Immunization with V987H-stabilized Spike glycoprotein protects K18-hACE2 and golden Syrian hamster upon SARS-CoV-2 infection.

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Article
Abstract

Safe and effective severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccines have been crucial to fight against the coronavirus disease 2019 pandemic. Most vaccines are based on a mutated version of the Spike glycoprotein [K986P/V987P (S-2P)] with improved stability, yield and immunogenicity. However, S-2P is still produced at low levels. Here, we described a novel V987H mutation that increases by two-fold the production of the recombinant Spike and the exposure of the receptor binding domain (RBD). S-V987H immunogenicity was similar to S-2P in K18-hACE2 mice and golden Syrian hamsters, and superior to a monomeric RBD. Immunization with S-V987H, but not with S-2P or RBD, conferred full protection against severe disease in both animal models after SARS-CoV-2 challenge (D614G and B.1.351 variants). Furthermore, S-V987H immunized K18-hACE2 mice showed a faster tissue viral clearance than RBD- or S-2P-vaccinated animals. Thus, S-V987H protein provides an alternative to S-2P for future SARS-CoV-2 vaccines development.

Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the etiological agent of coronavirus disease 2019 (COVID-19), an infectious disease that emerged at the beginning of December 2019 in Wuhan, China. COVID-19 was declared a pandemic by the World Health Organization (WHO) on March 11, 2020. As of April 17th 2023, over 760 million COVID-19-confirmed cases and 6.9 million deaths have been registered (WHO Coronavirus (COVID-19) Dashboard). Since the beginning of the pandemic, there has been a global effort to expedite the development of vaccines that could help control the COVID-19 pandemic. While more than 180 vaccine projects started being developed in 2020, four vaccines (Pfizer/BioNTech, Moderna, AstraZeneca and Janssen) received emergency use authorization by the Food & Drug Administration (FDA) and/or the European Medicines Agency (EMA) in record time. These four vaccines are based on the SARS-CoV-2 Spike (S) glycoprotein, since it is the main target of neutralizing antibodies (NAbs). In fact, previous studies showed that immunizations with SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV) whole S glycoproteins or their subunits (e.g., receptor binding domain (RBD)) protected from disease development in animal models.

The SARS-CoV-2 S glycoprotein is a homotrimeric protein and each monomer is composed of two subunits: the S1 surface/external subunit and the S2 membrane anchor domain. While S1 binds to the angiotensin converting enzyme 2 (ACE2) receptor, through the RBD, the S2 domain plays a crucial role in the membrane fusion process. The S glycoprotein is first synthetized in an inactive form and, only after priming by host proteases, it promotes the infection of susceptible cells by binding to ACE2 receptor. Therefore, the S glycoprotein undergoes drastic structural changes during the cell entry process. These changes, as well as its high glycosylation degree, may be affecting its immunogenicity. Studies performed with several surface proteins that are analogous to the SARS-CoV-2 S (e.g. F protein of respiratory syncytial virus) showed that protein stabilization in its prefusion conformation maintained the exposed neutralizing epitopes and increased yield and immunogenicity. In this line, Pallesen and
colleagues\textsuperscript{12} described that MERS-CoV S glycoprotein could be stabilized by two prolines (V1060P and L1061P). A similar strategy was applied to the S trimer of other coronaviruses, indicating that two proline substitutions (2P) between the heptad repeat region 1 (HR1) and the central helix could be a universal strategy for stabilizing coronavirus-derived S glycoproteins\textsuperscript{13}. Accordingly, Wrapp \textit{et al.} showed that the 2P strategy also stabilized the SARS-CoV-2 S in its prefusion conformation\textsuperscript{14}. Thus, the S-2P glycoprotein has been used as an immunogen in several COVID-19 vaccines, including those developed by Pfizer/BioNTech, Moderna, and Janssen\textsuperscript{3}. However, in the presence of the 2P mutations, the S glycoprotein remains some structural motility and the RBD can be found in an up (open) or down (close) conformation\textsuperscript{14}.

