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Supplementary Materials for

Antibody cocktail to SARS-CoV-2 spike protein prevents rapid mutational escape seen with individual antibodies

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Materials and Methods

Generation of recombinant VSV. Non-replicative pseudoparticles were generated using a VSV genome encoding the mNeonGreen fluorescent reporter gene (*13*) instead of the native viral glycoprotein (VSV-G). Infectious particles complemented with VSV-G ($VSV^{\Delta G:mNeon}$/VSV-G) were recovered and produced using standard techniques with minor modifications (*14-16*). Briefly, HEK293T cells (ATCC CRL-3216) were transfected with the genomic clone driven by a T7 promoter and helper plasmids expressing the VSV-N, VSV-P, VSV-G, VSV-L, and T7 RNA polymerase with Lipofectamine LTX reagent (Life Technologies). After 48 hours, the transfected cells were co-cultured with BHK-21 cells (ATCC CCL-10) transfected with VSV-G using the SE cell Line 4D-Nucleofector X Kit L (Lonza). Cells were monitored for mNeonGreen expression or cytopathic effect (CPE) indicative of virus replication. Virus was then plaque purified, expanded, and titered in BHK-21 cells transiently expressing VSV-G. Fully replicative VSV-SARS-CoV-2-S virus was generated by replacing the VSV glycoprotein with the native SARS-CoV-2 sequences encoding residues 1-1255 of the spike protein (MN908947.3). VSV-SARS-CoV-2-S virus was recovered as described above but the HEK293T cells were instead co-cultured with BHK-21 cells transfected with both VSV-G and hACE2. VSV-SARS-CoV-2-S virus was plaque purified and titered in Vero cells (ATCC CCL-81) and expanded in Vero E6 cells (ATCC CRL-1586). After collection, stocks of both viruses were centrifuged at 3000xg for 5 minutes to clarify, sucrose cushioned to concentrate 10-fold, aliquoted, and frozen at -80C.

Pseudotyping of VSV. Non-replicative pVSV-SARS-CoV-2-S (mNeon) pseudoparticles were generated using modified methods from those previously described (*17-19*). Human codon-optimized CoV-SARS-2 spike (MN908947.3) was synthesized (Genscript) and resulting product was cloned into an expression plasmid. A total of 1.2 x 10^7 HEK293T cells (ATCC CRL-3216) were seeded overnight in 15-cm dishes in DMEM high glucose media (Life Technologies) containing 10% heat-inactivated fetal bovine serum (Life Technologies), and Penicillin/-Streptomycin-L-Glutamine (Life Technologies). The following day, the cells were transfected with 15ug spike expression plasmid with Lipofectamine LTX (Life Technologies) following the manufacturer's protocol. At 24 hours post transfection, the cells were washed with phosphate buffered saline (PBS) and infected at a MOI of 1 with the $VSV^{\Delta G:mNeon}$/VSV-G virus diluted in 10mL Opti-MEM (Life Technologies). The cells were incubated 1 hour at 37C with 5% CO2. Cells were washed three times with PBS to remove residual input virus and overlaid with DMEM high glucose media (Life Technologies) with 0.7% Low IgG BSA (Sigma), sodium pyruvate (Life Technologies), and Gentamicin (Life Technologies). After 24 hours at 37C with 5% CO2, the supernatant containing pseudoparticles was collected, centrifuged at 3000xg for 5 minutes to clarify, aliquoted, and frozen at -80C. Amino acid substitutions detected in natural isolates and escape mutants were cloned into the spike expression plasmid using site-directed mutagenesis and pseudoparticles were produced as described above.

Neutralization assays with VSV based pseudoparticles and virus. Unless otherwise noted, all reagents obtained from Life Technologies. Vero cells (ATCC: CCL-81) were seeded in 96-well black, clear bottom tissue culture treated plated (Corning: 3904) at 20,000 cells/well in DMEM high glucose media containing 10% heat-inactivated fetal bovine serum, and 1X Penicillin/Streptomycin/L-Glutamine 24 hours prior to assay. Cells were allowed to reach approximately 85% confluence before use in assay. Antibodies were diluted in DMEM high

glucose media containing 0.7% Low IgG BSA (Sigma), 1X Sodium Pyruvate, and 0.5% Gentamicin (this will be referred to as "Infection Media") to 2X assay concentration and diluted 3-fold down in Infection media, for an 11-point dilution curve in the assay beginning at 10 ug/mL (66.67 nM). pVSV-SARS-CoV-2-S pseudoparticles were diluted 1:1 in Infection media for a fluorescent focus (ffu) count in the assay of ~1000 ffu. Antibody dilutions were mixed 1:1 with pseudoparticles for 30 minutes at room temperature prior to addition onto Vero cells. Cells were incubated at 37C, 5% CO_2 for 24 hours. Supernatant was removed from cells and replaced with 100 uL PBS, and fluorescent foci were quantitated using the SpectraMax i3 plate reader with MiniMax imaging cytometer. Exported values were analyzed using GraphPad Prism (v8.2.0).

