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Supplementary appendix

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Supplement to: Smit MJ, Sander AF, Ariaans MBPA, et al. First-in-human use of a modular capsid virus-like vaccine platform: an open-label, non-randomised, phase 1 clinical trial of the SARS-CoV-2 vaccine ABNCoV2. *Lancet Microbe* 2023; published online Jan 18. https://doi.org/10.1016/S2666-5247(22)00337-8.

Supplementary Appendix

Supplement to: M.J. Smit, A.F. Sander, M.B.P.A. Ariaans, et al. First-in-human use of a modular capsid virus-like vaccine platform: an open-label, non-randomised, phase 1 clinical trial of the SARS-CoV-2 vaccine ABNCoV2.

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Supplementary Methods

Enzyme-linked Immunosorbent Assay (ELISA)

Assay 1 (University Hospital Tübingen)

The humoral immune response against the SARS-CoV-2 antigen RBD (GenBank: QHD43416.1, amino acids R319 - F541) was determined by ELISA. First, the SARS-CoV-2 RBD antigen was diluted in 1x PBS to a final concentration of 2 µg/ml and 50 µl were added per well to coat Costar 96 well microtiter high binding plates (ref. 3590, Corning). After overnight incubation at 4°C, the wells were washed once with 1x PBS and blocked with 200 µl of The Blocking Solution (Condor Bioscience GmbH) for 2 hours at room temperature (RT) on a microplate shaker (700 rpm). Subsequent washing steps were repeated 3x with PBS/0.1% Tween20. Plasma was diluted 1:100 up to 1:7,812,500 dilution in The Blocking solution (1 to 5 dilution row). 100 µl of diluted sample was added to the ELISA plates and incubated for 1 h. IgG antibody presence was detected by 1:20,000 diluted HRP-coupled anti-human IgG (ref. 109-036-097, Jackson Immuno Research Laboratories). The detection antibody was diluted 1:5,000 in 1x ROTI Block buffer and incubated for 30 min (RT, 700 rpm). For visualization, 100 µl TMB substrate solution was added, and the reaction was stopped using 50 µl 1 M HCl. The plate was read at 450 nm and 620 nm with a microplate reader (CLARIOstar, BMG LABTECH). Data is given as concentration in ng/ml and was estimated by a respective dilution series of highly pure human IgG, which was precoated on separate wells on the same plates (ref. 31154, ThermoFisher).

Assay 2 (University of Copenhagen)

96-well plates (Nunc MaxiSorp) were coated with 0.002mg/mL recombinant ExpreS2 produced SARS-CoV-2 Spike (aa. 16-1208) protein. Plates were blocked for 1 hour using 3% skimmed milk in PBS+0.5%Tween20. Serum was diluted 1:30 in 1% skimmed milk in PBS+0.1%Tween20, and added to the plate in a 3-fold dilution, followed by incubation for 2 hours. Plates were washed three times in PBS+0.5%Tween20 in-between steps. WHO standard (NIBSC code: 20/136) was used to standardize each assay and for comparability to other studies. Total serum IgG were detected by polyclonal rabbit anti-human IgG-HRP (P0214 Dako) diluted 1:4000 in 1% skimmed milk in PBS+0.1%Tween20. Plates were developed with TMB X-tra substrate (Kem-En-Tec, 4800A) and absorbance was measured at 450nM.

Virus neutralisation assays

SARS-CoV-2 virus isolates, propagation and Plaque Reduction Neutralization Test (PRNT) (Aarhus University)

The Wuhan-Like, early-European B.1. isolate (Freiburg, FR-4286, GISAID accession no. EPI_ISL_852748) was kindly provided by Professor Georg Kochs, University of Freiburg. SARS-CoV-2 variant of concern Alpha (B.1.1.7, NCBI GenBank accession no. MZ314997) was kindly provided by Professor Arvind Patel, University of Glasgow, United Kingdom and Beta (B.1.351, GISAID accession no. EPI_ISL_678615) was kindly provided by Professor Alex Sigal, African Health Research Institute, South Africa. The delta variant (B.1.617.2, NCBI GenBank accession no. OM444216) was kindly provided by Statens Serum Institute, Denmark.

