Supplementary Information For

A ferritin-based COVID-19 nanoparticle vaccine that elicits robust, durable, broad-spectrum neutralizing antisera in non-human primates

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MATERIALS AND METHODS:

IgG plasmids

Antibody sequences and Fc-tagged ACE2 were cloned into the CMV/R plasmid backbone for expression under a CMV promoter. The antibodies with variable HC/LC were cloned between the CMV promoter and the bGH poly(A) signal sequence of the CMV/R plasmid to facilitate improved protein expression. The variable region was cloned into the human IgG1 backbone. This vector also contained the HVM06_Mouse (P01750) Ig HC V region 102 signal peptide to allow for protein secretion and purification from the supernatant.

Lentivirus plasmids

The 21-amino acid C-terminally truncated spike proteins with native signal peptides were cloned in place of the HDM-SARS2-spike-delta21 gene (Addgene plasmid, 155130). This construct contains a 21-amino acid C-terminal deletion to promote viral production, contained in all SARS-CoV-2 variants of concern. The SARS-CoV-1 spike contained an 18-amino acid C-terminal deletion. The other viral plasmids that were used have been previously described,[1] including pHAGE-Luc2-IRES-ZsGreen (NR-52516), HDM-Hgpm2 (NR-52517), pRC-CMV-Rev1b (NR-52519) and HDM-tat1b (NR-52518).

Other plasmids

An in-house pADD2 vector was used for all nanoparticle production. Sequences encoding DCFHP (residues 1-1146 of HexaPro)[2] and SΔC-Fer (residues 1-1143 as previously described)[3] were cloned into the pADD2 vector backbone using HiFi PCR (Takara) followed by In-Fusion (Takara) cloning with EcoRI/XhoI cut sites. This was followed by an amplicon containing H. pylori ferritin (residues 5–168) originally generated as a gene-block fragment from Integrated DNA Technologies (IDT). The spike and ferritin subunits were separated by a SGG linker as previously described.[4]

The SARS-CoV-2 RBD construct was kindly provided by Dr. Florian Krammer.[5] It contains the native signal peptide (residues 1–14) followed by residues 319–541 from the SARS-CoV-2 Wuhan-Hu-1 genome sequence (GenBank MN908947.3). There is a C-terminal hexa-histidine tag. The pCAGGS plasmid contains a CMV promoter for protein expression in mammalian cells.

Protein production

Transiently expressed proteins were expressed in Expi293F cells. Expi293F cells were cultured in media containing 66% Freestyle/33% Expi media (Thermo Fisher Scientific) and grown in TriForest polycarbonate shaking flasks at 37 °C in 8% CO_2. The day before transfection, cells were harvested by centrifugation and resuspended to a density of 3×10^6 cells / mL in fresh media. The next day, cells were diluted and transfected at a density of approximately $3–4 \times 10^6$ cells / mL. Transfection mixtures were made by combining the following components and adding the mixture to cells: maxi-prepped DNA, culture media and FectoPro (Polyplus) at a ratio of 0.5–0.8 µg:100 µl:1.3 µl to 900 µL cells. For example, for a 100-ml transfection, 50–80 µg of DNA would be added to 10 mL of culture media, and then 130 µl of FectoPro would be added to this. After mixing and a 10-minute incubation, the resultant transfection cocktail would be added to 90 mL of cells. The cells were harvested 3–5 days after transfection by spinning the cultures at >7,000g for 15 minutes. Supernatants were filtered using a 0.22-µm filter.

Protein purification — Fc Tag-containing proteins

All proteins containing an Fc tag (for example, IgGs and hFc-ACE2) were purified using a 5-ml MabSelect SuRe PRISM column on the ÄKTA pure fast protein liquid chromatography (FPLC) system (Cytiva). Filtered cell supernatants were diluted with a 1/10 volume of 10× PBS. The ÄKTA system was equilibrated with: A1 – 1× PBS; A2 – 100 mM glycine pH 2.8; B1 – 0.5 M NaOH; Buffer line – 1× PBS; and Sample lines – H_2O. The protocol washes the column with A1, followed by loading of the sample in Sample line 1 until air is detected in the air sensor of the sample pumps, followed by 5 column volume washes with A1 and elution of the sample by flowing of 20 ml of A2 (directly into a 50-ml conical containing 2 ml of 1 M Tris pH 8.0). The column was then washed

with 5 column volumes of A1, B1 and A1. The resultant Fc-containing samples were concentrated using 50-kDa or 100-kDa cutoff centrifugal concentrators.

Protein purification — His-tagged proteins

Expi293F expressed SARS-CoV-2 RBD, containing a hexa-His tag was purified using HisPur Ni-NTA resin (Thermo Fisher Scientific). Expi cell supernatants were diluted with 1/3 volume of wash buffer (20 mM imidazole, 20 mM HEPES pH 7.4 and 150 mM NaCl), and the Ni-NTA resin was added to diluted cell supernatants and then samples were incubated at 4 °C while stirring overnight. Resin-supernatant mixtures were added to chromatography columns for gravity flow purification. The resin in the column was washed with 20 mM imidazole in 1xPBS, and the proteins were eluted with 250 mM imidazole in 1xPBS. Column eluates were concentrated using centrifugal concentrators followed by size-exclusion chromatography on an ÄKTA pure system. ÄKTA pure FPLC with a Superose 6 Increase gel filtration column (S6) was used for further purification. One mL of sample was injected using a 2-ml loop and run over the S6, which had been pre-equilibrated in de-gassed 20 mM HEPES and 150 mM NaCl just before use.

Nanoparticle Purification – Transient Transfection

SΔC spike ferritin nanoparticles were isolated as previously described.[3] Briefly, they were purified using anion exchange chromatography, followed by size-exclusion chromatography using an SRT® SEC-1000 column. Expi293F supernatants were concentrated using a AKTA Flux S column (Cytiva). The buffer was then exchanged with 20 mM Tris, pH 8.0 via overnight dialysis at 4°C using 100 kDa molecular weight cut-off (MWCO) dialysis tubing. Dialyzed supernatants were filtered through a 0.22 µm filter and loaded onto a HiTrap® Q anion exchange column equilibrated in 20 mM Tris, pH 8.0. Spike nanoparticles were eluted using a 0 -1 M NaCl gradient. Protein-containing fractions were pooled and concentrated using a 100 kDa MWCO Amicon® spin filter, and subsequently purified on a AKTA Pure system (Cytiva) using an SRT® SEC-1000 SEC column equilibrated in 1X PBS. Fractions were pooled based on A_{280} signals and SDS-PAGE analysis on 4-20% Mini-PROTEAN® TGX™ protein gels stained with GelCode™ Blue Stain Reagent

(ThermoFisher). Prior to immunizations or freezing, the samples were supplemented with 10% glycerol, filtered through a 0.22 µm filter, snap frozen, and stored at -20°C until use.

DCFHP purification was done similar to above except via flowthrough anion exchange followed by size-exclusion. Two buffers were initially prepared (buffer A: 20mM Tris pH 8.0, buffer B: 20mM Tris pH 8.0, 1M NaCl). Filtered Expi293F or CHO supernatant was diluted with buffer B by 1/5 volume to a final concentration of 200mM NaCl. The HiTrap® Q anion exchange column was washed with 5 column volumes (CV) sequentially with buffers A, B, A, prior to sample loading. Diluted sample containing 200mM NaCl was added to the column and the flow through was collected. One 5mL HiTrap® Q anion exchange column was used for every 200mL of diluted media. Multiple columns were joined in series for larger sample volumes. 100 kDa MWCO Amicon® spin filters were used to concentrate and buffer exchange the sample with 2 washes with 20 mM Tris, 150 mM NaCl pH 7.5. After the final wash, the sample was concentrated and filtered with a .22µm filtered. The filtered sample was then loaded onto an SRT SEC-1000 column preequilibrated with 20mM tris pH 7.5, 150mM NaCl. The nanoparticle containing fractions were pooled as indicated in SI Fig 1. Samples were routinely concentrated to .5-1mg/mL and flash frozen in 20 mM Tris, 150 mM NaCl, 5% sucrose (weight:volume) buffer. Formulation with alum (Invivogen) was done by first extensively mixing (inversion 50 times) of the alum adjuvant and then mixing with appropriately diluted immunogen, at least 15 minutes prior to immunization.

