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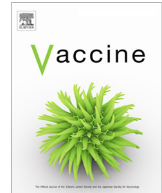

Pan-SARS neutralizing responses after third boost vaccination in non-human primate immunogenicity model

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ABSTRACT

The emergence of SARS-CoV-2 variants, especially Beta and Delta, has raised concerns about the reduced protection from previous infection or vaccination based on the original Wuhan-Hu-1 (D614) virus. To identify promising regimens for inducing neutralizing titers towards new variants, we evaluated monovalent and bivalent mRNA vaccines either as primary vaccination or as a booster in nonhuman primates (NHPs). Two mRNA vaccines, D614-based MRT5500 and Beta-based MRT5500β, tested in sequential regimens or as a bivalent combination in naïve NHPs produced modest neutralizing titers to heterologous variants. However, when mRNA vaccines were administered as a booster to pre-immune NHPs, we observed a robust increase in neutralizing titers with expanded breadth towards all tested variants, and notably SARS-CoV-1. The breadth of the neutralizing response was independent of vaccine sequence or modality, as we further showed either MRT5500 or recombinant subunit Spike protein (with adjuvant) can serve as boosters to induce broadly neutralizing antibodies in the NHPs primed with MRT5500. The data support the notion that a third vaccination is key to boosting existing titers and improving the breadth of antibodies to address variants of concern, including those with an E484K mutation in the Receptor Binding Domain (RBD) (Beta, Gamma).

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1. Introduction

SARS-CoV-2, a zoonotic Beta-coronavirus, is the cause of the current global COVID-19 pandemic [1]. In the United States, the implementation of public health measures and immunization campaigns with vaccines approved under emergency use authorization have lowered the incidence of hospitalization and death significantly [2]. However, the emergence of SARS-CoV-2 variants of concern (VoC), particularly the Beta (B.1.351) [3], Gamma (P.1) [4], and Delta (B.1.617.2) [5], as well as variant of interest Epsilon (B.1.429) [6], have threatened the success of current vaccines which are based on the original sequence from Wuhan-Hu-1 (D614) [7]. In addition to increased transmissibility due to the D614G mutation [8], both Beta and Gamma variants harbor key mutations such as K417N and E484K (or the functionally similar E484Q [9]) in the Receptor Binding Domain (RBD) of the viral Spike protein, which account for the observed reduction in neutralizing potency of convalescent sera and post-vaccination immune sera [10–13]. For example, trials of NVX-CoV2373, a subunit vaccine candidate with 89.3% efficacy against the Alpha variant, revealed a reduced vaccine efficacy of 61% against the Beta variant [14,15].

The question posed to vaccine researchers and developers is how to define vaccine candidates or compositions to best address

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the variants of concern already in circulation and those emerging in the future, especially viruses with the key E484K/Q mutation in the RBD resistant to neutralization by antibodies [10,16]. One approach to address new variants would be to update the composition to a multivalent vaccine to include new variants, analogous to seasonal influenza vaccine updates [17]. The drawback of this approach is the manufacturing requirement for regular updates to vaccine compositions to cover new variants. While mRNA vaccines offer speed and flexibility towards broad antigen production capabilities, this could still pose some challenges in vaccine manufacturing and distribution for some modalities. An alternative approach would be the development of pan-SARS-CoV-2 vaccines capable of sufficient cross-neutralizing breadth against the known variants of concern to circumvent the need for regular vaccines updates. Such efforts were discussed in a recent report on constructing pan-coronavirus vaccines [18]. Furthermore, a booster vaccination has been proposed based on the observations of waning neutralizing titers in subjects following vaccinations [19,20] or natural infection [21,22] with the question being whether such a booster vaccination should be inclusive of key variants of concern in its composition.

We previously reported on the pre-clinical immunogenicity evaluation of MRT5500 [23], an mRNA vaccine candidate based on the D614 sequence which is currently under clinical evaluation (NCT04798027). In the NHP studies described herein, we attempted to evaluate the optimal vaccine valency for coverage against VoC using the same technology and design principle as in the MRT5500 [23] to generate an additional vaccine candidate (MRT5500β). Using the widely accepted pseudovirus (PsV) neutralization assays to assess neutralizing titers against key VoC [24], we first compared vaccination regimens of monovalent or bivalent formulations for their ability to induce virus neutralizing responses in naïve NHPs, and then tested these compositions as a booster vaccination in NHPs primed with MRT5500. We also compared the boosting efficiency of an mRNA vs. a protein subunit (soluble pre-fusion-stabilized Spike trimers (SARS-CoV-2 preS dTM)) vaccine formulated with adjuvant in pre-immune NHPs. We found the third vaccination, regardless of vaccine composition or modality, appeared to be the key for eliciting broadly neutralizing antibodies against known variants of concern. Our findings have direct implications for vaccination regimens to address the known and emerging VoC in clinical settings.

2. Materials & methods

2.1. mRNA synthesis, lipid nanoparticle formulation and expression assay

Messenger RNA constructs incorporating coding sequences which contain both the stabilized pre-fusion mutation (2P) [25] and furin cleavage site mutation (GSAS) [26,27] to make the double mutant (2P/GSAS) of the full-length SARS-CoV-2 Spike glycoprotein were generated. mRNA was synthesized by *in vitro* transcription employing RNA polymerase and unmodified nucleotides to transcribe the mRNA from a plasmid DNA template encoding the desired gene. The resulting purified precursor mRNA was reacted further via enzymatic addition of a 5′ cap structure (Cap 1) and a 200 nucleotide 3′ poly(A) tail. The vaccine sequences are based on the D614 (Wuhan Hu-1) Spike sequence (Genbank accession MN908947) or on the Beta Spike sequence (Genbank accession QRI43207.1). Preparation of mRNA/lipid nanoparticle (LNP) formulations was described previously [28]. Briefly, an ethanolic solution of a mixture of lipids (ionizable lipid, phosphatidylethanolamine, cholesterol, and polyethylene glycol-lipid) at a fixed lipid to mRNA ratio were combined with an aqueous buffered solution of target mRNA at an acidic pH under controlled conditions to yield a suspension of uniform LNPs. After ultrafiltration and diafiltration into a suitable diluent system, the resulting nanoparticle suspensions were diluted to final concentration, filtered, and stored frozen at −80 °C until use.