SARS-CoV2 B.1, B.1.1.7 and B.1.617.2 was propagated in VeroE6 cells expressing human TMPRSS2 (kindly provided by Professor Stefan Pöhlmann, University of Göttingen)[1] by infection with MOI 0.05. SARS-CoV2 B.1.351 was propagated in A549 cells expressing human ACE-2.

Supernatant containing new virus progeny was harvested 72 hours post infection. The supernatant was first centrifuged 10 minutes at 3000 x g to pellet cellular debris. The virus containing supernatant was subsequently filtered through a 45 µm filter and concentrated by centrifugation in 100 kDa Amicon tubes (Merck) for 30 minutes at 4000 x g. Virus titer was determined by TCID50% assay and calculated by Reed-Muench method.

SARS-CoV-2 neutralizing antibodies in serum samples from I1-3, I1+14, I2-3, and I2+14 were measured by live virus Plaque Reduction Neutralization Test (PRNT) against B.1 FR-4286 isolate as previously described.[2] In short, serum were inactivated for 30-60 minutes at 56 degrees celcius, before mixing 2-fold serum dilutions with 100 TCID50% SARS-CoV2 and incubated overnight at 4 degrees celcius. The following day, the serum:virus mix was added to 2x104 VeroE6-hTMPRSS2 cells seeded in flat bottomed 96-well plate. Each serum dilution was represented in 8 wells. A no serum and a no virus sample were included as controls. In addition, for comparison, the WHO anti-SARS-CoV2 immunoglobulin standard (NBISC nr. 20-136) were included. PRNT50 titers were calculated using the drm command of the R package drc (version 3.0.1) with a two-parameter log-logistic function. Neutralization of SARS-CoV-2 variants of concern was assessed with serum samples obtained 2 weeks post second vaccination (I2+14), as previously described.[3]

SARS-CoV-2 virus isolates and virus reduction (50% inhibitory dilution [ID50]) neutralization (CO-HEP)

Three SARS-CoV-2 isolates were used to measure neutralization in this study, including a SARS-CoV-2 isolate representing the ancestral D614G SARS-CoV-2 variant (DK-AHH1, clade 20A, Genbank accession number MZ049597),[4] a SARS-CoV-2 isolate representing the delta variant (DK-AHH3, clade 21J, accession number OP271297) and a SARS-CoV-2 isolate representing the omicron BA.1 variant (DK-AHH4, clade 21K accession number OP271296). Neutralization experiments were performed as described previously.[5] In brief, the DK-AHH1, DK-AHH3 and DK-AHH4 isolates were incubated at multiplicity of infection [MOI] of 0.02, 0.1 and 0.06, respectively with 2-fold serially diluted plasma (starting at 1 in 10 dilution) at a 1:1 plasma/virus ratio at room temperature for 1h. As negative controls, plasma from pre-vaccine time points were included in each assay. Plasma that had been previously shown to neutralize all three variants was used as a positive control. Following 1h incubation, plasma/virus complexes were then added to Vero E6 cells (RRID: CVCL_0574) seeded 24 h prior (10^4 cells/well; 96 well plates) in quadruplicate. After 48 h incubation at 37°C and 5% CO_2, the cells were fixed using methanol and stained for viral surface antigen as described previously.[4] Spots representing virus infected cells were counted using an Immunospot series 5 UV analyser (Cellular Technologies). Single outliers of quadruplicates were calculated using a modified z-score system as previously described[6] and were removed from further analysis; thus, a minimum of triplicates was used for all assays. The percentage neutralization was calculated as:

$$\%\ Neutralization = 1 - \left(\frac{Spot\ count}{Average\ spot\ count\ \{pre - vaccine\ controls\}}\right)\ x\ 100$$

Any overall neutralization values (average of the triplicates/quadruplicates) that yielded higher than 100% were normalized to 100% and any overall neutralization values that yielded lower than 0% were normalized to 0%.