SDS-PAGE Analysis

For the SDS-PAGE quantitation of DCFHP, samples diluted 10X in 1xPBS were mixed with the 2X reducing SDS sample buffer (900 µL 4x SDS PAGE sample buffer (Biorad) + 100 µL ß-mercaptoethanol + 2 ml of water) at 1:1 ratio and 10 µL of the mixture was loaded the 10-well 4-20 % SDS-PAGE gradient gel. This loading is an equivalent to 0.5 µL of the starting media material. As a standard, each gel also included a mix of 1 ug of BSA + 1 µg of purified DCFHP from Expi293F cells. The quantitation was done with ImageJ software. The area of each band was determined, and the expression yield was calculated as Y=(Area of the band)/Area of the BSA standard*2 [g/L]

Western Blot Analysis

Samples were diluted in SDS-PAGE Laemmli loading buffer (Bio-Rad) and electrophoresed on a 4–20% Mini-PROTEAN TGX protein gel (Bio-Rad). Proteins were transferred to nitrocellulose membranes using a Trans-Blot Turbo transfer system. Blots were blocked in 5% milk/PBST (1× PBS [pH 7.4], 0.1% Tween 20) and then washed with PBST. In-house-made primary antibody, CR3022 as previously described[3,6] (approximate concentration 0.8–1.3 mg/mL), was added at a 1:10,000 dilution in PBST. Blots were washed with PBST, and secondary rabbit anti-human IgG H&L HRP (abcam ab6759) was added at 1:10,000 in PBST. Blots were developed using Pierce ECL substrate and imaged using a GE Amersham imager 600.

SEC-MALS of Ferritin-Antigen Nanoparticles

SEC-MALS was performed on an Agilent 1260 Infinity II HPLC instrument with Wyatt detectors for light scattering (miniDAWN) and refractive index (Optilab). The purified antigen (20 µg) was loaded onto a SRT SEC-1000 4.6 mm × 300 mm column and equilibrated in 1× PBS (pH 7.4). SRT SEC-1000 column was run at a flow rate of 0.35 mL/min, and molecular weights were determined using ASTRA version 7.3.2 (Wyatt Technologies). For quantification, 75 µL of media sample was filtered with 0.22 um 96 well plate filters and 5 µL was injected into SEC SRT-1000 column. DCFHP expressed in Expi293F cells was used to generate a standard curve of 2.5, 5, 10, and 20 µg DCFHP protein. The area under peak (UV) for each curve was determined with Agilent software. The amount of the protein in the nanoparticle peak was interpolated from a standard curve using Prism interpolation function.

BLI of mAbs Binding to SARS-CoV-2 Purified Antigens

BLI was performed on an OctetRed 96 system (ForteBio). Samples were assayed in “Octet buffer” (0.5% bovine serum albumin, 0.02% Tween, 1×DPBS (1xDPBS from Gibco)) in 96-well flat-bottom black-wall, black-bottom plates (Greiner). Biosensors were equilibrated in Octet buffer for at least 10 minutes and regenerated in 100 mM glycine (pH 1.5) prior to sample testing. Tips in experiments that involved regeneration were regenerated in 100 mM glycine [pH 1.5] prior to testing. Anti-Human Fc sensor tips (ForteBio, now Sartorius) were loaded with 200 nM mAb at a

threshold of 0.8nm and then submerged into wells containing 100 nM (protomer/monomer concentration) of each antigen. For samples where BLI was being used to quantitate concentrations, a standard curve was made using purified DCFHP in PBS, where the final association signal used to determine the standard curve.

Specifically, COVA 2-15 antibody was used for standard curve development and tested on day 13 media samples of stably expressing CHO cells. Media was diluted to a final dilution of 1:200. COVA2-15 antibody was diluted to 100 nM concentration in octet buffer and loaded on Fc-binding octet tip to threshold of 0.4 The tips was then moved into octet buffer for 30 seconds and then into well containing the 200X diluted media samples to obtain the end-point readout after 30 seconds of association (readout averaged at 29.5-29.9 sec). The tips were regenerated, as above, twice before starting the experiment. The yield was calculated using interpolation from the standard curve determined with DCFHP using Prism Version 9.4.1.

Differential scanning fluorimetry

Thermal melts were determined using the Prometheus NT.48 made by Nanotemper. Samples were loaded into Prometheus NT.Plex nanoDSF Grade High Sensitivity Capillary Chips and the laser intensity was set such that the discovery scan placed the auto fluorescence between the upper and lower bounds. Samples were let to melt using the standard melt program (1˚C/min). Melting temperatures were determined by peaks on the first derivatives of the ratio of F350/F330.

Stable cell line production

Stable cell line production was completed at ATUM (Newark CA) under GMP conditions as described previously[7,8]. Briefly, DNA sequences encoding DCFHP were chemically synthesized and transformed into *E. coli* hosts. In addition to DCFHP, the constructs were designed to express glutamine synthetase. Sequence confirmation of the DCFHP gene and whole plasmid sequence was routinely conducted. Additionally, Leap-In Transposase system ORF expression constructs were designed and constructed based on ATUM's proprietary technology. E. coli hosts containing

DCFHP genes were maxi-prepped (Macherey-Nagel). HD-BIOP3 cells were then transfected with both components and underwent selection by glutamine deprivation.[9] Resulting pooled cells were sorted and resulting single clones were analyzed as described throughout the manuscript. Optimal clones were selected for cell banking.

Lentivirus production

SARS-CoV-2 VOCs and SARS-CoV-1 spike pseudotyped lentiviral particles were produced. HEK293T cells were transfected with plasmids described above for pseudoviral production using BioT transfection reagent. Six million cells were seeded in D10 media (DMEM + additives: 10% FBS, L-glutamate, penicillin, streptomycin and 10 mM HEPES) in 10-cm plates 1 day before transfection. A five-plasmid system (plasmids described above) was used for viral production, as described in Crawford et al.[1] The spike vector contained the 21-amino acid truncated form of the SARS-CoV-2 spike sequence from the Wuhan-Hu-1 strain of SARS-CoV-2 or VOCs or 18-amino acid truncation for SARS-CoV-1. VOCs were based on wild-type (WT) (Uniprot ID: BCN86353.1); Alpha (sequence ID: QXN08428.1); Beta (sequence ID: QUT64557.1); Gamma (sequence ID: QTN71704.1); Delta (sequence ID: QWS06686.1, which also has V70F and A222V mutations); and Omicron (sequence ID: UFO69279.1) specific mutations shown in SI Table 3. The plasmids were added to D10 medium in the following ratios: 10 µg pHAGE-Luc2-IRS-ZsGreen, 3.4 µg FL spike, 2.2 µg HDM-Hgpm2, 2.2 µg HDM-Tat1b and 2.2 µg pRC-CMV-Rev1b in a final volume of 1,000 µl. To form transfection complexes, 30 µl of BioT (BioLand) was added. Transfection reactions were incubated for 10 minutes at room temperature, and 9 ml of medium was added slowly. The resultant 10 ml was added to plated HEK cells from which the medium had been removed. Culture medium was removed 24 hours after transfection and replaced with fresh D10 medium. Viral supernatants were harvested 72 hours after transfection by spinning at 300xg for 5 minutes, followed by filtering through a 0.45-µm filter. Viral stocks were aliquoted and stored at −80 °C until further use.