2.2. Non-human primate studies

Ethics statement: Animal studies were conducted in compliance with all pertinent US National Institutes of Health regulations as well as with all relevant local, state, and federal regulations. Animal protocols were approved by the Institutional Animal Care and Use Committees (IACUCs) of the facilities at New Iberia Research Center (New Iberia, LA) protocol code 2020-8733-013 last reviewed on April 28th, 2021.

Cynomolgus macaques of Mauritian origin 2–6 years of age and weighing in a range of 2–6 kg were administered 500 μL mRNA/LNP formulations via intramuscular (IM) route into the deltoid of the right forelimb on Day 0 and the opposite forelimb on Day 21. Animals were later randomized and re-grouped after the initial priming phase to ensure equivalent distribution of priming phase doses and male:female ratio into the boosting phase. The third dose was given either on D128 or D315. Sera were collected on pre-immunization day (D-4), and post-immunization days D14, D21, D28, D35, D42, and may also include D90, D125, D143, D157, D171, D308, D329, D343, D350, and D364. All immunizations and blood draws occurred under sedation with Ketamine HCl (10 mg/kg) or Telazol (4–8 mg/kg IM).

2.3. Convalescent human sera

Convalescent serum panel (N = 93) was obtained from commercial vendors (Sanguine Biobank, iSpecimen and PPD). These subjects had a PCR positive diagnosis of COVID-19, and the serum samples were collected within 3 months following diagnosis.

2.4. Enzyme-linked immunosorbent assay (ELISA)

For NHP sera, Nunc MaxiSorb plates (Thermo Scientific, Cat#439454) were coated with custom made Geneart SARS-CoV S-GCN4 protein at 0.5 μg/mL in PBS overnight at 4 °C. Plates were washed 3–5 times with PBS-Tween 0.1% before blocking with 1% BSA in PBS-Tween 0.1% for 1 h at ambient temperature. Samples were plated at a 1:450 initial dilution followed by 3-fold serial dilution in blocking buffer. Plates were washed 3–5 times after 1 h incubation at room temperature before adding 50 μL of 1:8000 Rabbit anti-human IgG (Jackson Immuno Research, Cat# 109-036-098) to each well. Plates were incubated at room temperature for 1hr and washed 3-5x. Plates were developed using Pierce 1-Step Ultra TMB-ELISA Substrate Solution for 0.1 h and stopped by TMB stop solution. Plates were read at 450 nm in SpectraMax plate readers. Antibody titers were reported as the highest dilution that is $\geq$ 0.2 OD cutoff.

2.5. Pseudovirus neutralization assay

Methods adapted from Kalnin et al 2021 for each pseudovirus Spike sequence [23]. In brief, Green Florescent Protein (GFP) expressing reporter virus particles (RVP) displaying SARS-CoV-2 or SARS-CoV-1 Spike proteins on their surface were obtained from Integral Molecular (catalog numbers: D614 RVP-701G, Alpha RVP-706G, Beta RVP-724G, Gamma RVP-708G, Delta custom order B.1.617.2 lineage, Epsilon RVP-713G, CoV-1 RVP-801G) (Table 3). Serum samples were diluted 1:4 in media (FluoroBrite phenol red free DMEM + 10% FBS + 10 mM HEPES + 1% PS + 1% Glutamax) and heat inactivated at 56 °C for 0.5 h. A further, 2-fold serial dilu-

tion of the heat inactivated serum was prepared and mixed with RVPs diluted to contain ~ 6 infectious particles per μL of the serum/RVP mixture and incubated for 1 h at 37 °C. 96-well plates of 50% confluent 293 T-hsACE2 clonal cells (Integral Molecular Cat. # C-HA102,) in 75 μL volume were inoculated with 50 μL of the serum/RVP mixtures and incubated at 37 °C for 72 h. At the end of the incubation, plates were scanned on a high-content imager and individual GFP expressing cells were counted. The inhibitory dilution titer (ID_{50}) was reported as the reciprocal of the dilution that reduced the number of virus plaques in the test by 50%. ID_{50} for each test sample was interpolated by calculating the slope and intercept using the last dilution with a plaque number below the 50% neutralization point and the first dilution with a plaque number above the 50% neutralization point. ID_{50} Titer = (50% neutralization point - intercept)/slope).

2.6. Statistical analysis

All statistical tests were two-sided, and the nominal level of statistical significance was set to α = 5%. The analyses were performed on SEG SAS v9.4®.

ELISA titers and neutralizing titers were $\log_{10}$ transformed prior to statistical analysis.

Statistical comparisons were performed among different groups and conducted for each readout and each strain separately (i.e., D614 or Beta). When applicable one-way ANOVA with the group as factor or Mixed effect models for repeated measures, including group, day and their interactions, where day is specified as repeated measure were performed. For Mixed model with repeated measures Tukey or Dunnett adjustment where performed, for one-way ANOVA no adjustment was performed as only 4 NHP per groups were available.

As the studies were not performed at the same time, for the comparison between the groups which received 5ug of MRT5500 at D0 and D21 and the groups which received MRT5500 with of MRT5500β, the characteristics at baseline (age, weight, and sex) were checked in order to verify that they were well balanced between the groups. Both studies were conducted in the same species of NHP, and the measurements were performed by the same laboratory and the same analysts.

3. Results

3.1. Monovalent or bivalent mRNA vaccines elicit a range of neutralizing antibody titers to variants of concern in naïve NHPs

The vaccine candidate MRT5500 was designed with mutations within the furin cleavage site (RRAR to GSAS) at positions 682 to 685 [29], in addition to the 2P pre-fusion stabilizing mutations [30]. Though not originally tested in our preclinical evaluation of MRT5500 in NHPs, reports about the reduced cross-neutralizing activity to the Beta variant [10–13] spurred the launch of a second-generation vaccine candidate. To improve coverage against the Beta variant, we utilized the same mutational design scheme on the Beta variant Spike sequence MRT5500β (monovalent 2P/GSAS Beta variant mRNA). Next, we evaluated whether two vaccinations at days 0 (D0) and 21 (D21), with different mRNA-LNP (lipid nanoparticle) combinations, would elicit similar binding and neutralizing antibody (NAb) responses to both D614 and Beta variants in naïve NHPs. Groups of four cynomolgus macaques received combinations of 5 μg each of either MRT5500 or MRT5500β at D0 and D21 (Groups 1, 2, 3), or 5 μg of MRT5500 at D0 and a bivalent formulation (10 μg total mRNA) of co-encapsulated MRT5500 and MRT5500β injected at D21 (Group 4) (Fig. 1**a**). In addition, we evaluated 10 μg (total mRNA) of bivalent formulations dosed as either a co-encapsulated mRNA-LNP (Group 5) or an admix of two separate mRNA-LNPs (Group 6) at D0 and D21. The neutralizing titers were measured using lentivirus particles pseudotyped with Spike proteins of the D614 or Beta SARS-CoV-2 variants [3,23].