Cellular immune responses (Immunitrack)

Peptides

Peptides for peptide pools were produced in house by solid phase synthesis using Fluorenylmethyloxycarbonyl protecting group (Fmoc)/Tert-butoxycarbonyl protecting group (Boc) chemistry and were produced as C terminal amides. Afterwards, the peptides were dissolved in DMSO (10 mM) and stored at -20°C.

Long-term peptide stimulation of PBMCs

PBMCs were thawed in warm x-vivo (Lonza) and washed twice before counted and seeded with a density of 1,5-3e6 cells/well into 48-well plates in 1 ml without serum. Peptide pools were added in a concentration of 5-10 uM and incubated in bench wrapped in parafilm for 2 hours before 1 ml x-vivo+10% human serum (HS) (sigma-aldrich) was added and the PBMCs were incubated at 37°C with 5% CO2 in a humidified atmosphere. On day 1, 120 U/ml IL-2 (Proleukin) was added. The cells were incubated for 12-14 days with media change on day 10 if necessary. The cells were counted before harvest.

Intracellular cytokine staining (ICS)

Harvested long-term stimulated PBMCs were washed (300g, 5 min) in warm RPMI-1640 (Life Technologies) by centrifugation. Then seeded in a round bottom 96-well plate in duplicates ~0,5e6 cells/well. The cells were stimulated with 5uM peptide pools and costimulatory factors 1:1000x of 1mg/ml anti-CD28 (BD Biosciences) and anti-CD49d (BD Biosciences) for 1h, before adding GolgiPlug (BD Biosciences), GolgiStop (BD Biosciences), and anti-CD107a-BV421 (BD Biosciences) and incubated for additional 5 hours. Afterwards, the cells were transferred to a 96-v-shaped plate and washed (5min, 300g) twice in FACS buffer (FB) (PBS, 2mM EDTA, 0,05%NaN_3, 0,5%bovine serum albumin (Sigma-Aldrich). The cells were stained for 10 min at 4°C with Invitrogen™ LIVE/DEAD™ Fixable Near-IR Dead Cell Stain Kit, before pre-titrated surface antibodies (BV510-CD4, APCR700-CD8, BV786-CD3, BV605-CD56 (BD Bioscience)) were added and incubated for 20 additional min at 4 °C. The cells were washed (5min, 300g) with cold PBS and permeabilized and fixed with Fixation/Permabilization kit (ebiosciences) in the dark for 30 min at room temperature (RT) according to the protocol provided by the supplier. The cells were washed (5min, 300g) in freshly prepared permeabilization buffer (ebioscence) and stained with intracellular antibodies (APC-TNFa, PeCy7-IFNy, PE-CD137 (BD Bioscience)) for 20 min at 4 °C. The cells were washed (5min, 300g) twice in permeabilization buffer, before run on a NovoCyte Quanteon Flow Cytometer (Agilent Technologies)