Neutralization

The target cells used for infection in viral neutralization assays were from a HeLa cell line stably overexpressing the SARS-CoV-2 receptor, ACE2, as well as the protease known to process SARS-CoV-2, TMPRSS2. Production of this cell line is described in detail in ref.[10] with the addition of stable TMPRSS2 incorporation. ACE2/TMPRSS2/HeLa cells were plated 1 day before infection at 10,000 cells per well. Ninety-six-well white-walled, clear-bottom plates were used for the assay (Thermo Fisher Scientific) and a white seal was placed on the bottom prior to readout. As previously described,[3] on the day of the assay, dilutions of heat inactivated serum were made into sterile D10 medium. Samples were analyzed in technical duplicate in each experiment. Virus-only wells and cell-only wells were included in each assay. Additionally, a COVA2-15 positive neutralization control was included in each assay to confirm replicability of the experiment.

A virus dilution was made containing the virus of interest (for example, SARS-CoV-2) and D10 media (DMEM + additives: 10% FBS, L-glutamate, penicillin, streptomycin and 10 mM HEPES). Virus dilutions into media were selected such that a suitable signal (>1,000,000 RLU) would be obtained in the virus-only wells. Polybrene was present at a final concentration of 5 µg/mL in all samples. 50 µL of heat inactivated sera was mixed with 50 µL viral dilution to make a final volume of 120 µl In each well.

The inhibitor (serum dilution) and virus mixture was left to incubate for 1 hour at 37 °C. After incubation, the medium was removed from the cells on the plates made 1 day prior. This was replaced with 100 µl of inhibitor/virus dilutions and incubated at 37 °C for approximately 48 hours. Infectivity readout was performed by measuring luciferase levels. 48 hours after infection, the medium was removed from all wells and cells were lysed by the addition of 100 µl of a 1:1 dilution of BriteLite assay readout solution (Perkin Elmer) and 1xDPBS (Gibco) into each well. Luminescence values were measured using a BioTek Synergy HT (BioTek) or Tecan M200 microplate reader. Each plate was normalized by averaging cell-only (0% infectivity) and virus-only (100% infectivity) wells. Cell-only and virus-only wells were averaged. Normalized values were fit with a four-parameter non-linear regression inhibitor curve in Prism to obtain 50%

neutralizing titer (NT_{50}) values. Half-life values were calculated starting from the maximum serum titer obtained for each sample. Half-life values were first calculated via a 1 phase decay, which, when unconstrained, demonstrated high plateau values of serum neutralization around 10^3, demonstrating the importance of constraining the plateau value to close to 0. Constraining the plateau to $\log(10^0)$, we tested both one-phase (monophasic) and two-phase (biphasic) decay. The fast phase of the two-phase decay fit well for all animals (SI Table 5) but given the slow decline of neutralizing titers, there was not enough data to fully fit the slow phase decay for some animals, specifically those in group B. in such cases where estimated half-life was >5000 days, 5000 days was used for average calculations shown in SI Table 5. LOQ was set as the neutralizing titer of day 0 serum or the lowest serum dilution tested, whichever was higher. For samples with different LOQs on the same graph, the average value was used.

ELISA

RBD (5 µg/mL) was plated in 50 µl in each well on a MaxiSorp (Thermo Fisher Scientific) microtiter plate in 1xPBS and left to incubate for at least 1 hour at room temperature. These were washed 3 times with 300 µl of ddH_2O using an ELx 405 Bio-Tex plate washer and blocked with 150 µl of ChonBlock (Chondrex) for at least 1 hour at room temperature. Plates were washed 3x with 300 µl of 1x PBST. Mouse serum samples, serially diluted in diluent buffer (1x PBS, 0.1% Tween) starting at 1:50 serum dilution was then added to coated plates for 1 h at room temperature. This was then washed 3x with PBST. HRP goat anti-mouse (BioLegend 405306) was added at a 1:10,000 dilution in diluent buffer for 1 h at room temperature. This was left to incubate at room temperature for 1 hour and then washed 6x with PBST. Finally, the plate was developed using 50 µl of 1-StepTM Turbo-TMB-ELISA Substrate Solution (Thermo Fisher Scientific) per well, and the plates were quenched with 50 µl of 2 M H_2SO_4 to each well. Plates were read at 450 nm and normalized for path length using a BioTek Synergy HT Microplate Reader.

Live SARS-CoV-2 virus isolation and passages

Variants were obtained from two sources. WA-1/2020 was obtained from the WRCEVA collection. BA.1 and BA.2 were isolated from de-identified nasopharyngeal (NP) swabs sent to

the California Department of Public Health from hospitals in California for surveillance purposes. To isolate from patient swabs, 200 µl of an NP swab sample from a COVID-19-positive patient that was previously sequence-identified was diluted 1:3 in PBS supplemented with 0.75% BSA (BSA-PBS) and added to confluent Vero E6-TMPRSS2-T2A-ACE2 cells in a T25 flask, allowed to adsorb for 1 hour, inoculum removed, and additional media was added. The flask was incubated at 37 °C with 5% CO_2 for 3-4 days with daily monitoring for cytopathic effects (CPE). When 50% CPE was detected, the contents were collected, clarified by centrifugation and stored at -80 °C as a passage 0 stock. 1:10 diluted passage 0 stock was used to inoculate Vero E6-TMPRSS2-T2A-ACE2 grown to confluency in T150 flasks and harvested at approximately 80% CPE. All viral stocks were sequenced to confirm lineage, and 50% tissue culture infectious dose ($TCID_{50}$) was determined by titration.

Live SARS-CoV-2 virus 50% CPE endpoint neutralization

CPE endpoint neutralization assays were performed following the limiting dilution model using sequence-verified viral stocks of WA-1, BA.1 and BA.2 in Vero E6-TMPRSS2-T2A-ACE2. Three-fold serial dilutions of inhibitor (antisera) were made in BSA-PBS and mixed at a 1:1 ratio with 100 $TCID_{50}$ of each virus and incubated for 1 hour at 37 °C. Final inhibitor dilutions ranged from 500 nM to 0.223 nM. Then, 100 µl of the plasma/virus mixtures were added in duplicate to flat-bottom 96-well plates seeded with Vero E6-TMPRSS2-T2A-ACE2 at a density of 2.5×10^4 per well and incubated in a 37 °C incubator with 5% CO_2 until consistent CPE was seen in the virus control (no inhibitor added) wells. Positive and negative controls were included as well as cell control wells and a viral back titration to verify $TCID_{50}$ viral input. Individual wells were scored for CPE as having a binary outcome of 'infection' or 'no infection', and the ID_{50} was calculated using the Spearman–Karber method. All steps were done in a Biosafety Level 3 laboratory using approved protocols.

Cryo-EM Data Acquisition

Previously frozen DCFHP protein was quick-thawed at room temperature and subjected for gel filtration and concentrated to 0.4 mg/mL in a buffer of 150 mM NaCl, 20 mM HEPES pH 7.4. 3 µL

of the DCFHP proteins were applied onto a glow-discharged Quantifoil R 1.2/1.3 Cu 300-mesh grid (Quantifoil). The grids were blotted for 2 sec at 20°C and 100% humidity and rapidly cryocooled in liquid ethane using a Vitrobot Mark IV instrument (Thermo Fisher Scientific). The DCFHP proteins were imaged at 0.86Å per pixel by a Titan Krios cryo-electronmicroscope, TEM Beta (Thermo Fisher Scientific) at Stanford-SLAC Cryo-EM CenterS2C2, operated at 300 kV. Micrographs were recorded with EPU (Thermo Fisher Scientific) with a Gatan K2 Summit direct electron detector. Each movie was composed of 40 frames with an exposure time of 2.4 sec and 50.187 electron dose. A data set of 8,750 movie stacks was collected.