For replicative VSV-SARS-CoV-2-S virus neutralization assays, antibodies were diluted as described above but in VSV media (DMEM high glucose media containing 3% heat-inactivated fetal bovine serum and Penicillin/-Streptomycin-L-Glutamine). An equal volume of media containing 2000 pfu of VSV-SARS-CoV-2-S virus was mixed with the antibody dilutions and incubated for 30 minutes at room temperature. The mixture was then added onto Vero cells and incubated at 37C, 5% CO_2 for 24 hours. The cells were fixed (PBS with 2% paraformaldehyde) for 20 minutes, permeabilized (PBS with 5% fetal bovine serum and 0.1% Triton-X100) for 15 minutes and blocked (PBS with 3% bovine serum albumin) for 1 hour. Infected cells were immunostained with a rabbit anti-VSV serum (Imanis Life Sciences) and an Alexa Fluor® 488 secondary antibody in PBS + 3% bovine serum albumin. Fluorescent foci were quantitated using the SpectraMax i3 plate reader with MiniMax imaging cytometer.

Antibody escape studies. Antibodies were serially diluted 1:5 starting at 100ug/mL in 500 uL of VSV media. A no antibody control was included to account for any tissue culture adaptations and quasispecies variability that may occur during virus replication. A total of 1.5 x 10^6 plaque forming units (pfu) of VSV-SARS-CoV-2-S virus in 500ul of media was added to each dilution and incubated at room temperature for 30 minutes. After the incubation, the mixture was added to 3 x 10^5 Vero E6 cells and incubated for 96 hours at 37C with 5% CO_2. Virus replication was monitored by screening for cytopathic effect. The supernatants and cellular layers were collected from wells with the highest antibody concentration with evident viral replication. The total RNA, including the viral RNA, was extracted from the cells using TRIzol (Life Technologies) following the manufacturer's protocol for next gen sequencing. For a second round of selection, 100uL of supernatant containing the virus was brought to 500ul with VSV media and passed under the same or greater antibody concentrations as before. Again, the supernatants were collected, and RNA was extracted from cells in wells with the highest antibody concentration with evident viral replication.

Virus RNA Sequencing

RNA was quantified using the Qubit RNA HS Assay Kit (ThermoFisher). 1 to 50 ng RNA was treated by FastSelect-rRNA HMR (Qiagen) to remove host rRNA, and the incubation condition for fragmentation was followed as 85ºC for 6 minutes, 75ºC for 2 minutes, 70ºC for 2 minutes, 65ºC for 2 minutes, 60ºC for 2 minutes, 55ºC for 2 minutes, 37ºC for 5 minutes, 25ºC for 5 minutes, and hold at 4ºC. Strand-specific RNA-seq libraries were prepared from the treated RNA using KAPA RNA HyperPrep Kit (Roche Sequencing). UDI with UMI Adapters (IDT) were

ligated. Sixteen-cycle PCR was performed to amplify libraries. Sequencing was run on MiSeq (Illumina) by multiplexed paired-read run with 2X70 cycles.

Variant calling from SARS-CoV-2 public genomes. SARS-CoV-2 complete genome sequences were downloaded from GISAID Nucleotide database (https://www.gisaid.org) on March 20th, 2020. Sequences were curated and genetic diversity of the spike-encoding gene was assessed across high quality genome sequences using custom pipelines. In brief, Blastn was used to align the Whuan_Hu_1 spike nucleotide sequence (accession: MN908947) against each individual genome. Results were analyzed and presence of the gene was validated if alignment length was greater than 95% with an identity percentage greater than 70%. Homologous spike protein sequences were extracted, translated and aligned to identify amino-acid changes with respect to the reference.