While all groups developed Spike binding antibodies detectable by ELISA (**Supplemental** Fig. 1), NHPs did not develop robust NAb titers against either the D614 or Beta variants in PsV assays after one injection with either the monovalent MRT5500 or MRT5500β formulation (Fig. 1**b** and **Supplemental** Fig. 2). For example, Group 2 which received MRT5500 at D0 and MRT5500β at D21 developed only modest NAb titers to both viruses. The D35 geometric mean titer to D614 was 13.4-fold lower (p = 0.0324) than Group 1 which received MRT5500 at both D0 and D21 (GMT 771.8), while the GMT for Group 2 to the Beta variant (GMT 21) was comparable to Group 1 (GMT 15) with only two out of four or one out of four NHPs seroconverting, respectively. The NHPs that received two vaccinations of MRT5500β (Group 3) developed neutralizing titers to D614 (GMT 35) that were 4.7-fold lower than the titers against Beta (GMT 164) (p = 0.0572). The group immunized with MRT5500 at D0 followed by a bivalent formulation at D21 (Group 4) developed neutralizing titers to D614 (GMT 313) comparable to the Group 1 (p = 0.4248). However, the titers that developed to Beta (GMT 21) were 52.4-fold and 15.1-fold lower than the titers to D614 for Groups 1 and 4, respectively. These data indicated two immunizations with homologous mRNA vaccines at D0 and 21 were needed for NHPs to develop potent neutralizing titers against the homologous variant. Mixing heterologous vaccine candidates between D0 and D21 did not provide equivalent neutralizing titers against both the parental D614 and Beta variants, rather neutralizing potency was shifted towards the priming (D0) variant.

In addition to dosing with different combinations of separate monovalent formulations, we tested if bivalent vaccine formulations could generate a better balance in neutralizing titers in the same study. The bivalent formulations of MRT5500 + MRT5500β were dosed as either co-encapsulated mRNAs (2 mRNAs formulated in the same LNP, Group 5) or as an admixture (separate mRNA-LNP formulations admixed before injection, Group 6). On D35, NAb GMTs against D614 were 300 for the admixed Group 6 vs. 120 for the co-encapsulated Group 5 (p = 0.8366) (Fig. 1**b**), while their GMTs against Beta were 279 for the admixed Group 6 vs. 187 for the co-encapsulated Group 5 (p = 0.8996). Both bivalent formulations induced titers against D614 and Beta similar to the priming monovalent two-dose series of each respective sequence (Group 1 vs Group 5 p = 0.1116, Group 1 vs Group 6 p = 0.4045, Group 3 vs Group 5 p = 0.9914, Group 3 vs Group 6 p = 0.8631).

Taken together, the bivalent formulations and heterologous priming series on Days 0 and 21 did not appear to offer synergistic responses in the naïve animals. Although vaccination on day 21 with heterologous mRNA did not increase monovalent titers above the initial dose 1 response, the bivalent formulations given for both dose 1 and 2 generated titers on par with monovalent formulations for each homologous variant administered at the same dose level of each individual mRNA. In addition, these data demonstrate that admixing variant mRNA-LNPs is possible as no interference was detected in the bivalent compared to the monovalent formulations. Admixing could potentially offer the option to manufacture variant mRNA vaccines as needed to maximize viral variant coverage for later admixture.

3.2. A third vaccination as a booster is effective in expanding variant coverage in pre-immune NHPs

Due to the low cross-reactivity between the variant monovalent vaccines, a two-shot of either MRT5500 or MRT5500β vaccine administration was needed to reach substantial neutralizing anti-