MHC/epitope in vitro binding assay

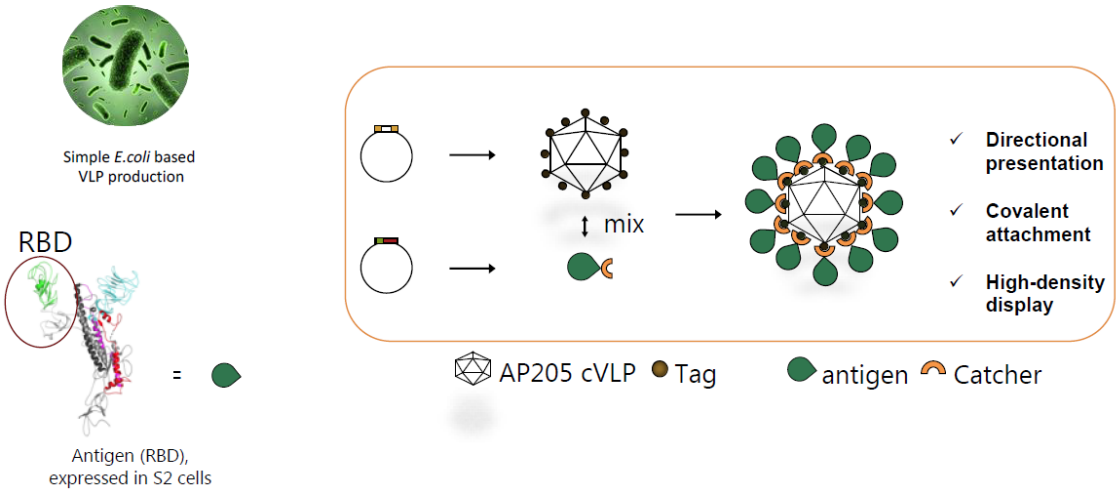
NeoScreen stability assay works by measuring the decay of MHC/epitope complexes using stress induced conditions, in this case urea denaturation. Reference peptides were included in all assays and the data yielded from the test epitopes was expressed in a percentage of the reference peptide. For this study 9 mer epitopes overlapping by 8 amino acids and spanning the RBD region of spike were found to bind to one or several of the following alleles: HLA class I A*0201, A*0301, A*1101,

A2402, B*0801, B*0702, C*0401, C*0702 and HLA class II: DRB*0301, DRB1*0401, DRB1*0801, DRB1*1101, DRB1*1301, DRB5. More information on the HLA alleles applied in stability binding assay on RBD can be found here https://www.immunitrack.com/vaccines/spike/.

