Driving potent neutralization of a SARS-CoV-2 Variant of Concern with a heterotypic boost.

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The emergence of SARS-CoV-2 Variants of Concern (VOCs) with mutations in key neutralizing antibody epitopes threatens to undermine vaccines developed against the pandemic founder variant (Wu-Hu-1). Widespread vaccine rollout and continued transmission are creating a population that has antibody responses of varying potency to Wu-Hu-1. Against this background, it is critical to assess the outcomes of subsequent immunization with variant antigens. It is not yet known whether heterotypic vaccine boosts would be compromised by original antigenic sin, where pre-existing responses to a prior variant dampen responses to a new one, or whether the primed memory B cell repertoire would bridge the gap between Wu-Hu-1 and VOCs. Here, we show that a single adjuvanted dose of receptor binding domain (RBD) protein from VOC 501Y.V2 (B.1.351) drives an extremely potent neutralizing antibody response capable of cross-neutralizing both Wu-Hu-1 and 501Y.V2 in rhesus macaques previously immunized with Wu-Hu-1 spike protein. Passive immunization with plasma sampled following this boost protected K18-hACE2 mice from lethal challenge with a 501Y.V2 clinical isolate, whereas only partial protection was afforded by plasma sampled after two Wu-Hu-1 spike immunizations.

Introduction

At least 20 candidate SARS-CoV-2 vaccines have already entered phase 3 clinical trials. A number of these demonstrated high efficacy1–5, significantly reducing morbidity and mortality, and are being rolled-out globally. This first generation of vaccines all encode or deliver a spike glycoprotein derived from the pandemic founder strain, Wu-Hu-1.6

Driven by multiple evolutionary forces8, SARS-CoV-2 is evading immune responses and undermining our prevention and mitigation strategies. Globally, a number of VOCs are rising in frequency (see Fig 1), each harbouring spike mutations that confer resistance to prior immunity. Of particular concern is the surge of variant 501Y.V29, with multiple mutations in dominant neutralizing antibody epitopes making it several fold more resistant to antibodies elicited by current vaccines10–12. This underpins the substantially reduced vaccine efficacies in South Africa, where this variant is circulating at high frequency13,14. Updated vaccines are likely required to protect against current and future mutated variants. Importantly, by the time these are rolled out, a significant proportion of the global population are likely to be seropositive as a result of either infection, or immunization with Wu-Hu-1 based vaccines. A relevant question now is whether a single dose will be sufficient to induce robust neutralising antibody responses to VOCs in seropositive individuals, and whether these boosts are sufficient to confer protection. Importantly, the first exposure to a pathogen can shape future responses to mutated variants. This immunological imprinting or original antigenic sin15 is well-described for influenza A virus, where protection is highest against the first strain encountered and diminished against those encountered later in life16,17. It is crucial for the design of updated vaccines and regimens to determine if existing immunity dampens antibody responses to new VOCs, or if a heterotypic boost can efficiently recruit cross-protective memory responses.

Results

To address this, we immunized three rhesus macaques with two doses of soluble prefusion-stabilized Wu-Hu-1 spike protein (2 µg), adjuvanted with saponin-based Matrix-M™ (Novavax AB, Uppsala, Sweden), with a one-month interval be-
between doses, mimicking an immunization schedule for approved SARS-CoV-2 vaccines. After a single dose, neutralizing antibodies were detectable against Wu-Hu-1 but not against 501Y.V2 (Fig. 2). Neutralizing antibody responses against Wu-Hu-1 were substantially boosted by the second immunization (“post S”, GMT = 3980 at peak), and then waned over the following months (Fig. 2), as also reported in immunized humans. Notably, VOC 501Y.V2 was on average 9-fold (range: 5.6 - 12.2 fold) less potently neutralized (GMT = 451 at peak), consistent with the responses observed in humans following vaccination.

Six months after their first immunization, macaques were boosted with either 2 µg (H05), 10 µg (H06), or 50 µg (H07) of soluble 501Y.V2 RBD in 50 µg Matrix-M™ adjuvant. One macaque (H05) was terminated 5 days after immunization, due to an unrelated illness that had begun prior to the third immunization, and was sampled for detailed follow-up studies of antibody specificities. The two other macaques (H06 and H07) were followed for 4 weeks. In all three animals, 501Y.V2 RBD efficiently boosted responses that potently cross-neutralized both Wu-Hu-1 and 501Y.V2, with similar titers (Fig. 2a-c; Wu-Hu-1 GMT = 11795, 501Y.V2 GMT = 12595). In contrast, for macaques previously immunized with three doses of Wu-Hu-1 spike, the reduced neutralization of 501Y.V2 compared to Wu-Hu-1 remained after the third homotypic spike immunization (Supp. Fig. 1).