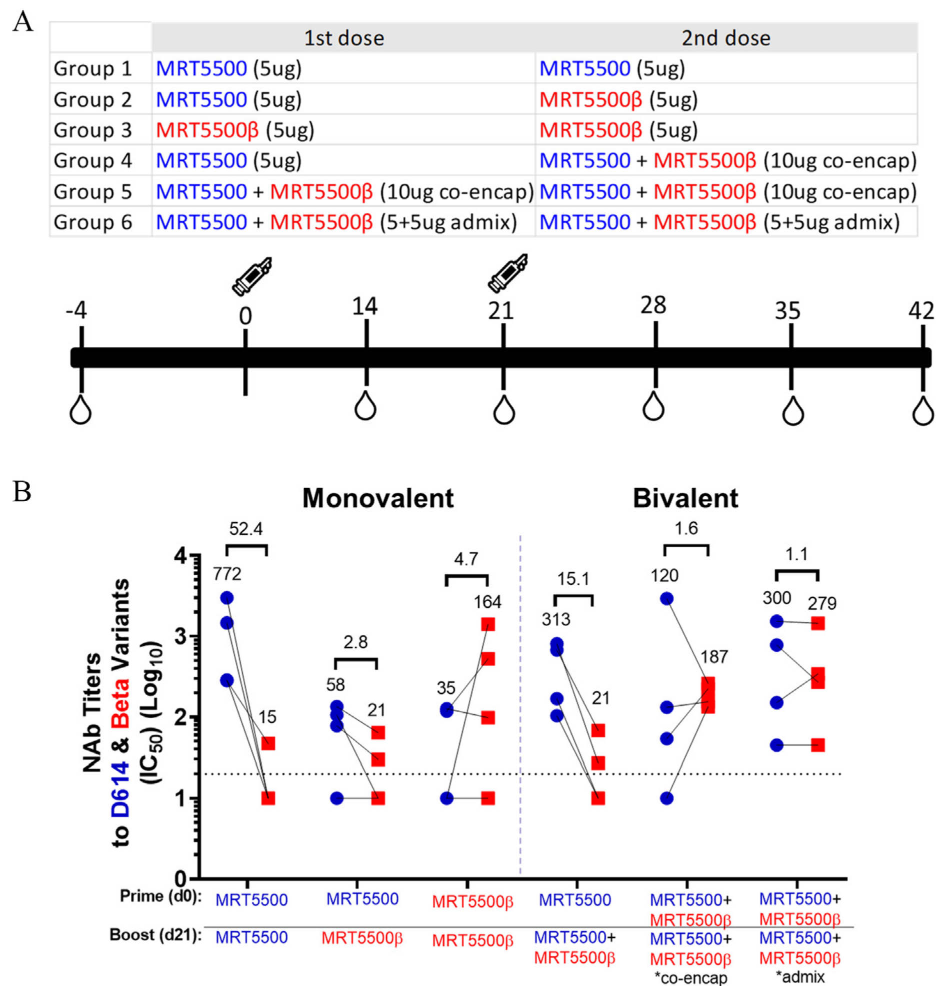

Fig. 1. A two-dose primary vaccination in naïve animals with different SARS-CoV-2 variants does not generate cross-neutralizing antibodies to D614 or Beta variant. A) Cynomolgus monkeys (n = 24) were immunized at D0 and D21 with 5 µg of the MRT5500 formulation, MRT5500β, or 10 µg (total mRNA) of bivalent formulations dosed as a co-encapsulated mRNA-LNP or an admix of separate mRNA-LNPs. B) Sera samples were tested in PsV neutralization assays against D614 (blue circles) and Beta variants (red squares). 50% inhibitory dilution titers (ID_{50}) from D35 sera with lines connecting each individual animal are shown. Each dot represents an individual serum sample, and the lines represent same animal across variant PsV assays. GMTs are above each group with fold-difference between GMTs of D614 and Beta shown above the bracket. The dotted line represents the lower limit of assay readout. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

body titers against D614 or Beta in naïve NHPs, and a bivalent formulation (MRT5500 + MRT5500β) was needed to generate titers against both. Next, we investigated if in the mRNA vaccine-primed animals an additional boost with a variant mRNA sequence could be used to not only boost pre-existing titers, but also to further expand the breadth of neutralizing activity. We sought to address whether a booster would need to be a bivalent formulation comprised of both parental D614 and Beta or simply a monovalent Beta variant. Thus, two mRNA-LNP booster formulations were prepared of either monovalent MRT5500β mRNA (10 µg) (Group A) or bivalent of MRT5500 + MRT5500β mRNAs (each at 5 µg, co-encapsulated) (Group B). Twelve NHPs that received the MRT5500 vaccine approximately ten months previously [23] were randomized to receive these booster vaccinations (Fig. 2**A**). NAb titers in these animals were measured using D614 PsV (Fig. 2**B**). The priming phase with the MRT5500 formulation elicited comparable NAb titers for both groups against the D614 virus on D35 ($p = 0.4432$); GMTs were 1683 (Group A) and GMT 1085 (Group B) that waned 27.1-fold ($p < 0.001$) to GMT 62 and 26.3-fold ($p < 0.001$) to GMT 41, respectively by D308. Booster administration on D315 resulted in an anamnestic response with the antibody titers measured 14 days post-boost on D329 rising 39.8 -fold above D308 ($p < 0.001$) for Group A and 62.4 -fold ($p < 0.001$) for Group B. The peak titers at D329 were similar with GMT 2471 and 2576 for the monovalent (Group A) and bivalent formulations Group B) ($p = 0.9419$), respectively. Binding antibody ELISA titers showed the same trends in significant waning over time and significant boosting response after the third dose with Group B GMT being 4.3-fold higher than that of Group A on D329 ($p = 0.0045$) (**Supplemental** Fig. 3). These results demonstrate that the monovalent MRT5500β formulation, despite lacking the MRT5500 mRNA sequence, was able to efficiently boost the memory neutralizing responses to the D614 Spike.

To evaluate the effect of the booster on the breadth of neutralization, we tested the sera against a panel of pseudoviruses consisting of Alpha (B.1.1.7) [31], Beta (B.1.351) [3], Gamma (P.1) [4], Delta (B.1.617.2) [5], and Epsilon (B.1.429) [6] variants, and SARS-CoV-1 [32] (Fig. 2**C** and Table 1). The pre-boost NAb GMTs at D35, against variants lacking the E484K/Q mutation (D614, Alpha, Delta, and Epsilon) were generally comparable to each other, with GMTs to D614, Alpha, and Epsilon on the order of 3 $\log_{10}$. GMTs to Delta were lower than those to D614 (1683 and 1085 respectively for Group A and B), with GMTs 382 (4.4-fold lower) for Group A and GMT 499 (2.2-fold lower) for Group B. At D329 (308 days post-2nd vaccination in the primary series), the GMTs against D614 increased by 1.5-fold and 2.4-fold, to Alpha