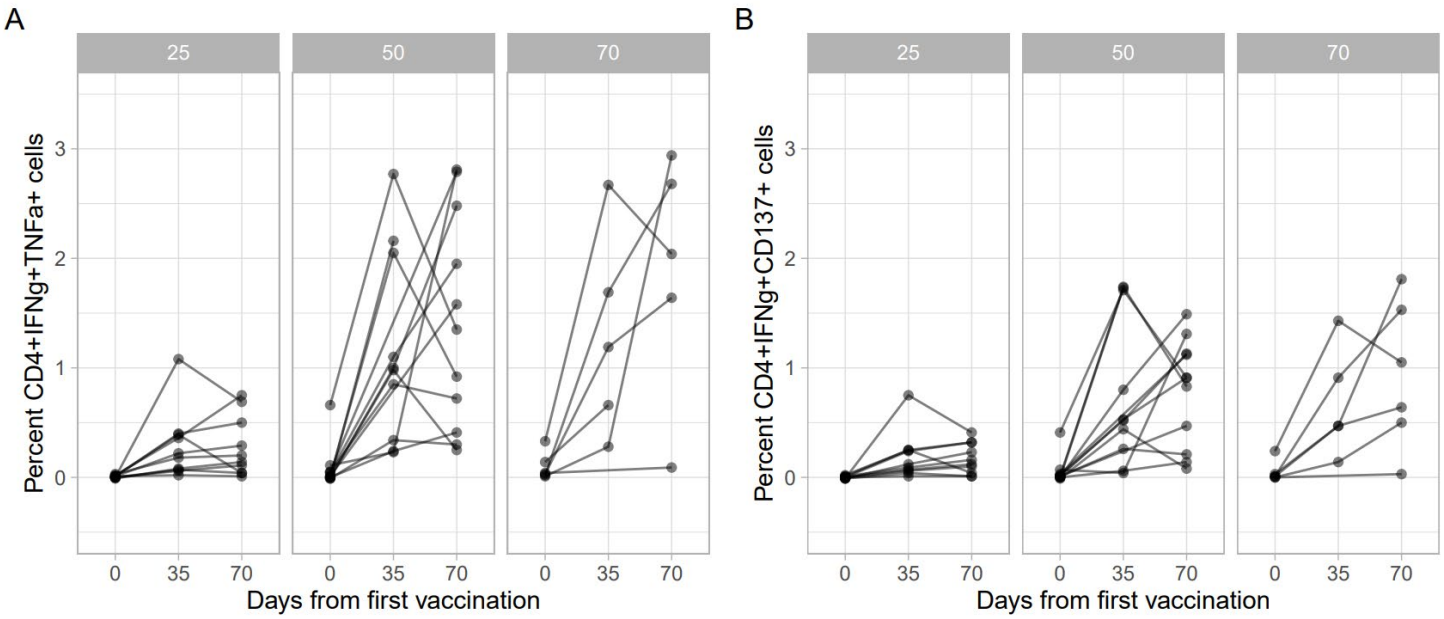
Supplementary Figures

Supplementary Figure S1. Structural elements of ABNCoV2: Engineered AP205 VLP and Spike RBD Antigen.

AP205: Acinetobacter Phage 205; cVLP: capsid virus-like particle; RBD: receptor binding domain; S2 cells: drosophila S2 cells; VLP: virus-like particle.