Here, we explored new stabilization approaches of the SARS-CoV-2 S glycoprotein. Through extensive computational modelling, we identified a mutation (V987H) that improved the production of the recombinant S trimer and the exposure of the RBD. Even though both V987H Spike (S-V987H) and S-2P variants showed equivalent immunogenicity, S-V987H showed a better prophylactic activity than S-2P in K18-hACE2 mice and golden Syrian hamsters (GHS) against both SARS-CoV-2 D614G and B.1.351 (Beta) variants.

\textbf{Results}

\textit{Identification of S glycoprotein mutations that constrain the motility of RBD.}

The native SARS-CoV-2 S trimer possess some structural flexibility that affects its stability and immunogenicity\textsuperscript{10,14,15}. In addition to pre- and post-fusion S conformations, each RBD displays a dynamic equilibrium between open (up) and closed (down) configurations. In this regard, we aimed to design S variants with a preference for adopting the closed state, and thus, showing limited opening motion and RBD exposure. To this end, we envisioned a computational pipeline involving the three-dimensional modeling of all possible single mutations for both open and closed states, followed by the estimation of changes in their Gibbs free energy ($\Delta \Delta G$) (Fig. 1\textit{a}). We focused on all single mutations showing a strong predicted preference for the closed-state ($\Delta \Delta G<-1$ kcal/mol) and among them, only those that clearly generated well-defined interactions (hydrogen bonds, ionic interactions of filling hydrophobic pockets) between the RBDs of the trimer were screened. We selected a total of 11 single mutations (A372W, K386R, G416R, D420R, D420Y, D427I, L517Y, S982F, D985L, V987H, and V987W) (Fig. 1\textit{a}). We also included one double mutation (A372W-D420R) referred as 2M, and a combined quintuple mutation (D198F-G232L-A372W-N394Q-D420R) named 5M. Locations of the selected mutations are represented in Fig. 1\textit{b}. Recombinant mutant proteins were expressed by transient transfection in Expi293F cells, and their production was evaluated by ELISA (Fig. 1\textit{c}). Most variants displayed a substantial decrease in production when compared to the S-2P trimer (Fig. 1\textit{c}). Then, we analyzed the exposure of the RBD by ELISA using a Fc fusion protein containing the extracellular portion of the human ACE2 receptor fused to the human IgG1-Fc domain. The results confirmed that most of the variants were in fact promoting a closed trimer conformation (Fig. 1\textit{d}) as it was predicted by our \textit{in silico}
pipeline. Moreover, variants associated with a reduced exposure of the RBD also resulted in a very low production. In contrast, the V987H mutation promoted the exposure of the RBD (Fig. 1d) and showed higher production than the S-2P protein (2.5-fold). Thus, our results suggest that the RBD exposure may be associated with S production levels.

**S-V987H trimer vaccination protects K18-hACE2 mice from SARS-CoV-2 infection-associated disease.**

It has been described that K986P and V987P mutations stabilize and increase the expression and immunogenicity of the Spike glycoprotein\(^{14,15}\). Since the V987H mutation improved Spike trimer production and RBD exposure, we evaluated whether it could impact the Spike antigenicity *in vivo*. Thus, we compared the immunogenicity and protective capacity of the recombinant S-2P, S-V987H and RBD (Fig. 2a) after SARS-CoV-2 D614G challenge in K18-hACE2 mice (Fig. 2b).