Single-Particle Image Processing and 3D Reconstruction

All 8,750 movies were imported into cryoSPARC 3.2. [11] Motion correction was performed in patch motion correction and the contrast transfer function (CTF) was determined in patch CTF estimation.[12] 601 DCFHP single particles were manually picked from 93 micrographs in Manual Picker. Four 2D classes were determined in 2D classification, which were used as templates in Template picker and a total of 1,590,688 particles was picked from 8,750 movies.

By 2D classification, 127,630 particles of DCHPF were selected and used for Ab-initio reconstruction and homogeneous refinement with C1 symmetry, the CryoEM density of the ferritin core was well resolved, while that of the Spike trimer are not, suggesting that the conformational orientation of the Spike trimer displayed on the 3-axis symmetry of ferritin is flexible. Through iterative 2D classifications, 67,324 particles showing relatively homogenous Spike density from five 2D classes were used for generating an initial model in Ab-initio reconstruction. All eight spike trimers were resolved by homogeneous refinement enforced with octahedral symmetry. A Gaussian low-pass filter was applied to the CryoEM maps displayed in UCSF Chimera.[13]

Mouse Immunizations

Balb/c female mice (6–8 weeks old) were purchased from The Jackson Laboratory. All mice were maintained at Stanford University according to the Public Health Service Policy for “Humane Care

and Use of Laboratory Animals" following a protocol approved by Stanford University Administrative Panel on Laboratory Animal Care (APLAC-33709). Mice were immunized intramuscularly with antigen doses indicated in figure legends. With the exception of the high dose alum and alum/CpG (Fig 1 and SI Fig 1 and 2 which contain 500ug alum ± 20ug CpG), all antigen doses were formulated with 150 µg alum in Tris Buffer (20mM, pH 7.5, 150mM NaCl, 5% sucrose) in a total volume of 100 µL per injection. DCFHP was produced in Expi293F cells via transient transfection for all mouse experiments excluding the temperature stability experiment where the sample was produced in the described CHO cell-line. Mice immunization schedules were as described in the Figure legends. Serum was collected and processed using Sarstedt serum collection tubes. Mouse serum was centrifuged at 10,000xg for 15 min and heat inactivated for 30 min at 56 °C.

NHP Studies

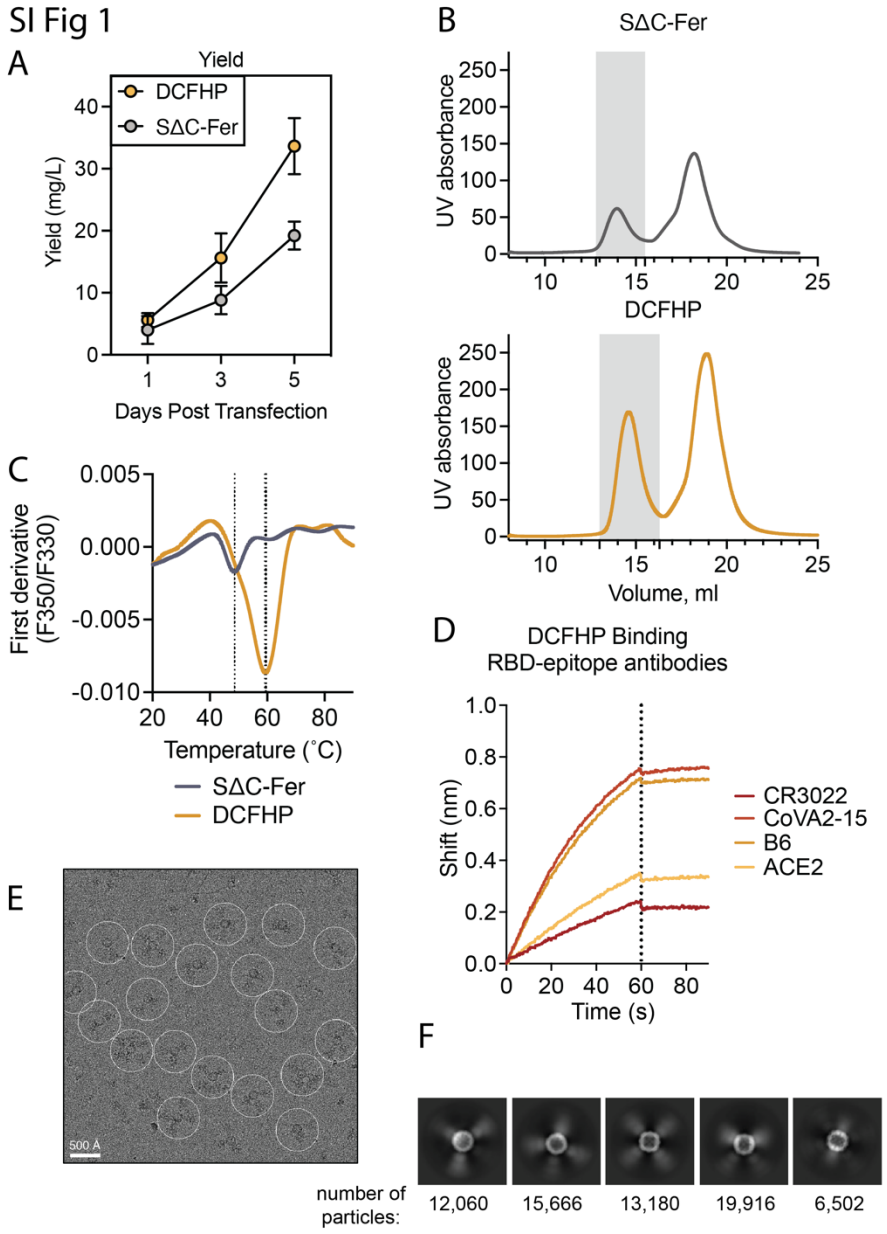
10 male rhesus macaques (Macaca mulatta) of Indian origin, aged 3-9 years, were assigned to the study (SI Table 1). The animals were distributed between the two groups such that the age and weight distribution were comparable across them. Animals were housed and maintained at the New Iberia Research Center (NIRC) of the University of Louisiana at Lafayette in accordance with the rules and regulations of the Committee on the Care and Use of Laboratory Animal Resources. The entire study (IACUC approval number: 2021-012-8738) was reviewed and approved by the University of Louisiana at Lafayette Institutional Animal Care and Use Committee (IACUC) and Stanford University APLAC committee (Protocol # 34139). All animals were negative for simian immunodeficiency virus, simian T cell leukemia virus and simian retrovirus. Animals were immunized according to the schedule outlined in SI Table 2. DCFHP for NHP vaccines was isolated from a pool of the top 24 expressing CHO cell lines.