To determine whether restoration of neutralizing antibody titers to 501Y.V2 afforded a biologically relevant improvement in protective immunity, mice transgenic for human ACE2 (K18-hACE2) were passively immunized intraperitoneally (i.p.) with plasma samples taken either 2 weeks following the second spike immunization (N=8) (“post S”), or 1-2 weeks following the RBD booster immunization (N=8) (“post vRBD”). Passive immunization conferred titers approximately 10-fold lower than donor plasma (Supp. Fig 2), and macaque polyclonal antibodies were not rapidly cleared following xenotransfusion with an unchallenged mouse still maintaining titers >1400 after 5 days (data not shown). Mice were then challenged intranasally with 2.4x10⁶ RNA copies of either 501Y.V2 or ‘wild-type’ (encoding a spike matching Wu-Hu-1) virus (corresponding to 100 PFU of 501Y.V2 or 86 PFU of wild-type), and weight — a reliable proxy for disease severity — was monitored daily.

Across all groups, protection was strongly correlated with the neutralizing antibody titers to the challenge virus on the day of challenge (Spearman’s ρ = 0.822, p<1x10⁻⁸, Fig. 3a). All control mice that did not receive plasma (PBS only) succumbed to disease when challenged with either variant, showing precipitous weight loss starting around three days post-challenge (Fig. 3b-d). Passive transfer of post S serum conferred protection from WT virus (Fig. 3c) but not from 501Y.V2 (Fig. 3d), clearly demonstrating that evasion of the antibody response by this VOC was sufficient to cause disease. Notably, passive transfer of post vRBD plasma protected against both WT and 501Y.V2 (Fig. 3c,d).

**Discussion**

For many licensed vaccines, reduced efficacy has been observed against the 501Y.V2 variant. Moreover, the decay of vaccine-elicited antibody titers suggests that over
Fig. 3. Heterotypic RBD boost restores protection against 501Y.V2 in passively immunized k18-hACE2 mice. (a) Pseudovirus neutralizing antibody titers against the challenge spike (ID$_{50}$) in passively immunized mice on the day of challenge are associated with infection, and disease severity summarized as weight loss 6 days following challenge. Titers below the limit of detection of the assay (20) are plotted as 10. (b) Weight loss at day 6 for each group. Unchallenged littermates housed in the same cages (grey); PBS, mock immunized mice (black). Post S, passive immunization with plasma following the second spike immunization (6 week plasma); post vRBD, passive immunization with plasma from macaques boosted with variant (501Y.V2) RBD (31 or 32 week plasma). Statistical comparisons are summarized as: ∗∗, p ≤ 0.01; ∗∗∗, p ≤ 0.001; ns, not significant. Groups displaying significant weight loss compared to uninfected mice are annotated above the points for that group. (c-d) Weight loss following challenge with either (c) ‘wild-type’ (‘WT’) or (d) 501Y.V2 virus for K18-hACE2 mice passively immunized with NHP plasma sampled post S or post vRBD. Control mice mock immunized with PBS and subsequently challenged (‘PBS’) are shown in black, while uninfected littermates housed in the same cages (‘uninfected’) are shown in grey.

The ability of vaccines to broaden existing responses to new variants is still largely unclear. Despite weak immunogenicity of soluble, monomeric RBD as a priming antigen, heterotypic RBD administered as a boost elicited a potent recall response in non-human primates. This was robust to the boosting dose, and effective as low as 2 µg, possibly aided by a dose-sparing effect of Matrix-M. While reduced neutralization of 501Y.V2 was evident following 2 doses of Wu-Hu-1 spike, both Wu-Hu-1 and 501Y.V2 were potently neutralized following heterotypic (501Y.V2) RBD boost. In animal challenge models, neutralizing antibodies following passive immunization represented a robust correlate of protection such that the restoration of neutralizing antibody titers to 501Y.V2 also translated into protective immunity.