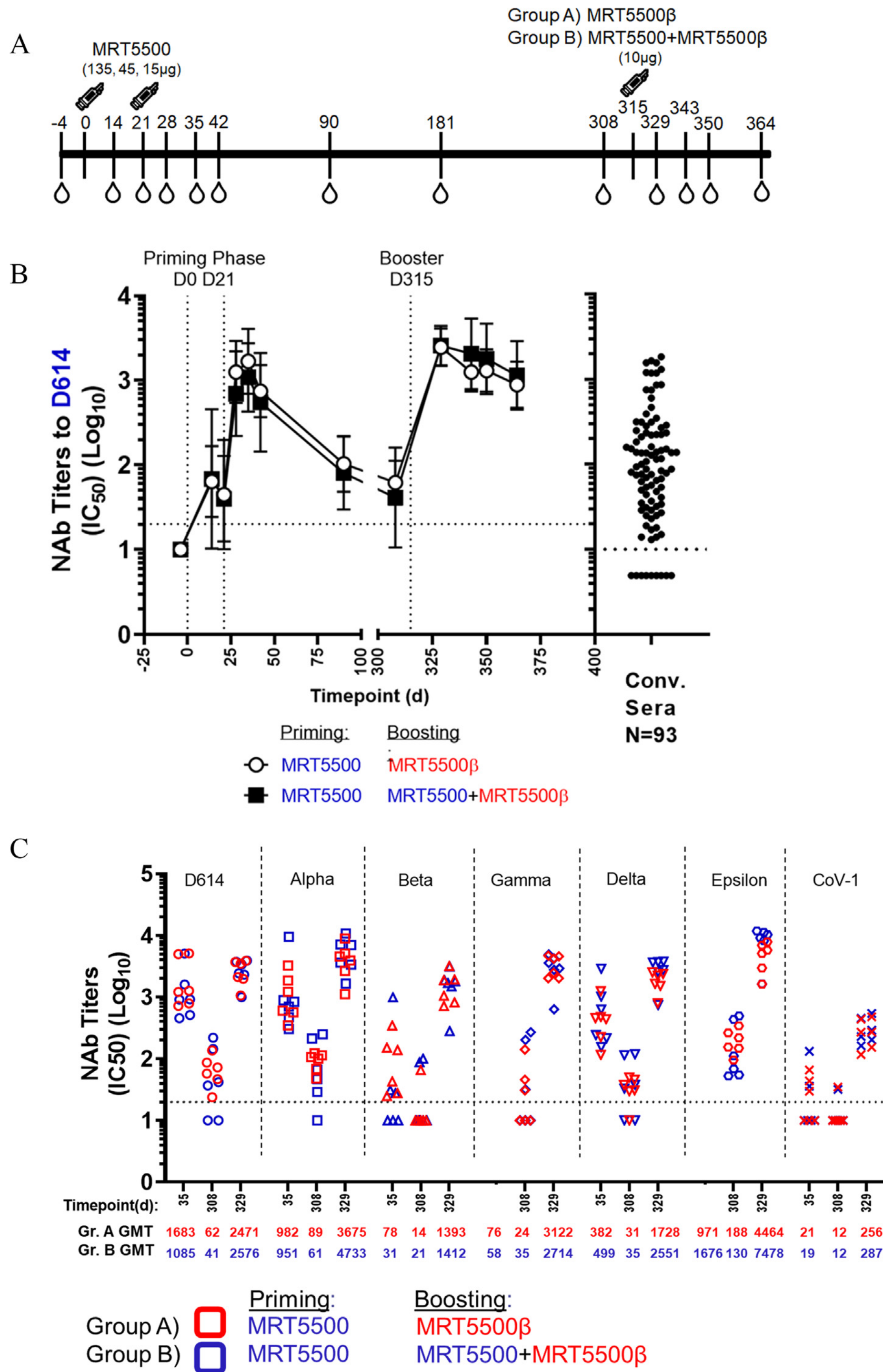

Fig. 2. Boosting with either MRT5500β or MRT5500 + MRT5500β formulations 10 months after MRT5500 prime immunizations induced high neutralizing antibody titers across variants. A) Cynomolgus monkeys (n = 12) were immunized at D0 and D21 with 15 μg, 45 μg or 135 μg of the MRT5500 formulation. Based on D35 D614 titers, the animals were distributed into 2 groups (n = 6 per group) and immunized on D315 with either MRT5500β (Group A) or MRT5500 + MRT5500β (Group B) formulations at 10 μg total mRNA per dose. B) Sera samples from specified timepoints were tested in a pseudovirus (PsV) D614 neutralization assay. Each dot represents a group, and error bars represent geometric means and geometric standard deviations. C) Sera samples from D35, D308, and D329 were collected and tested in a PsV assay against D614, Alpha, Beta, Gamma, Delta and Epsilon SARS-CoV-2 variants and against SARS-CoV-1. Each dot represents an individual serum sample with Group A titers in red and Group B titers in blue. GMTs for the group are written below each timepoint. The dotted line below for each panel represents the lower limit of assay readout. The convalescent human serum panel (n = 93) was included in PsV D614 neutralization, and the titers are shown separately in the same scale on Y-axis as other samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

by 3.7-fold and 5.0-fold, to Delta by 4.5-fold and 5.1-fold, and to Epsilon by 4.6-fold and 4.5-fold compared to D35 GMTs for Groups A and B, respectively. For the variants with the E484K/Q mutation (Beta and Gamma), lower GMTs compared to D614 were demonstrated at D35 with GMTs to Beta at 78 (21.7-fold) and 31 (35.2-fold) and to Gamma at 76 (22.1-fold) and 58 (18.7-fold) for Groups A and B, respectively. However, at D329, booster immunization had increased GMTs to Beta by 17.9-fold ($p < 0.001$) and 45.8-

Table 1
Fold difference of GMTs from Fig. 2C.

Boost	Comparison	D614	Alpha	Beta	Gamma	Delta	Epsilon	Cov-1
MRT5500β	D35:D308	/27.1	/11.0	/5.7	/3.1	/12.5	/5.2	/1.7
MRT5500β	D308:D329	x39.8	x41.2	x101.7	x128.8	x56.6	x23.8	x20.8
MRT5500β	D35:D329	x1.5	x3.7	x17.9	x41.0	x4.5	x4.6	x12.2
MRT5500+MRT5500β	D35:D308	/26.3	/15.7	/1.5	/1.7	/14.4	/12.9	/1.6
MRT5500+MRT5500β	D308:D329	x62.4	x78.2	X66.8	x78.1	x73.7	x57.4	x23.6
MRT5500+MRT5500β	D35:D329	x2.4	x5.0	x45.8	x46.7	x5.1	x4.5	x15.1

"/ Fold difference": Response for Day1 is 'x' folds lower than response for Day2
"x Fold difference": Response for Day1is 'x' folds higher than response for Day2

"/ Fold difference": Response for Day1 is 'x' folds lower than response for Day2
"x Fold difference": Response for Day1is 'x' folds higher than response for Day2

fold ($p < 0.001$) and to Gamma by 41-fold ($p < 0.001$) and 46.7-fold ($p < 0.001$) compared to D35 titers for Groups A and B, respectively. Interestingly, the booster vaccination also elicited considerable neutralizing titers against SARS-CoV-1 with D329 GMTs rising from near baseline levels at D35 by 12.2-fold from 21 (3/6 NHP responders) to 256 (6/6 NHP responders) and 15.1-fold from 19 (2/6 NHP responders) to 287 (6/6 NHP responders) for Groups A and B, respectively. These results collectively demonstrated the benefit of a third vaccine dose in expanding the coverage breadth against the SARS-CoV-2 variants, regardless of the key E484K/Q mutation, and to a lesser extent NAbs to SARS-CoV-1.