Intracellular cytokine staining assay

Antigen-specific T cell responses were measured using the intracellular cytokine staining assay as previously described[14]. Live frozen PBMCs were thawed, counted and resuspended at a density of 10^6 live cells/mL in complete RPMI (RPMI supplemented with 10% FBS and antibiotics). The

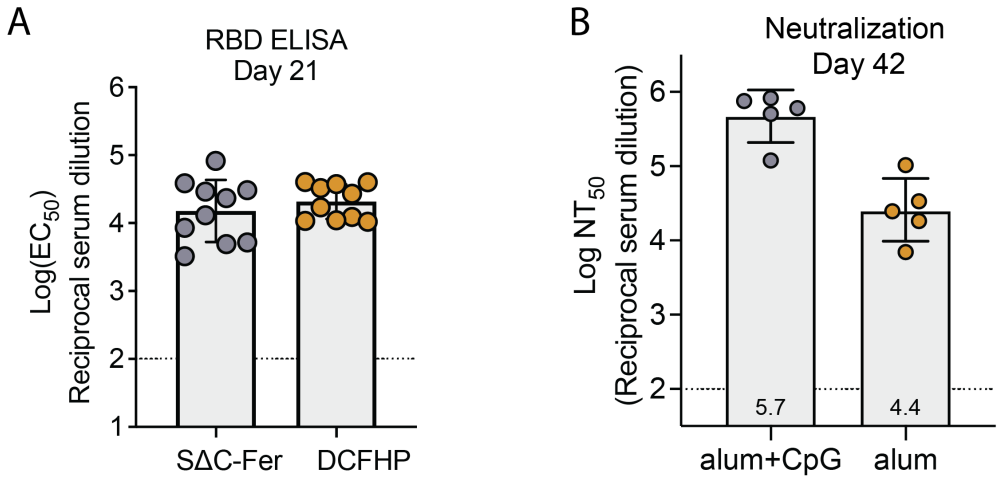
cells were rested overnight at 37 °C in a CO_2 incubator. The following day the cells were counted again, resuspended at a density of 15 × 10^6 cells/mL in complete RPMI and 100 µL of cell suspension containing 1.5 × 10^6 cells was added to each well of a 96-well round-bottomed tissue culture plate. Each cell sample was treated with three conditions: no stimulation (DMSO), a peptide pool spanning the spike protein at a concentration of 1.2 µg/mL of each peptide, and a peptide pool spanning the spike protein of Omicron BA.1 (1.2 µg/mL of each peptide). This was done in the presence of 1 µg/mL of anti-CD28 (clone CD28.2, BD Biosciences) and anti-CD49d (clone 9F10, BD Biosciences) as well as anti-CXCR3 and anti-CXCR5. The peptides were custom-synthesized to 90% purity using GenScript, a commercial vendor. All samples contained 0.5% (v/v) DMSO in total volume of 200 µL per well. The samples were incubated at 37 °C in CO_2 incubators for 2 hours before addition of 10 µg/mL brefeldin A. The cells were incubated for an additional 4 hrs. The cells were washed with PBS and stained with Zombie UV fixable viability dye (Biolegend). The cells were washed with PBS containing 5% FCS, before the addition of surface antibody cocktail. The cells were stained for 20 min at 4 °C in 100 µL volume. Subsequently, the cells were washed, fixed and permeabilized with cytofix/cytoperm buffer (BD Biosciences) for 20 min. The permeabilized cells were stained with intracellular cytokine staining antibodies for 20 min at room temperature in 1× perm/wash buffer (BD Biosciences). Cells were then washed twice with perm/wash buffer and once with staining buffer before acquisition using the BD Symphony Flow Cytometer and the associated BD FACS Diva software. All flow cytometry data were analysed using Flowjo software v10 (TreeStar Inc.).

SUPPLEMENTARY FIGURES:

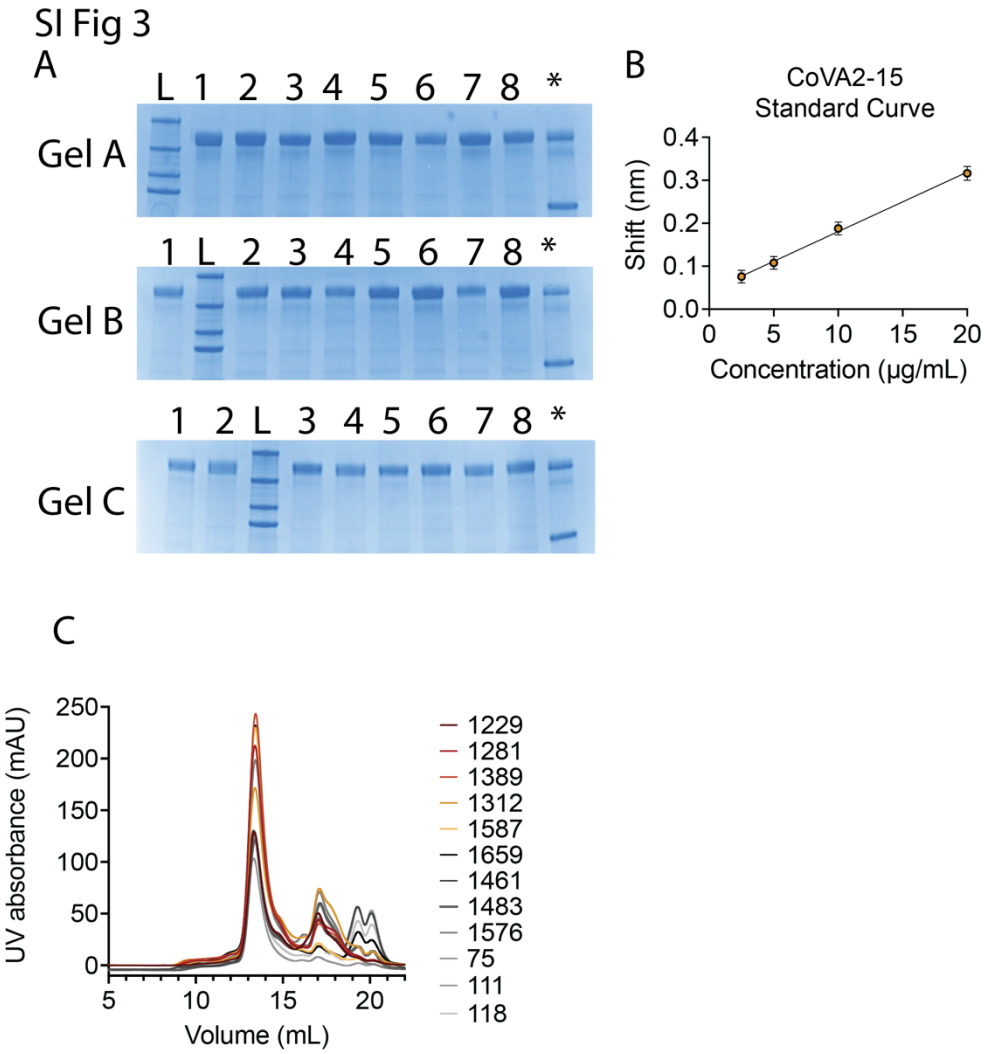


SI Figure 1 – DCFHP shows improved expression compared to SΔC-Fer, as well as proper conformation compared to SΔC-Fer. (A) Yield of DCFHP (yellow) compared to SΔC-Fer (grey) as measured by a normalized BLI assay shows improved expression in an Expi-293F transient transfection model. n=2, mean and STD are shown. (B) SEC purification on an SRT-1000 column of SΔC-Fer (grey) or DCFHP (yellow) and measurement of UV absorbance at 210 nm on the Akta Pure shows improved nanoparticle yield (grey box indicates pooled fractions). (C) DSF melting profiles of DCFHP (yellow) are substantially altered compared to SΔC-Fer (grey), consistent with previous reports of stabilization conferred by HexaPro mutations (ref[2]). (D) Monitoring binding of antibodies and Fc-ACE2 to DCFHP by BLI indicates proper epitope exposure. (E) Representative motion-corrected Cryo-EM micrograph of DCFHP. White circles indicate single particles that were manually picked and subsequently used as template for particle auto-picking. Scale bar, 500 Å. (F) Reference-free 2D class averages with the number of particles used in each class. Five 2D classes were used for generating an initial model.