NGS Data Analysis. NGS analysis was perform using Array Studio software package platform (Omicsoft). Quality of paired-end RNA Illumina reads was assessed using the "raw data QC of RNA-Seq data suite". Minimum and maximum read length, total nucleotide number, and GC% were calculated. Overall quality report was generated summarizing the quality of all reads in each sample, along each base pair. Paired-end RNA Illumina reads were then mapped against VSV-SARS-CoV-2-S virus genome using Omicsoft Sequence Aligner (OSA) version 4. Reads were trimmed by quality score using default parameters (when aligner encountered nucleotide in the read with a quality score of 2 or less, it trimmed the remainder of the read). OSA outputs were analyzed and annotated using Summarize Variant Data and Annotate Variant Data packages (Omicsoft). Target coverage was calculated for each sample. SNPs calling was performed using samples with average target coverage greater than 500 reads. SNPs with a minimum frequency of 1% and a coverage greater than 50 were identified and annotated.

Fig. S1.

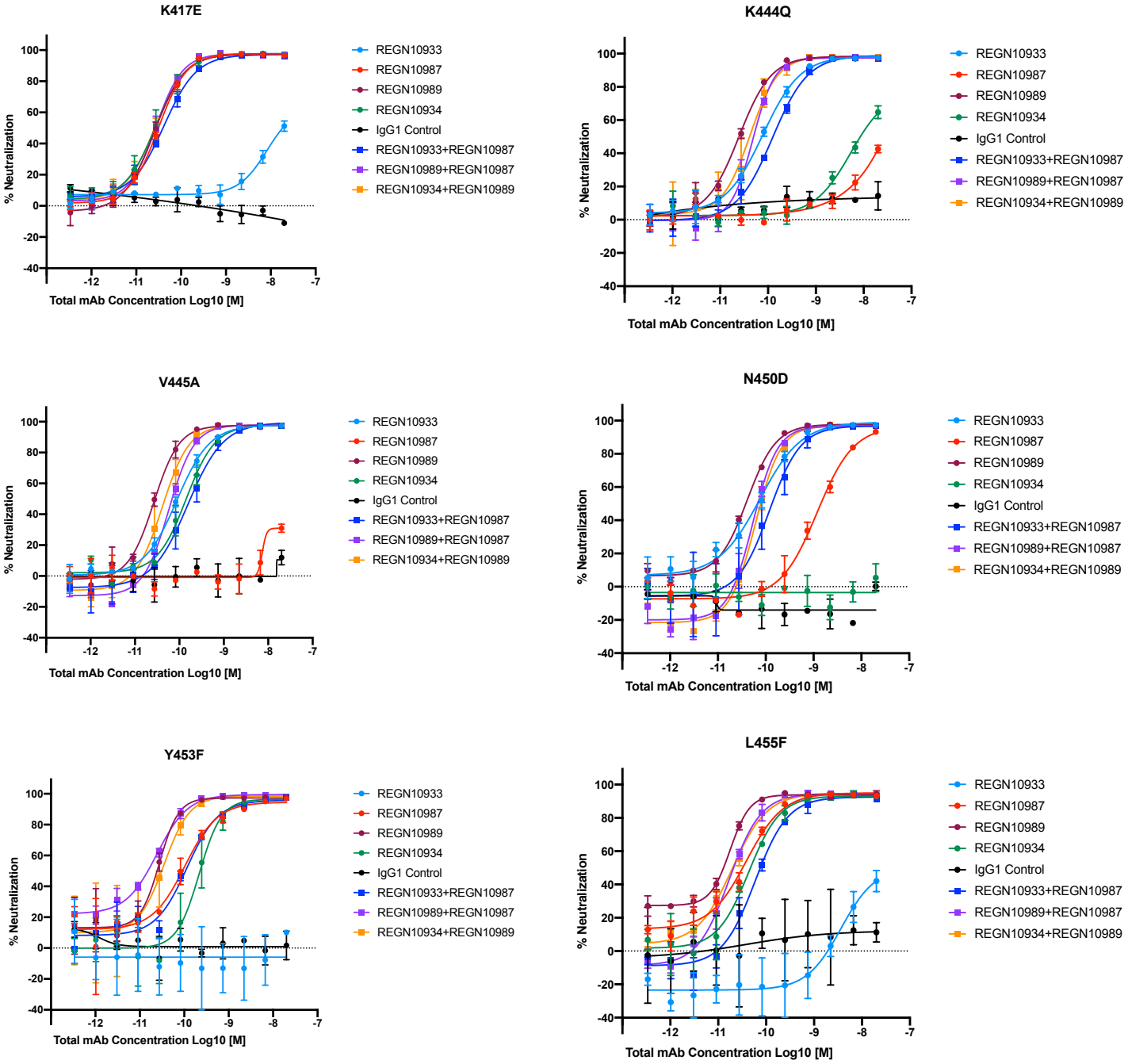
K417E
K444Q
V445A
N450D
Y453F
L455F
% Neutralization
Total mAb Concentration Log10 [M]
REGN10933
REGN10987
REGN10989
REGN10934
IgG1 Control
REGN10933+REGN10987
REGN10989+REGN10987
REGN10934+REGN10989

Fig. S1.