3.3. Both mRNA and subunit formulations are effective as boosters to enhance broadly neutralizing antibodies in pre-immune NHPs

Intrigued by the results of breadth development after the third vaccination, we evaluated whether such expansion of neutralization across diverse VoC could be accomplished with vaccine formulations comprising just the original D614 sequence. We selected twelve NHPs that had been primed with two administrations of the MRT5500 vaccine approximately 4 months prior and boosted them with either 15 μg of the MRT5500 (Group 1) or with 2.6 μg of the subunit D614 SARS-CoV-2 pre-fusion S protein trimers (SARS-CoV-2 preS-dTM) [33] formulated with the Sanofi Pasteur proprietary oil-in-water emulsion adjuvant AF03 [34] (Group 2) (Fig. 3**A**). The longitudinal titers against the D614 PsV showed both formulations were effective in increasing binding and NAb titers measured 14 days after the booster vaccination (D143) **(**Fig. 3**B)**. The D143 titers were comparable to those at D35 ($p = 0.9709$ and $p = 0.2401$ for Groups 1 and 2, respectively) after the first two vaccinations and increased 10.9-fold ($p < 0.001$) and 37.6-fold ($p < 0.001$) from the pre-boost D125 levels for Groups 1 and 2, respectively. These results demonstrated that either mRNA or subunit formulations were able to efficiently boost the homologous neutralizing responses to the D614 Spike. While the peak titers 2 weeks after the third dose (D143) for the subunit group were slightly higher than those of mRNA the difference was not statistically significant ($p = 0.2073$). The binding antibody ELISA titers showed similar trends in significant waning over time and significant boosting after a third dose with group 2 about 10.8-fold higher than group 1 ($p < 0.001$) by D143 (**Supplemental Fig. 4**).

To evaluate the effect of these boosters on the neutralization breadth, we tested the sera at selected timepoints against the same panel of pseudoviruses as described in Fig. 2 (Fig. 3**C** and Table 2). At D35, neutralizing titers against Alpha, Delta, and Epsilon variants, without the key E484K/Q mutation, were comparable to those against D614 with GMTs to D614, Alpha, and Epsilon all around the order of 3.3–3.5 $\log_{10}$. However, D35 GMTs to Delta were 2.2-fold and 2.7-fold lower than those to D614 for Group 1 and Group 2 respectively. Consistent with the results of the previous section, GMTs by D143 (122 days after the 2nd immunization of the primary vaccination series) were boosted by 1.0- and 2.0-fold to D614, 1.9- and 4.7-fold to Alpha, 1.6- and 3.9-fold to Delta, and 1.5- and 4.1-fold to Epsilon for Groups 1 and 2, respectively. Also consistent with Fig. 2**, D35** GMTs against variants with the E484K/Q mutation were lower than those against D614 by 13.9- and 34.8-fold to Beta and by 8.4- and 20.5-fold to Gamma for Groups 1 and 2, respectively. However, booster administration induced increased D143 GMTs by 5.6–($p = 0.0084$) and 22.7–fold ($p < 0.001$) to Beta and by 5.5–($p = 0.0034$) and 27.6–fold ($p < 0.001$) to Gamma compared to their D35 GMTs for Groups 1 and 2, respectively. Additionally, the booster vaccination induced a 3.0-fold ($p = 0.055$) and 11.3-fold ($p < 0.001$) rise in neutralizing titers against SARS-CoV-1 virus by D143 from those observed at D35 for Groups 1 and 2, respectively. These results with those shown in Fig. 2, collectively demonstrated that the third booster vaccination, even with the vaccines containing the original D614 Spike protein sequence, can effectively improve the quality of vaccine-induced neutralizing antibody responses by expanding the breadth of coverage against VoC, especially those harboring the key E484K/Q mutation. More encouragingly, cross-neutralizing activities against SARS-CoV-1 were observed, which signifies that the antibodies being generated have broad neutralizing activity even to a more distantly related coronavirus. Lastly, the effect of such booster vaccination appeared to be dependent neither on the mRNA nor on subunit formulation modality, nor on the vaccine sequence (D614 vs Beta variant).

4. Discussion

SARS-CoV-2 variants represent serious challenges to the immune protection provided by the vaccines and previous infection. The Beta and Gamma variants are the most resistant to monoclonal antibodies (mAbs) and convalescent plasma from SARS CoV-2-infected individuals, and the resistance profiles correspond to several deletions in the N-terminal domain and the K417N/T, E484K/Q, and N501Y mutations in the RBD of SARS-CoV-2 Spike protein [35]. The E484 position was identified as a dominant residue for escape from neutralizing activity by sera from COVID-19 convalescent patients [36] with the E484K mutation in the SARS-CoV-2 Beta variant as the key factor responsible for the reduced neutralization potency of convalescent immune sera and human

