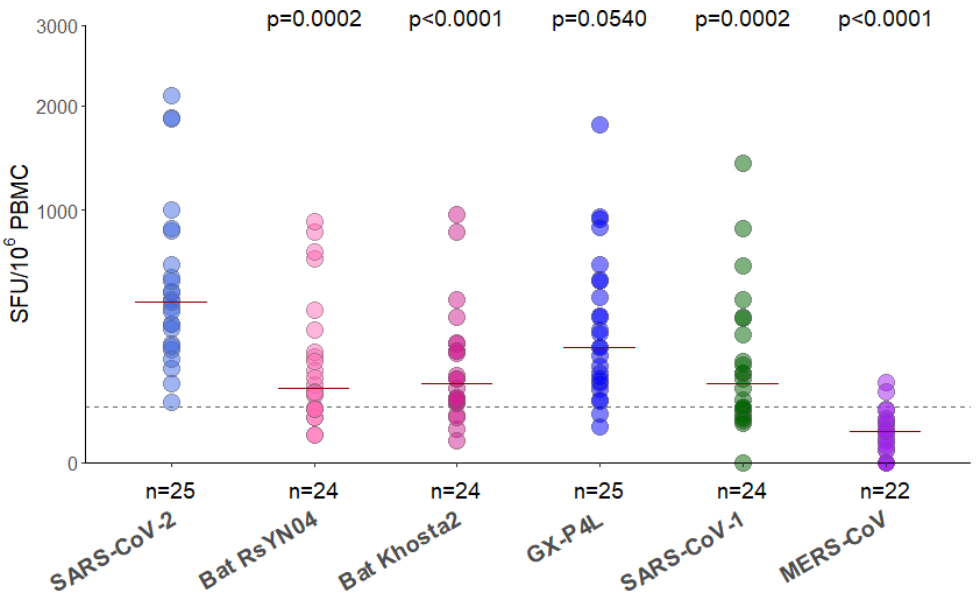
GX-P4L
SARS-CoV-1
MERS-CoV

Figure S4B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3000
2000
1000
0
SFU/10^6 PBMC
n=25
n=24
n=24
n=25
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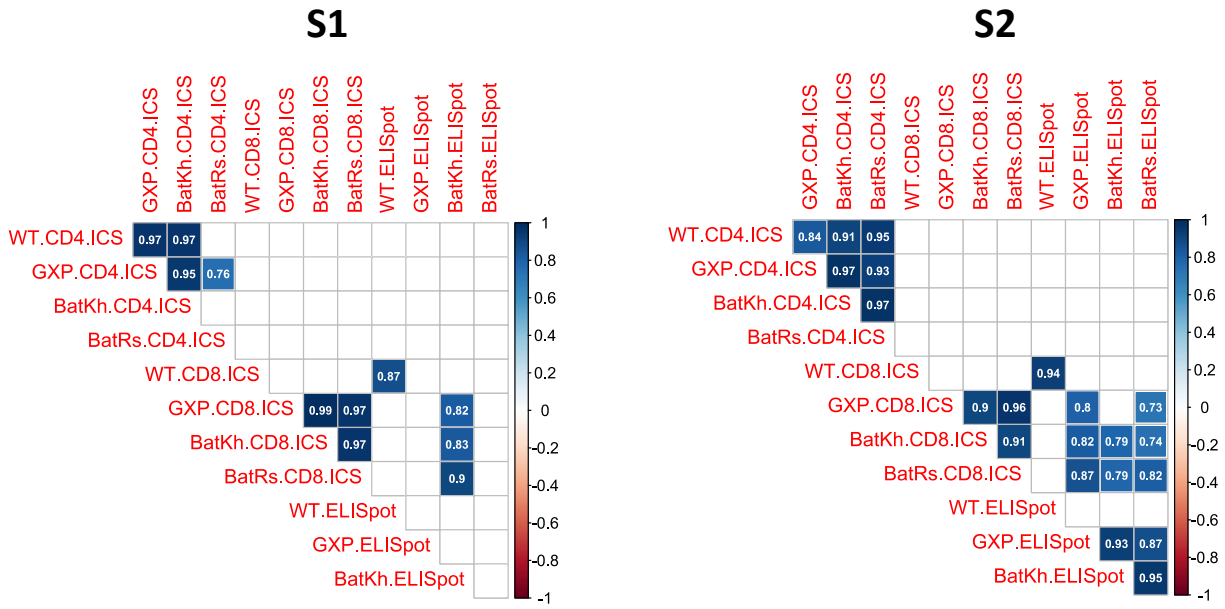
Bat RsYN04
Bat Khosta2
GX-P4L
SARS-CoV-1
MERS-CoV

Figure S4C


S1
GXP.CD4.ICS
BatKh.CD4.ICS
BatRs.CD4.ICS
WT.CD8.ICS
GXP.CD8.ICS
BatKh.CD8.ICS
BatRs.CD8.ICS
WT.ELISpot
GXP.ELISpot
BatKh.ELISpot
BatRs.ELISpot
WT.CD4.ICS 0.97 0.97
GXP.CD4.ICS 0.95 0.76
BatKh.CD4.ICS
BatRs.CD4.ICS
WT.CD8.ICS 0.87
GXP.CD8.ICS 0.99 0.97 0.82
BatKh.CD8.ICS 0.97 0.83
BatRs.CD8.ICS 0.9
WT.ELISpot
GXP.ELISpot
BatKh.ELISpot
1 0.8 0.6 0.4 0.2 0 -0.2 -0.4 -0.6 -0.8 -1
S2
GXP.CD4.ICS
BatKh.CD4.ICS
BatRs.CD4.ICS
WT.CD8.ICS
GXP.CD8.ICS
BatKh.CD8.ICS
BatRs.CD8.ICS
WT.ELISpot
GXP.ELISpot
BatKh.ELISpot
BatRs.ELISpot
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GXP.CD4.ICS 0.97 0.93
BatKh.CD4.ICS 0.97
BatRs.CD4.ICS
WT.CD8.ICS 0.94
GXP.CD8.ICS 0.9 0.96 0.8 0.73
BatKh.CD8.ICS 0.91 0.82 0.79 0.74
BatRs.CD8.ICS 0.87 0.79 0.82
WT.ELISpot
GXP.ELISpot 0.93 0.87
BatKh.ELISpot 0.95
1 0.8 0.6 0.4 0.2 0 -0.2 -0.4 -0.6 -0.8 -1

Figure S5