SI Fig 2

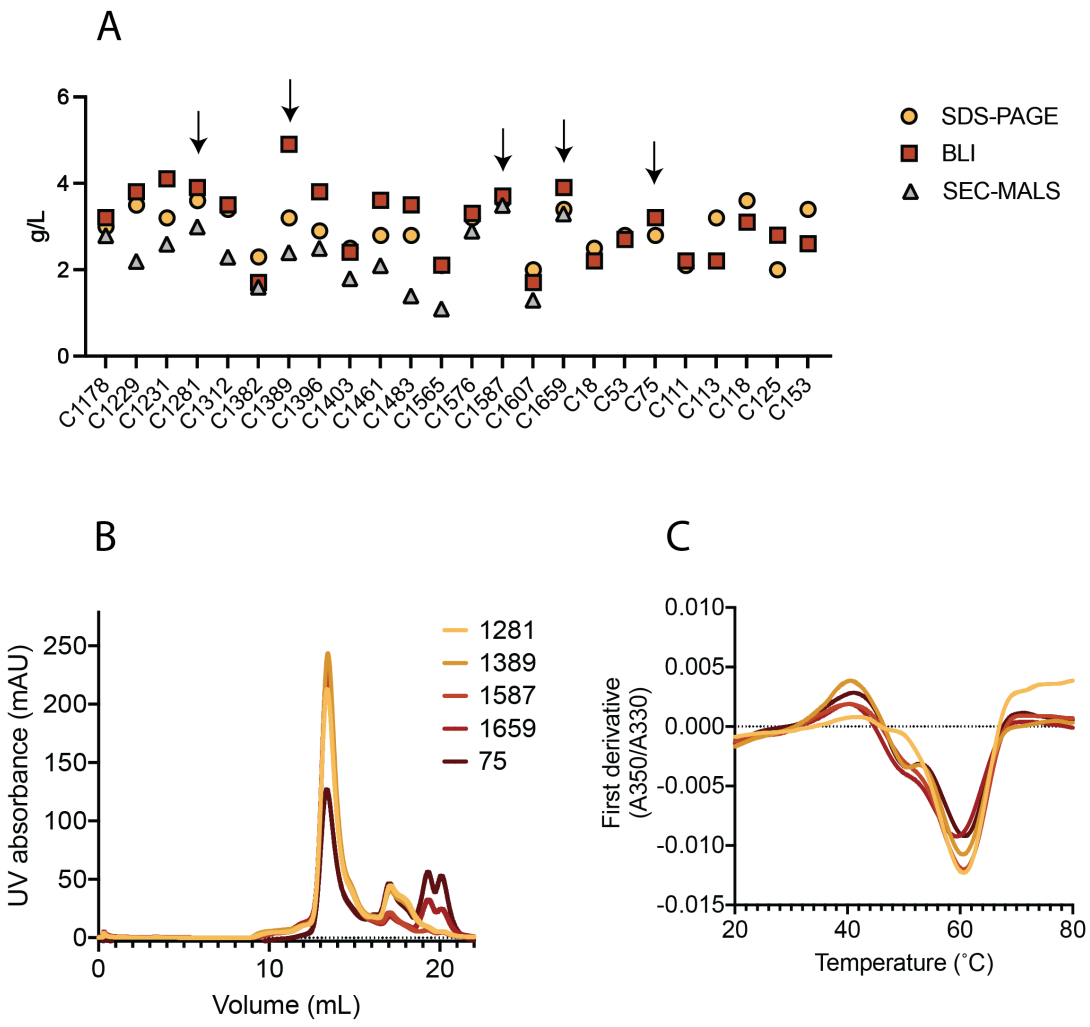


SI Figure 2 – DCFHP is immunogenic and with only alum adjuvant provides robust neutralization. (A) Serum from mice as in Fig 1E tested by ELISA binding to the SARS-CoV-2 RBD by sera from mice isolated at day 21 shows comparable binding between SΔC-Fer and DCFHP. Immunization was done with high-dose alum/CpG. Points are individual titers from each animal, GMT and STD are shown (B) Immunization with DCFHP with alum adjuvant alone retains robust 50% neutralization titer (NT50) against Wuhan-1 SARS-CoV-2 pseudovirus compared to alum and CpG. Serum analyzed on day 42 post a single prime. Individual data points are shown for each animal titer. Assay limit of quantitation are shown as dotted horizontal lines.



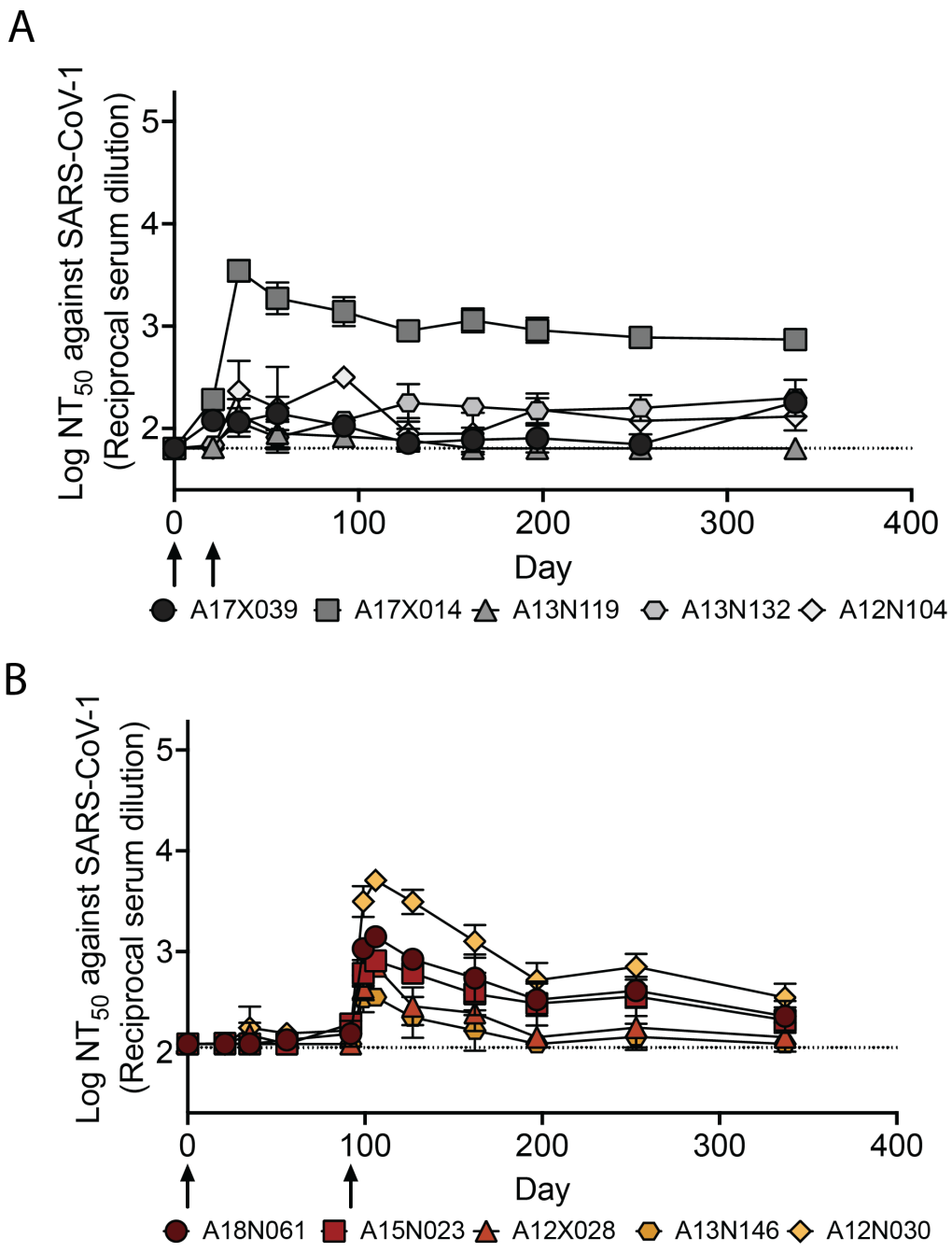
SI Figure 3 – Characterization of 24 lead, single-cell CHO clones expressing DCFHP. (A) SDS-PAGE gels from 0.5µL supernatants of each clone show robust protein expression. L = molecular weight ladder, * = 1µg DCFHP + 1µg BSA. MW lanes are, top to bottom, 250kDa, 150kDa, 100kDa, 75kDa. Gel A lanes 1-8 = C1178, C1229, C1231, C1281, C1312, C1382, C1389, C1396. Gel B lanes 1-8 = C1403, C1461, C1483, C1565, C1576, C1587, C1607, C1659. Gel C lanes 1-8 = C18, C53, C75, C111, C113, C118, C125, C153. (B) The standard curve of DCFHP binding to antibody COVA2-15 shows what was used to convert nm shifts to g/L of clones as described in the Methods. (C) SEC-MALS traces for 12 samples shown in Gel A, Gel B, and Gel C in panel A define the predominant nanoparticle peak. AUC was used to estimate g/L. as described in the Methods.