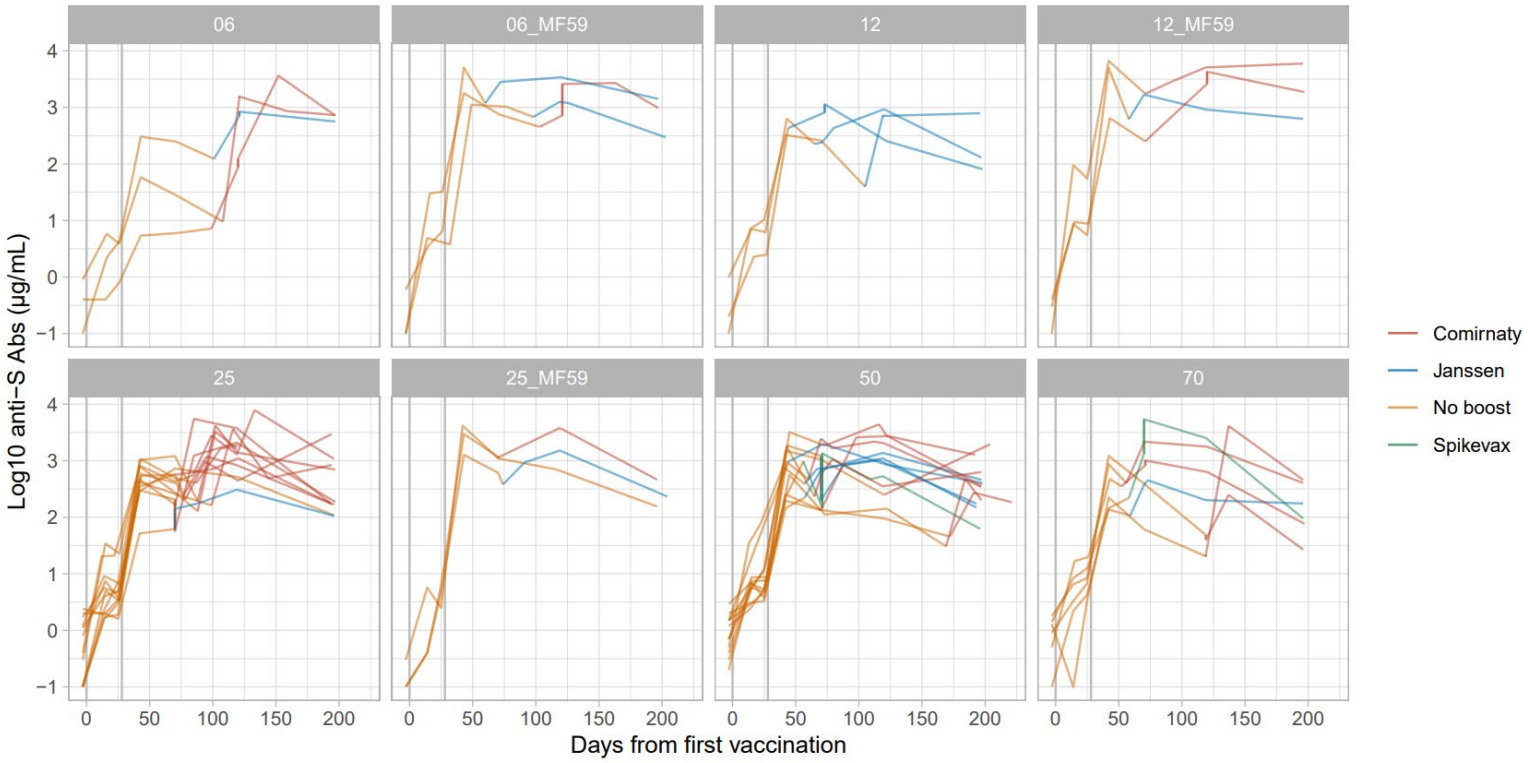


Supplementary Figure S2. Phenotype RBD-specific CD4+ T cells.



Supplementary Figure S3. SARS-CoV-2 RBD-specific antibodies until end of study visit.

Vertical lines indicate the first and second ABNCoV2 vaccination (28 days after first vaccination). Different colors indicate different types of licensed SARS-CoV-2 vaccines that participants received during the follow up period.



Supplementary Tables

Supplementary Table S1. Solicited local and systemic adverse events

Percentage of participants experiencing related solicited adverse events by symptom, vaccination number, dose group, and maximum severity. The US Food and Drug Administration (FDA) toxicity grading scale was used for solicited adverse events.[7]

Adverse event	Vaccination	ABNCoV2 dose	N	% Mild	% Moderate	% Severe
Pain	1	6 µg	3	66.7	0	0
		6 µg + MF59	3	100	0	0
		12 µg	3	66.7	33.3	0
		12 µg + MF59	3	100	0	0
		25 µg	12	75.0	16.7	0
		25 µg + MF59	3	66.7	0	0
		50 µg	12	50.0	41.7	0
		70 µg	6	66.7	33.3	0
	2	6 µg	3	0	0	0
		6 µg + MF59	3	66.7	0	0
		12 µg	3	0	33.3	0
		12 µg + MF59	3	100	0	0
		25 µg	12	66.7	16.7	0
		25 µg + MF59	3	0	100	0
		50 µg	12	50.0	33.3	0
		70 µg	6	50.0	33.3	0
Tenderness	1	6 µg	3	0	0	0
		6 µg + MF59	3	66.7	0	0
		12 µg	3	66.7	0	0
		12 µg + MF59	3	33.3	0	0
		25 µg	12	16.7	0	0
		25 µg + MF59	3	66.7	33.3	0
		50 µg	12	50.0	0	0
		70 µg	6	33.3	0	0
	2	6 µg	3	0	0	0
		6 µg + MF59	3	66.7	0	0
		12 µg	3	33.3	0	0
		12 µg + MF59	3	66.7	0	0
		25 µg	12	66.7	8.3	0
		25 µg + MF59	3	33.3	0	0
		50 µg	12	50.0	0	0
		70 µg	6	16.7	16.7	0
Erythema	1	6 µg	3	0	0	0
		6 µg + MF59	3	0	0	0
		12 µg	3	0	0	0
		12 µg + MF59	3	0	0	0
		25 µg	12	8.3	0	0
		25 µg + MF59	3	0	0	0
		50 µg	12	16.7	0	0
		70 µg	6	33.3	0	0
	2	6 µg	3	0	0	0
		6 µg + MF59	3	0	0	0
		12 µg	3	0	0	0
		12 µg + MF59	3	0	0	0
		25 µg	12	25.0	0	0
		25 µg + MF59	3	0	0	0
		50 µg	12	33.3	0	0
		70 µg	6	16.7	0	0
Induration	1	6 µg	3	0	0	0
		6 µg + MF59	3	0	0	0
		12 µg	3	0	0	0
		12 µg + MF59	3	0	0	0
		25 µg	12	16.7	0	0
		25 µg + MF59	3	33.3	0	0
		50 µg	12	16.7	0	0
		70 µg	6	16.7	0	0
	2	6 µg	3	33.3	0	0
		6 µg + MF59	3	33.3	0	0
		12 µg	3	0	0	0
		12 µg + MF59	3	33.3	0	0
		25 µg	12	33.3	0	0
		25 µg + MF59	3	0	0	0
		50 µg	12	33.3	0	0
		70 µg	6	16.7	0	0