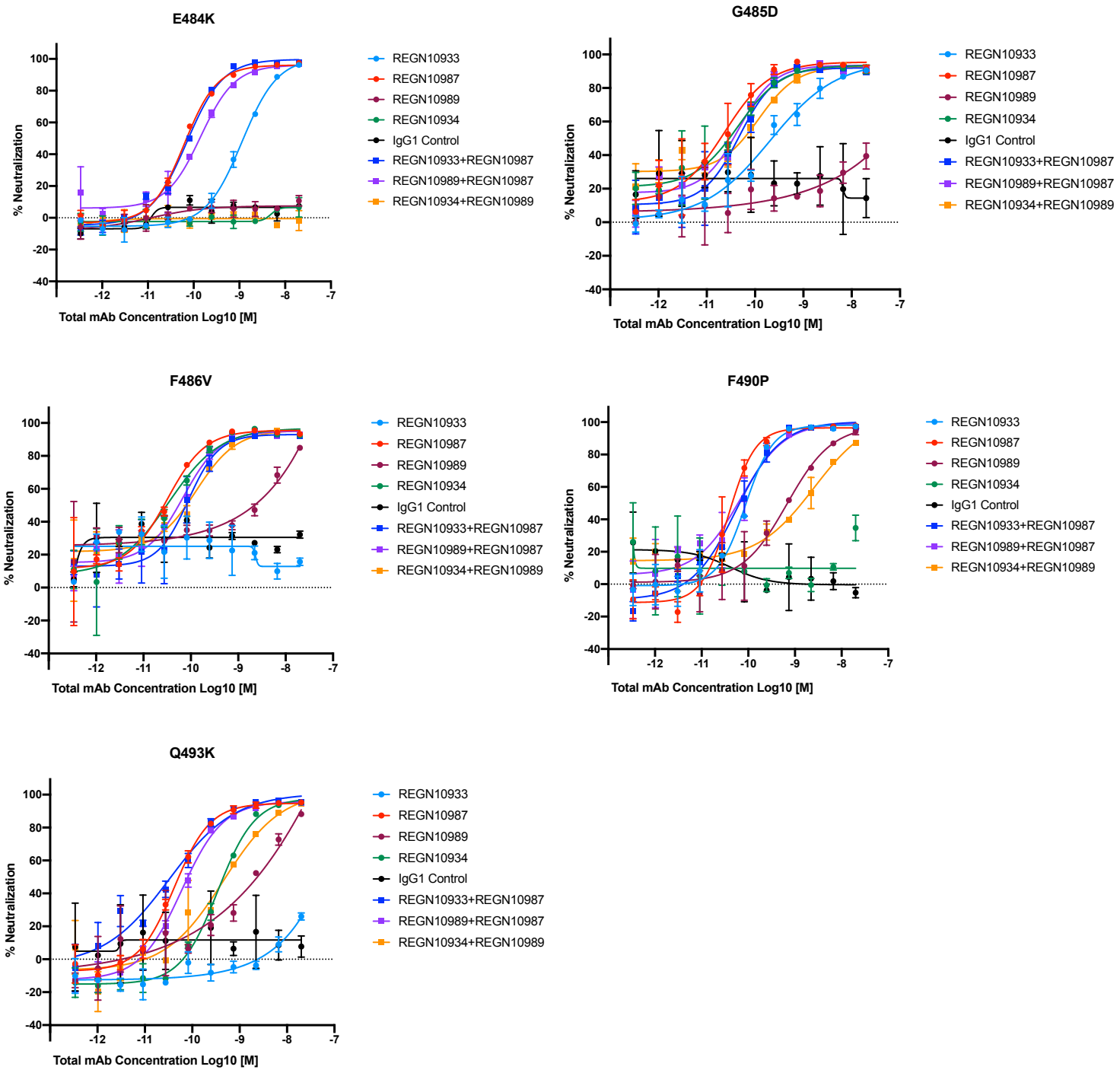

Fig. S1. Neutralization potency of individual anti-spike antibodies and antibody combinations against pseudoparticles encoding individual escape mutants-full curves. Escape mutations identified by RNAseq analysis within the RDB domain were cloned and expressed on pseudoparticles to assess their impact on mAb neutralization potency. Complete loss of neutralization or dramatic shift in potency can be observed for all mutations identified by RNAseq analyses.

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