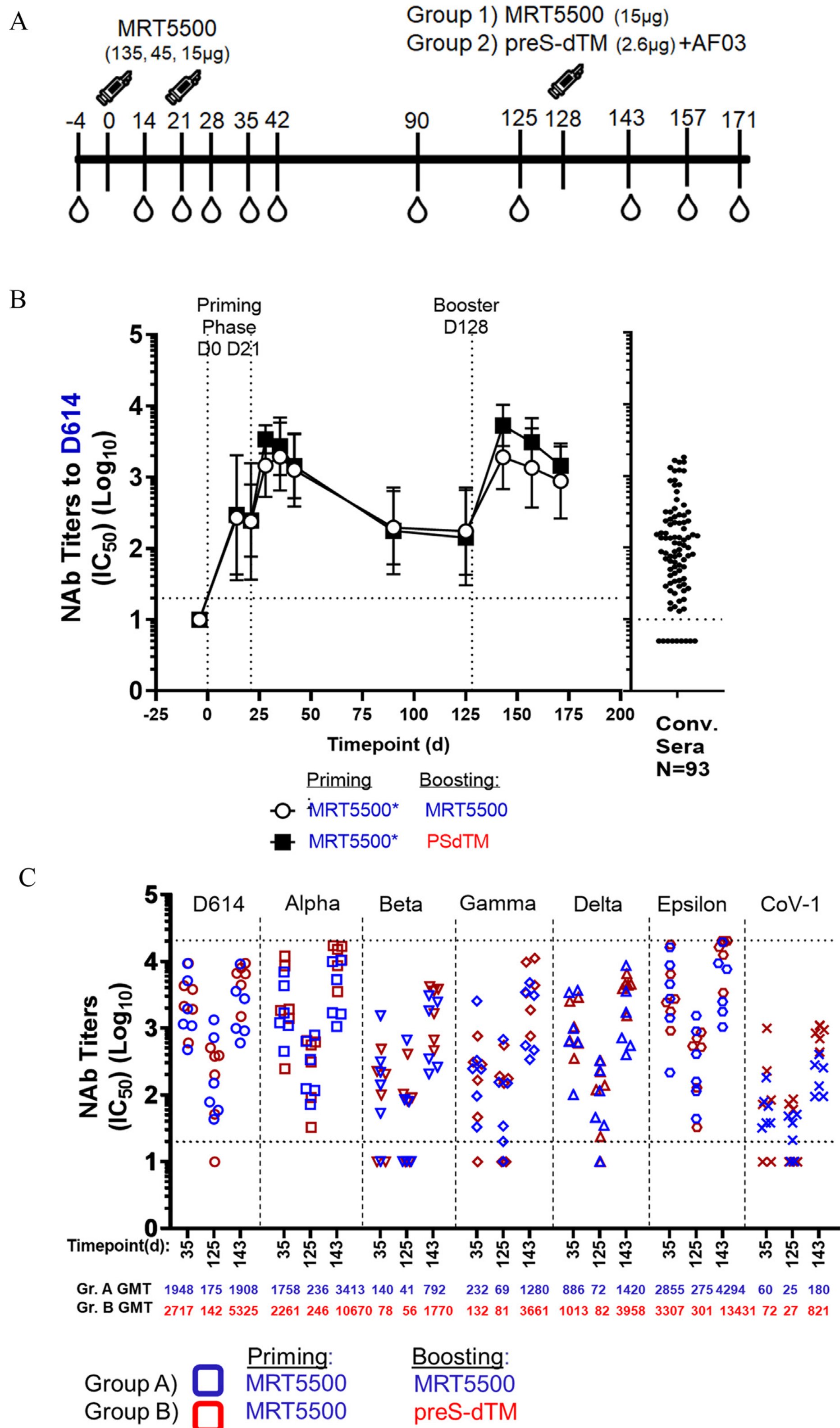

Fig. 3. Boosting with either MRT5500 or preS-dTM formulations induced high neutralization potencies and breadth 4 months after MRT5500 prime. A) Cynomolgus monkeys (n = 12) were immunized at D0 and D21 with 15 μg, 45 μg or 135 μg of MRT5500. On D128 animals were randomized to 2 groups (n = 6 per group) and immunized with either 15 μg of mRNA MRT5500 (Group 1) or 2.6 μg of protein subunit SARS CoV-2 preS-dTM with adjuvant AF03 (Group 2) per dose. B) Sera collected on the indicated timepoints were tested in a PsV D614 neutralization assay. Each dot represents a group, and error bars represent the geometric means and geometric standard deviations. C) Sera samples collected on D35, D125, and D143 were further tested in a PsV assay against Alpha, Beta, Gamma, Delta and Epsilon SARS-CoV-2 variants and against SARS-CoV-1. Each dot represents an individual serum sample, with the Group 1 titers in blue and Group 2 titers in red. GMTs for the group are written below each timepoint. The dotted line below for each panel represents the limit of assay readout. The convalescent human serum panel (n = 93) was included in PsV D614 neutralization, and the titers are shown separately in the same scale on Y-axis as other samples. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2
Fold difference of GMTs from Fig. 3C.

Boost	Comparison	D614	Alpha	Beta	Gamma	Delta	Epsilon	Cov-1
MRT5500	D35:D125	/11.2	/7.5	/3.5	/3.4	/12.3	/10.4	/2.3
MRT5500	D125:D143	x10.9	x14.5	x19.6	x18.6	x19.7	x15.6	x7.1
MRT5500	D35:D143	/1.0	x1.9	x5.6	x5.5	x1.6	x1.5	x3.0
preS-dTM	D35:D125	/19.2	/9.2	/1.4	/1.6	/12.4	/11.0	/2.7
preS-dTM	D125:D143	x37.6	x43.4	x31.5	x45.1	x48.4	x44.6	x30.3
preS-dTM	D35:D143	x2.0	x4.7	x22.7	x27.6	x3.9	x4.1	x11.3

"/ Fold difference": Response for Day1 is 'x' folds lower than response for Day2
"x Fold difference": Response for Day1is 'x' folds higher than response for Day2

"/ Fold difference": Response for Day1 is 'x' folds lower than response for Day2
"x Fold difference": Response for Day1is 'x' folds higher than response for Day2

Table 3
Pseudovirus Neutralization Variant Strains.

Strain	Pango Lineage	WHO Name	Sequence source	Catalog number	Lot	Mutations relative to D614
Wuhan-D614 (WIV04)	B.1	NA	QHD43416.1	RVP-701G	113A	N/A
UK (VUI202012/01)	B.1.1.7	alpha	QQH18545.1	RVP-706G	CG-135A	ΔH69/V70, ΔY144, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H
South African (20H/501Y.V2)	B.1.351	beta	Tegally et al. 2020	RVP-724G	CG-180A	L18F, D80A, D215G, ΔL242/A243/L244, R246I, K417N, E484K, N501Y, D614G, A701V
Brazilian (P.1)	B.1.128	gamma	QQX12069.1	RVP-708G	CG-160A	L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G H655Y, T1027I, V1176
Indian (double variant)	B.1.617.2	delta	cov-lineages.org	Custom	CG-233A	T19R, G142D, E156G, ΔF157/R158, L452R, T478K, D614G, P681R, D950N
Californian (452R)	B.1.429	epsilon	QPJ72086.1	RVP-706G	CG-135A	S13I, W152C, L452R
SARS-CoV-1 (Urbani)	NA	NA	P59594.1	RVP-801G	SG-115B	28%

mAbs [10,11]. These data prompted the evaluations of monovalent Beta or bivalent D614/Beta mRNA formulation in NHPs to define the vaccination regimens with optimal breadth of neutralizing activities against VoC.