The potent, cross-neutralizing antibody response that arises following a heterotypic boost indicates that original antigenic sin does not represent a significant barrier to the acquisition of protective immunity against current SARS-CoV-2 VOCs. In the immunized animal sampled only 5 days post vRBD boost, neutralizing titers (against both Wu-Hu-1 and 501Y.V2) were already elevated suggesting these titers are the product of a rapidly activated population of antibody secreting cells. Further, this time course indicates that successive rounds of affinity maturation likely were not required...
for neutralization of 501Y.V2, but rather that vRBD-specific antibody responses could be boosted from the pool of existing memory B cells primed by Wu-Hu-1. These responses are largely consistent with recently reported results from 501Y.V2 spike mRNA (mRNA1273.351, Moderna) booster vaccinations.28,29 The observation that immunization with RBD (and not whole spike) was capable of inducing robust neutralizing antibody responses is particularly promising as RBD is a small, stable protein that can be easily synthesized and efficiently expressed. Taken together, these data indicate that potent, cross-neutralizing and cross-protective antibody responses can be recruited with heterotypic SARS-CoV-2 immunogens following a primary exposure, and identify soluble RBD booster immunizations as an attractive strategy to broaden vaccine protection from new SARS-CoV-2 variants.

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METHODS
Ethics statement. The animal work was conducted with the approval of Stockholms Länsstyrelsen’s ethical committee (10513-2020, 1842-2019 and 10895-2020). All animal procedures were performed according to approved guidelines.

Animal models. Rhesus macaques (Macaca mulatta) of Chinese origin, 5-6 years old, were housed at the Astrid Fagraeus Laboratory at Karolinska Institutet. Housing and care procedures complied with the provisions and general guidelines of the Swedish Board of Agriculture. The facility has been assigned an Animal Welfare Assurance number by the Office of Laboratory Animal Welfare (OLAW) at the National Institutes of Health (NIH). The macaques were housed in groups in enriched cages with a daily 14 m3 cages. They were habituated to the housing conditions for more than six weeks before the start of the experiment and subjected to positive reinforcement training in order to reduce the stress associated with experimental procedures. The macaques were weighed at each sampling. All animals were confirmed negative for simian immunodeficiency virus, simian T cell lymphotropic virus, simian retrovirus type D and simian herpes B virus. Mice transgenic for human ACE2 under control of the cytokeratin 18 (K18) promoter30 were obtained from the Jackson Laboratory. Mice were maintained as a hemizygous line, with hACE2 transgene presence confirmed in each quadricep. All immunizations and blood samplings were performed under sedation with 10-15 mg/kg ketamine (Ketamin, Intervet, Sweden) administered i.m. Blood plasma was isolated by centrifugation, and heat inactivated at 56°C for 60 minutes.
Pseudotyped virus neutralization assays. All plasma and serum samples were heat-inactivated at 56°C for 60 minutes. Pseudotyped lentiviruses displaying either the SARS-CoV-2 pandemic-related vRBD variant spike31 and packaging a firefly luciferase reporter gene were generated by the co-transfection of HEK293T cells using Lipofectamine 3000 (Invitrogen) per the manufacturer’s protocols. Media was changed 12-16 hours after transfection, and pseudotyped viruses were harvested at 48- and 72-hours post-transfection, clarified by centrifugation, and stored at -80°C until use. Pseudotyped viruses sufficient to generate 50,000 relative light units (RLUs) were incubated with serial dilutions of plasma for 60 min at 37°C in a 96-well plate, and then 15,000 HEK293F/AACE2 cells were added to each well. Pseudotyped virus was determined by luciferase expression. For 501Y.V2 neutralization assays, we gratefully acknowledge all data contributors i.e. the Authors and their Originating Laboratories responsible for obtaining the specimens, and their Submitting Laboratories that generated the genetic sequence and metadata and shared via the GISAID initiative the data on which the variant frequency estimates in this research are based. This project has received funding from the European Union’s Horizon 2020 research and innovation programme under grant agreement No. 101003653 (CoroNAb), to GM, GKH, and BM, from the Swedish Research Council to GM, JMC, GKH, GM, and from Karolinska Institutet Development Office and Karolinska Institutet’s President’s Fund to GM, GKH, and BM. EU is supported by a Wenner Gren Fellowship.