SI Fig 4



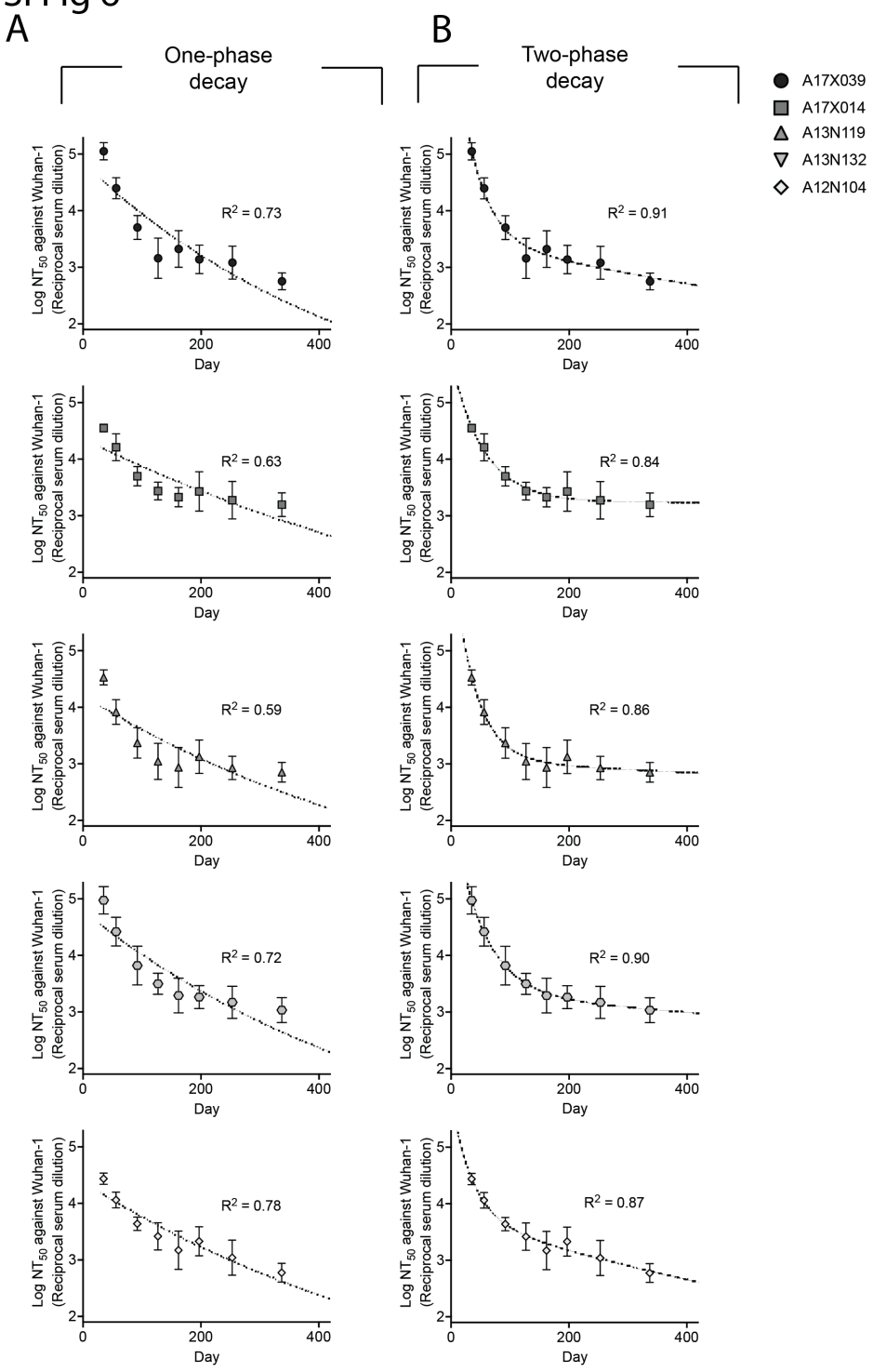
SI Figure 4 – Single-cell clonal selection of DCFHP-expressing, stably integrated CHO K1 cells. (A) The 24 top cell clones were analyzed for DCFHP expression on day 13 of culture by SDS-PAGE, BLI, and SEC-MALS (as in SI Fig 3). Final, calculated g/L are plotted, estimated by densitometry compared to purified DCFHP, a BLI standard curve using CoVA2-15 antibody developed against purified DCFHP, and area under the curve analysis for the nanoparticle peak, respectively. The five clones with the most favorable parameters are indicated by arrows. (B) SEC-MALS traces of the five selected clones shows predominant nanoparticle peaks at ~13.5mL. (C) DSF melting curves of the five selected clones shows similar profiles, with peaks at 40˚C and 61˚C.

SI Fig 5



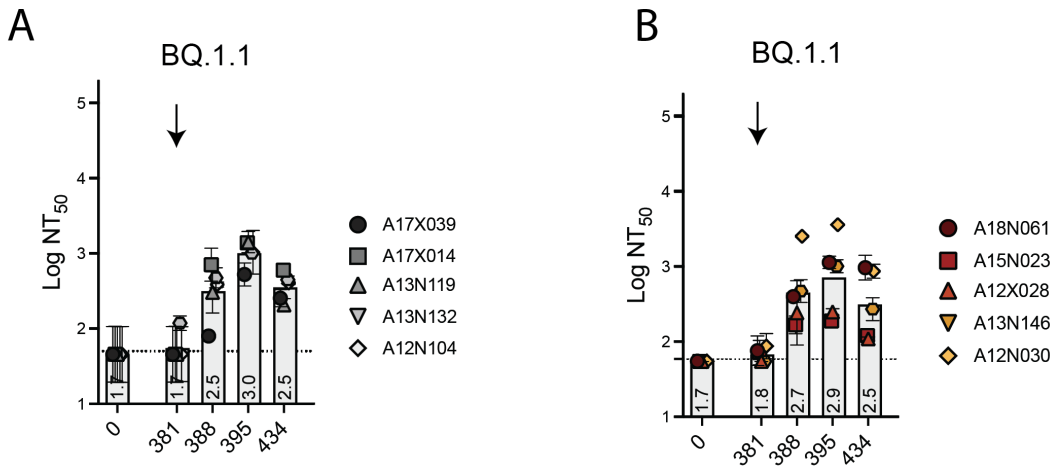
SI Figure 5 – Serum neutralizing titers over 337 days for animals in groups A and B shows longevity of the neutralizing response against SARS-CoV-1 for animals in group B. (A) as in Fig 4A but with SARS-CoV-1 pseudovirus. (B) as in Fig 4C but with SARS-CoV-1 pseudovirus. Average and standard deviation for biological replicates are shown, n = 2 for group A and n = 3 for group B, all biological replicates of the entire experiment were conducted on different days.