Adverse event	Vaccination	ABNCoV2 dose	N	% Mild	% Moderate	% Severe
Headache	1	6 µg	3	33.3	0	0
		6 µg + MF59	3	0	33.3	0
		12 µg	3	33.3	0	0
		12 µg + MF59	3	66.7	0	0
		25 µg	12	16.7	8.3	0
		25 µg + MF59	3	33.3	0	0
		50 µg	12	41.7	8.3	0
		70 µg	6	33.3	0	0
	2	6 µg	3	0	0	0
		6 µg + MF59	3	66.7	33.3	0
		12 µg	3	0	33.3	0
		12 µg + MF59	3	33.3	0	0
		25 µg	12	41.7	16.7	0
		25 µg + MF59	3	66.7	0	0
		50 µg	12	25.0	8.3	0
		70 µg	6	16.7	0	16.7
Fatigue	1	6 µg	3	33.3	0	0
		6 µg + MF59	3	0	33.3	0
		12 µg	3	0	66.7	0
		12 µg + MF59	3	33.3	33.3	0
		25 µg	12	8.3	16.7	0
		25 µg + MF59	3	0	66.7	0
		50 µg	12	8.3	41.7	0
		70 µg	6	33.3	0	0
	2	6 µg	3	0	0	0
		6 µg + MF59	3	33.3	33.3	0
		12 µg	3	0	0	0
		12 µg + MF59	3	0	66.7	0
		25 µg	12	8.3	16.7	0
		25 µg + MF59	3	0	33.3	0
		50 µg	12	16.7	0	0
		70 µg	6	0	33.3	0
Fever	1	6 µg	3	0	0	0
		6 µg + MF59	3	0	0	0
		12 µg	3	0	0	0
		12 µg + MF59	3	0	0	0
		25 µg	12	0	0	0
		25 µg + MF59	3	0	0	0
		50 µg	12	16.7	0	0
		70 µg	6	16.7	0	0
	2	6 µg	3	0	0	0
		6 µg + MF59	3	0	0	0
		12 µg	3	0	0	0
		12 µg + MF59	3	0	0	0
		25 µg	12	0	0	0
		25 µg +MF59	3	0	0	0
		50 µg	12	0	0	0
		70 µg	6	0	16.7	0
Drowsiness	1	6 µg	3	0	0	0
		6 µg + MF59	3	0	0	0
		12 µg	3	0	0	0
		12 µg + MF59	3	0	0	0
		25 µg	12	16.7	0	0
		25 µg + MF59	3	33.3	0	0
		50 µg	12	16.7	0	0
		70 µg	6	0	0	0
	2	6 µg	3	0	0	0
		6 µg + MF59	3	0	0	0
		12 µg	3	0	0	0
		12 µg + MF59	3	0	0	0
		25 µg	12	0	0	0
		25 µg + MF59	3	0	0	0
		50 µg	12	16.7	8.3	0
		70 µg	6	33.3	0	0
Chills	1	6 µg	3	0	0	0
		6 µg + MF59	3	0	0	0
		12 µg	3	0	0	0
		12 µg + MF59	3	0	0	0
		25 µg	12	0	0	0
		25 µg + MF59	3	0	0	0
		50 µg	12	0	25.0	0
		70 µg	6	16.7	0	0
	2	6 µg	3	0	0	0
		6 µg + MF59	3	33.3	0	0
		12 µg	3	0	0	0
		12 µg + MF59	3	0	0	0
		25 µg	12	8.3	8.3	0
		25 µg + MF59	3	0	0	0
		50 µg	12	25.0	8.3	0
		70 µg	6	0	0	0

Supplementary Table S2. Unsolicited adverse events

Number and severity of unsolicited, non-serious, adverse events classified by MedDRA® System Organ Class. The US Food and Drug Administration (FDA) toxicity grading scale was used for laboratory adverse events.[7]