Our data showed that NHPs can develop neutralizing titers to either D614 or Beta vaccine strain if they were immunized twice with the same mRNA in the formulations either as monovalent or as bivalent. Similar to recent data [37], immunizing with heterologous mRNA sequences for the first and second dose on D0 and D21 did not provide improvement in cross-neutralizing activity against D614 and Beta variant, suggesting that bivalent mRNA formulations would be needed to elicit sufficient neutralizing titers in naïve population using the current two dose pandemic regimens. These data further suggest the need for updated vaccine sequences or multivalent formulations for naïve individuals, at least to cover those strains with key mutations, such as E484K/Q, which can escape the vaccine-induced antiviral responses.

One solution to address potential waning efficacy of vaccine titers over time and low cross-protection to variants is administration of a booster vaccination in the immunized population [38,39]. To evaluate a booster strategy, particularly regarding the question of vaccine valency, we compared monovalent MRT5500β (Beta) or bivalent MRT5500 + MRT5500β (D614 + Beta) mRNA vaccine candidates in NHPs previously inoculated with monovalent D614 mRNA vaccine nearly ten months prior [23]. Remarkably, both booster formulations were able to restore neutralizing titers against D614 to the levels seen on D35 after the primary vaccination series and expanded breadth to other variants where previously there had not been cross-neutralizing antibodies. Furthermore, the improved breadth by the booster vaccination was produced with either an mRNA vaccine or a recombinant subunit vaccine, both based on D614 sequence in pre-immune NHPs. The data from both studies demonstrate that a third dose booster not only restores neutralizing titers to D614 to the levels post-first two vaccinations, but also improves the quality of immune responses for the cross-neutralization of the key variants, including the Beta and Delta variants of concern which is in agreement with recent publications [37,38]. Importantly, the expanded breadth on VoC appeared to not depend on vaccine modality (mRNA or subunit) or vaccine antigen sequence (D614 or Beta, or both). This finding, which is beginning to be confirmed clinically in humans [40,41], can dramatically reduce the burden on vaccine development and implementation as the vaccine composition of an annual booster, if needed, may not need to be updated based on viral evolution.

Notably, we detected the emergence of neutralizing titers to SARS-CoV-1, which is analogous to the results reported by Stamatatos *et al.* that human subjects with histories of natural SARS-CoV-2 infection could develop broadly neutralizing antibodies to the Beta variant and even SARS-CoV-1 after mRNA vaccination [40]. Although previous work demonstrated that neutralizing mAbs to the SARS-CoV-1 RBD could not neutralize SARS-CoV-2 [25], the expanded breadth we observed may be explained by antibodies targeting regions outside the RBD, analo-

gous to selected human mAbs previously reported [42,43]. Such antibody species are rare since the majority of neutralizing mAbs isolated from human subjects post-infection or post-vaccination target the RBD region [16,44]. Thus, antibodies to neutralizing epitopes outside the RBD may be critical for broad neutralization. Further exploration is needed to understand the immunological and biochemical basis for the broad neutralizing antibodies to coronaviruses. In fact, studies have already begun to identify cross-neutralizing antibodies that arise after infection or vaccination in order to determine conserved neutralizing epitopes which may be used to generate a pan-coronavirus vaccine (or therapeutic mAb) [45–47]. However, our study and others suggest that vaccine redesign may not be necessary as a 3rd dose with a homologous vaccine is sufficient to generate the breadth needed to cross-neutralize current VoC [44,48], which would greatly simplify the vaccine supply chain as the formulations would not need to be constantly updated to cover new variants.

In summary, our studies showed that a third vaccination administered as a booster, regardless of composition, to the currently approved two dose regimens [49,50] could effectively promote broadly neutralizing antibodies against all variants of concern. Understanding the immune mechanism underlying this breadth expansion of neutralizing responses may form the foundation for the development of a pan-SARS-CoV vaccine.

CRediT authorship contribution statement

Kirill V. Kalnin: Conceptualization, Writing – original draft, Supervision, Project administration. **Timothy Plitnik:** Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Michael Kishko:** Methodology, Formal analysis, Investigation, Data curation, Writing – review & editing. **Dean Huang:** Methodology, Formal analysis, Investigation, Data curation. **Alice Raillard:** Software, Formal analysis, Data curation. **Julie Piolat:** Software, Formal analysis, Data curation. **Natalie G. Anosova:** Methodology, Formal analysis, Writing – review & editing, Supervision. **Timothy Tibbitts:** Writing – review & editing. **Joshua DiNapoli:** Methodology, Formal analysis. **Shrirang Karve:** Resources. **Rebecca Goldman:** Resources. **Hardip Gopani:** Resources. **Anusha Dias:** Resources. **Khang Tran:** Resources. **Minnie Zacharia:** Resources. **Xiaobo Gu:** Resources. **Lianne Boeglin:** Resources. **Jonathan Abysalh:** Resources. **Jorel Vargas:** Resources. **Angela Beaulieu:** Resources. **Monic Shah:** Resources. **Travis Jeannotte:** Resources. **Kimberly Gillis:** Resources. **Sudha Chivukula:** Writing – review & editing, Supervision. **Ron Swearingen:** Supervision. **Victoria Landolfi:** Supervision. **Tong-Ming Fu:** Resources, Supervision. **Frank DeRosa:** Conceptualization, Writing – review & editing. **Danilo Casimiro:** Conceptualization, Resources, Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kirill V. Kalnin, Michael Kishko, Dean Huang, Alice Raillard, Julie Piolat, Natalie G. Anosova, Tim Tibbitts, Joshua DiNapoli, Sudha Chivukula, Victoria Landolfi, Tong-Ming Fu, and Danilo Casimiro are current or former employees of Sanofi Pasteur and may hold stock in Sanofi company. Frank DeRosa, Shrirang Karve, Rebecca Goldman, Hardip Gopani, Anusha Dias, Khang Tran, Minnie Zacharia, Xiaobo Gu, Lianne Boeglin, Jonathan Abysalh, Jorel Vargas, Angela Beaulieu, Monic Shah, Travis Jeannotte, Kimberly Gillis, and Ron Swearingen are employees of Translate Bio and may hold stock in the company (TBio). Timothy Plitnik declares no competing interests. The research is funded by Translate Bio and Sanofi Pasteur.

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Appendix A. Supplementary material

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