AUTHOR CONTRIBUTIONS
Conceptualization: DJS, MM, EU, JMC, GKH, BM; Formal Analysis: DJS, BM; Funding acquisition: GMM, JMC, GKH, BM; Investigation: DJS, MM, EU, CK, AP, NLS, XDC; Methodology: DJS, MM, EU, AP, NLS, JMC, GKH, BM; Resources: CK, LH, LPV, Software: BM; Supervision: LH, GMM, JMC, GKH, BM; Visualization: DJS, AP, BM; Writing – original draft: DJS, BM; Writing – review editing: all authors; Funding acquisition: GMM, JMC, GKH, BM; Investigation: DJS, MM, EU, AP, NLS, JMC, GKH, BM; Resources: CK, LH, LPV, Software: BM; Supervision: LH, GMM, JMC, GKH, BM; Visualization: DJS, AP, BM; Writing – original draft: DJS, BM; Writing – review editing: all authors; Funding acquisition: GMM, JMC, GKH, BM; Investigation: DJS, MM, EU, AP, NLS, JMC, GKH, BM; Resources: CK, LH, LPV, Software: BM; Supervision: LH, GMM, JMC, GKH, BM; Visualization: DJS, AP, BM; Writing – original draft: DJS, BM; Writing – review editing: all authors; Funding acquisition: GMM, JMC, GKH, BM; Investigation: DJS, MM, EU, AP, NLS, JMC, GKH, BM; Resources: CK, LH, LPV, Software: BM; Supervision: LH, GMM, JMC, GKH, BM; Visualization: DJS, AP, BM; Writing – original draft: DJS, BM; Writing – review editing: all authors; Funding acquisition: GMM, JMC, GKH, BM; Investigation: DJS, MM, EU, AP, NLS, JMC, GKH, BM; Resources: CK, LH, LPV, Software: BM; Supervision: LH, GMM, JMC, GKH, BM; Visualization: DJS, AP, BM; Writing – original draft: DJS, BM; Writing – review editing: all authors; Funding acquisition: GMM, JMC, GKH, BM; Investigation: DJS, MM, EU, AP, NLS, JMC, GKH, BM; Resources: CK, LH, LPV, Software: BM; Supervision: LH, GMM, JMC, GKH, BM; Visualization: DJS, AP, BM; Writing – original draft: DJS, BM; Writing – review editing: all authors;
linear combination of 400 randomly drawn Fourier basis features (aka. a “Randon Kitchen Sink” [34]) to allow frequencies to vary non-linearly as a function of time. We estimate the model parameters with an L2 norm on the random feature coefficients, using the GLMnet.jl package, plotting the map with Cartopy (https://github.com/MurrellGroup/VOCfreq). Code available at https://github.com/MurrellGroup/VOCfreq.

ID50 titers. Neutralizing antibody ID50 titers were calculated in Prism 9 (GraphPad Software) by fitting a four-parameter logistic curve bounded between 0 and 100, allowing the fit to extrapolate beyond the plasma/serum dilution where RLUs were reduced by 0.5% to 99.5%. The association between neutralizing antibody titers and weight loss was assessed with a Spearman’s rank correlation in Prism 9 (GraphPad Software).

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Fig. SI1. (left) Longitudinal neutralizing antibody responses against Wu-Hu-1 (blue) and 501Y.V2 (red) for plasma samples from Mandolesi et al.\textsuperscript{20}, where three rhesus macaques (NHP1-NHP3) were immunized with three doses of Wu-Hu-1 spike (100 µg) in Matrix-M\textsuperscript{™} adjuvant. Vertical blue lines indicate the timing of immunizations (at 0, 4, and 9 weeks). (right) Comparison of the titers at 6 weeks (post 2) and 11 weeks (post 3) illustrating that reduced titers to 501Y.V2 (red) compared to Wu-Hu-1 (blue) were maintained after a third homotypic spike boost.

Fig. SI2. Relationship between donor and recipient titers following passive immunization. Mice were passively immunized with 200 µl of plasma, resulting in titers approximately 10-fold lower than in the donor. Correcting for weight, Spearman’s $r = 0.98$ (after a variance-stabilizing square-root transform).
20A Calu-3 72 hpi

20A Vero E6

20H/501Y.V2 isolate

20H/501Y.V2 Calu-3 72 hpi

20H/501Y.V2 Vero E6

Fig. S13. Expansion of SARS-CoV-2 isolates in Vero E6 but not Calu-3 cells rapidly selected for mutations and deletions proximal to the furin cleavage site. Challenge stocks used in this study were produced in Calu-3 cells, and confirmed by Sanger sequencing to harbour no high frequency cell culture adaptation mutations in spike. Electropherograms spanning the furin cleavage site from sanger sequencing of amplified viral RNA are shown for virus cultured in Vero E6 or Calu-3 cells demonstrating the rapid loss of the furin recognition sequence upon culture in Vero E6 cells but not Calu3 cells. Received stock of 501Y.V2 (“20H/501Y.V2 isolate”) had a mixture of intact/knocked-out furin site.