MedDRA System Organ Class	Severity	Related to vaccination	Not related to vaccination
General disorders and administration site conditions	Mild	22	47
	Moderate	5	22
	Severe	-	4
Nervous system disorders	Mild	9	42
	Moderate	4	19
	Severe	-	1
Musculoskeletal and connective tissue disorders	Mild	4	52
	Moderate	-	17
	Severe	-	-
Gastrointestinal disorders	Mild	14	29
	Moderate	6	15
	Severe	-	1
Infections and infestations	Mild	-	19
	Moderate	-	12
	Severe	-	2
Respiratory, thoracic and mediastinal disorders	Mild	9	14
	Moderate	2	3
	Severe	-	-
Skin and subcutaneous tissue disorders	Mild	8	8
	Moderate	1	2
	Severe	-	-
Injury, poisoning and procedural complications	Mild	-	12
	Moderate	-	4
	Severe	-	-
Metabolism and nutrition disorders	Mild	4	2
	Moderate	2	1
	Severe	-	-
Reproductive system and breast disorders	Mild	1	7
	Moderate	-	1
	Severe	-	-
Immune system disorders	Mild	-	8
	Moderate	-	-
	Severe	-	-
Blood and lymphatic system disorders	Mild	2	3
	Moderate	-	-
	Severe	-	-
Cardiac disorders	Mild	-	3
	Moderate	-	-
	Severe	-	-
Ear and labyrinth disorders	Mild	-	3
	Moderate	-	-
	Severe	-	-
Psychiatric disorders	Mild	1	2
	Moderate	-	-
	Severe	-	-
Investigations	Mild	-	2
	Moderate	-	-
	Severe	-	-
Renal and urinary disorders	Mild	-	1
	Moderate	-	-
	Severe	-	-
Vascular disorder	Mild	1	-
	Moderate	-	-
	Severe	-	-

Supplementary Table S3. RBD peptide list

SARS-Cov-2 RBD peptides used for PBMC stimulation prior to flow cytometry analysis of cytokine (TNF-a and IFN-g), activation marker CD137 and degranulation marker CD107a on CD4 and CD8 T cells

Peptide sequence	Class I and Class II alleles
CYFPLQSYG	C*0702
FRKSNLKPF	B*0801
KIADYNYKL	A*0201
KPFERDIST	B*0702
KVGGNYNYL	A*0201, B*0702
NYLYRLFRK	A*2402
NYNYLYRLF	A*0201, A*2402
QAGSTPCNG	C*0401, C*0702
QPYRVVVLS	B*0702, B*0801
RLFRKSNLK	A*0301, A*1101, B*0702
VVLSFELLH	A*0301, A*1101
VYADSFVIR	A*2402
YFPLQSYGF	A*2402
YLYRLFRKS	A*2402
YQPYRVVVL	B*0801
YRLFRKSNL	B*0801
GKIADYNYKLP	DRB1*0301
GGNYNYLYRLFRKSNL	DRB1*0801,DRB1*1101, DRB1*1301, DRB5*0101
LKPFERDISTEI	DRB1*0401
CYFPLQSYGFQPTN	DRB1*0401

Supplementary Table S4. WHO standard ELISA

The First WHO International Standard (20/136) for anti-SARS-CoV-2 immunoglobulin is the freeze-dried equivalent of 0.25 mL of pooled plasma obtained from eleven individuals recovered from SARS-CoV-2 infection. The intended use of the International Standard is for the calibration and harmonisation of serological assays detecting anti-SARS-CoV-2 neutralising antibodies. The preparation can also be used as an internal reference reagent for the harmonisation of binding antibody assays.[8]

Sample ID	Classification	Mean (µg/ml)	Mean (BAU/ml)	BAU/ml (WHO)
20/136	high	266	1000	1000
20/150	high	183	688	817
20/148	median	63	236	205
20/144	low	13	48	66
20/140	low	7	28	45
20/142	negative	0	0	0

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