SI Fig 6



SI Figure 6 – Serum NT_{50} values are best fit with a two-phase decay. Constrained with a plateau value of 0 (NT_{50} value = 1 on log plot), monophasic (A) or biphasic (B) decay models were used to fit the NT_{50} values for individual animal in group A starting 14 days post boost. R^2 values shown on plots. Unconstrained monophasic decay shows acceptable fits, but results in plateau values of ~10^3.

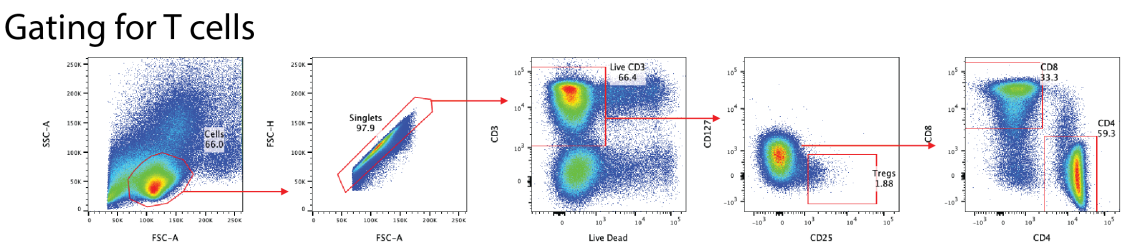
SI Fig 7



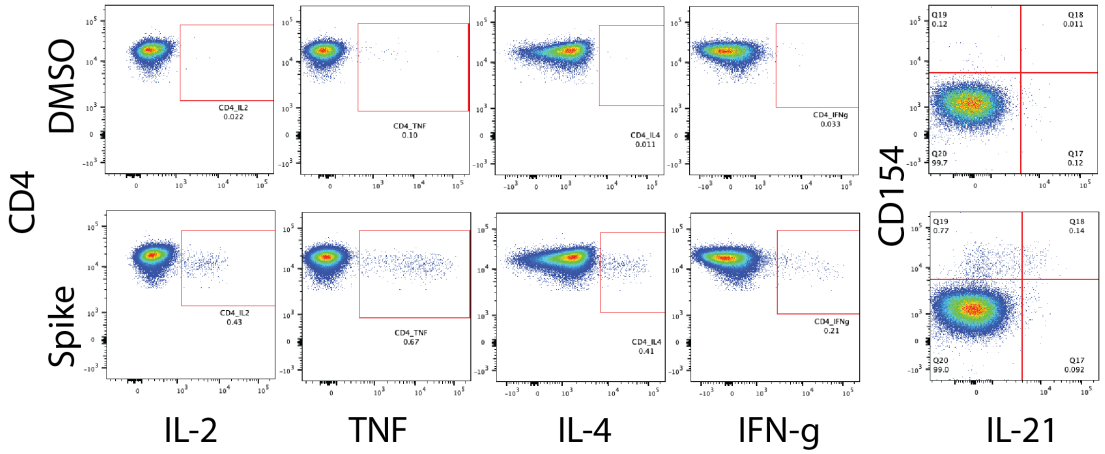
SI Figure 7 – Anamnestic responses against BQ.1.1 is seen following a second booster of DCFHP-alum after ~one year in NHPs. Neutralization against BQ.1.1 by antisera from NHPs in group A (A) or B (B) following a boost at day 381. NHP identification provided correlate with SI table 1. (n = 2 biological replicates throughout). Assay limits of quantitation is indicated by horizontal dotted lines.

SI Fig 8

A



B



SI Figure 8 – Representative flow cytometry gating scheme for T-cell analysis. (A) Gating scheme for CD4+ T-cells is shown, showing selection of live, single cell T cells which are positive for either CD4+ or CD8+. No CD8+ cells showed stimulation. (B) Representative flow plots from NHP PBMCs stimulated with DMSO (top) or spike protein peptides (bottom), gated as in A were further gated based on intracellular cytokine activity. Stimulation results in positive populations when staining for intracellular cytokines (bottom). Percent positive cells denoted on plots. Percent positive was determined by subtracting the percent positive in the DMSO sample to that in the stimulated sample.

SUPPLEMENTARY TABLES

SI Table 1: NHP Information

Group	Animal ID	Birthdate	Sex	Body Weight (kg) – Day 0 (10/04/2021)
A	A17X039 Rhm	5/16/17	Male	7.5
A	A17X014 Rhm	4/23/17	Male	6.5
A	A13N119 Rhm	6/5/13	Male	8.5
A	A13N132 Rhm	6/19/13	Male	12.5
A	A12N104 Rhm	6/23/12	Male	13.1
B	A18N061 Rhm	4/25/18	Male	5.2
B	A15N023 Rhm	4/9/15	Male	10.3
B	A12X028 Rhm	11/22/12	Male	10.7
B	A13N146 Rhm	6/28/13	Male	12.5
B	A12N030 Rhm	5/1/12	Male	10.8

SI Table 2: NHP Immunizations

Group	Vaccine	Dose (mg)	Adjuvant – Alhydrogel (mg)	Administration Volume (µL)	# of Animals	Immunization Schedule (days)
A	DCFHP	50	750	500	5	0, 21, 381
B	DCFHP	50	750	500	5	0, 92, 381

SI Table 3: Variant of Concern Mutations used in Pseudoviral Assay

Variant	Strain name	Mutations
D614G	D614G	D614G
Alpha	B.1.1.7	Δ69-70, Δ144, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H
Beta	B.1.351	L18F, D80A, D215G, Δ242-244, R246I, K417N, E484K, N501Y, D614G, A701V,
Gamma	P1	L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, T1027I
Delta	B.1.617.2	T19R, T95I, G142D, Δ156-157, R158G, L452R, T478K, D614G, P681R, D950N
Omicron	BA.1	A67V, Δ69-70, T95I, Δ142-Δ144, Y145D, Δ211, L212I, G339D, S371L, S373P, S375F, K417N, N440K, G446S, S477N, T478K, E484A, Q493R, G496S, Q498R, N501Y, Y505H, T547K, D614G, H655Y, N679K, P681H, N764K, D796Y, N856K, Q954H, N969K, L981F
Omicron	BA.2	T19I, Δ24-26, A27S, G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, S477N, T478K, E484A, Q493R, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K
Omicron	BA.4/5	T19I, Δ24-26, A27S, G142D, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, L452R, S477N, T478K, E484A, F486V, Q493R, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K
Omicron	BQ.1	T19I, Δ24-26, A27S. Δ69-70, G142D, Δ144, V213G, G339D, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, K444T, L452R, N460K, S477N, T478K, E484A, F486V, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K
Omicron	BQ.1.1	T19I, Δ24-26, A27S. Δ69-70, G142D, V213G, G339D, R346T, S371F, S373P, S375F, T376A, D405N, R408S, K417N, N440K, K444T, L452R, N460K, S477N, T478K, E484A, F486V, Q498R, N501Y, Y505H, D614G, H655Y, N679K, P681H, N764K, D796Y, Q954H, N969K

SI Table 4: Estimated one-phase and two-phase decay half-life values against Wuhan-1 pseudovirus for NHPs

Animal ID	Half-Life One-Phase (days)	Half-Life Two Phase, Fast Phase (days)	Percent in Fast Phase (Two Phase)	Half-Life Two Phase, Slow Phase (days)
Group A (Day 21 boost)				
A17X039	340	26	54%	1100
A17X014	580	38	44%	>5000
A13N119	450	26	55%	3,900
A13N132	390	36	50%	2,600
A12N104	450	25	36%	800
Group B (Day 92 boost)				
A18N061	360	47	72%	>5000
A15N023	870	58	48%	>5000
A12X028	200	34	84%	860
A13N146	470	25	84%	1,800
A12N030	380	16	95%	700
Averages for all NHPs	64 weeks ± 25 weeks	4.7 weeks ± 1.8 weeks	47% Group A 77% Group B	7.3 years ± 5.1 years

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