Forty-five K18-hACE2 mice were immunized using a prime-boost immunization strategy (Fig. 2b). In addition to S-V987H (n = 14), we determined the immunogenicity of S-2P (n = 16), and a recombinant monomeric RBD protein (n = 15). Mice were first immunized by DNA electroporation with 40 µg of plasmid. Two weeks later, animals were boosted with the corresponding purified recombinant protein (15µg) formulated with aluminum phosphate as adjuvant. Prior to challenge, four mice from S-2P and control groups, two mice from S-V987H and three mice from RBD groups were euthanized to collect tissue samples. Then, 12 vaccinated mice for each group, and 16 unvaccinated controls were intranasally challenged with SARS-CoV-2 D614G (Fig. 2b). Four mice (two male/two female) from each group were euthanized on days 2, 4 and 7 (end of the experiment) post-challenge (Fig. 2b) to analyze the humoral immune responses, viral replication in target organs, and tissue damage. Mice that developed severe SARS-CoV-2 induced disease and/or showed a weight reduction higher than 20% of the initial weight were euthanized before the end of the experiment (day 7) as a humane endpoint. Four additional unvaccinated mice were used as uninfected controls.

The humoral responses elicited against the RBD (Fig. 2c), and the S protein (Supplementary Fig. 1a) were evaluated before each immunization (days – 28 and – 14) and viral challenge (day – 2), and in the mice euthanized on days 2, 4 and 7 after infection. At all-time points, mice immunized with S-V987H and S-2P showed similar levels of anti-RBD and anti-Spike IgG antibodies, which were greater than those observed in RBD vaccinated animals (\(p < 0.001\), Conover-Iman test) (Fig. 2c and Supplementary Fig. 1a, b and c). For simplification purposes, challenged animals were grouped as a “post-challenge” group (Fig. 2c). The levels of anti-RBD and anti-Spike IgG antibodies in the S-2P and S-V987H groups increased after boost and viral challenge (\(p < 0.05\), Conover-Iman test) (Fig. 2c and Supplementary Fig. 1a), while mice immunized with RBD only presented increased levels of these antibodies after viral challenge (\(p < 0.05\), Conover-Iman test), indicating that infection may further boost humoral responses in vaccinated mice (Fig. 2c and Supplementary Fig. 1a, b and c).

In addition, we evaluated level of NAbs against the Wuhan-Hu-1 (WH1) strain and Beta (B.1.351) variant after SARS-CoV-2 challenge. Both S-2P or S-V987H mice groups developed equivalent titers of NAbs
against SARS-CoV-2 WH1, but significantly higher than those measured in the RBD immunized group (Fig. 2d \( p < 0.05 \), Conover-Iman test). However, only S-2P and S-V987H vaccinated mice had systemic neutralizing activity against the Beta VoC prior to challenge (Fig. 2e). Despite this, the titers of NAbs against WH1 strain were higher than those targeting the Beta VoC (Supplementary Fig. 1d).

The progressive increase in the levels of total IgG antibodies and/or NAbs observed in both RBD and control-challenged groups after infection (Fig. 2c-d) also supports the idea of a boosting of the humoral response after virus challenge.

To assess the ability of each immunogen to prevent SARS-CoV-2 infection-associated disease, we measured weight evolution in all groups as an indicator of disease progression in this model\(^{16}\). We identified weight reduction on day 5 post-infection in mice belonging to infected-control and RBD groups, which is opposed to S-2P and S-V987H vaccinated groups (\( p < 0.05 \), Kruskal-Wallis corrected by Dunn’s test) (Fig. 2f). On day 6 and 7, all animals from the inoculated-control group (\( n = 4 \)), one out of four S-2P vaccinated mice, and two out of four RBD immunized mice had to be euthanized due to disease development (Fig. 2g; \( p < 0.05 \) compared to control-infected group, Log-rank test) Mice from the S-V987H group did not show clinical signs of disease during the entire experimental procedure (\( p < 0.01 \) compared to control-infected group, Log-rank test) (Fig. 2f and Fig. 2g).

In addition to the clinical course, we measured the levels of viral replication by RT-qPCR in four samples (Fig. 3a-d): oropharyngeal swab, nasal turbinate, lung, and brain. Although the levels of genomic RNA (gRNA) in oropharyngeal swabs decreased in all groups over time, only S-V987H vaccinated mice became undetectable on day 7. On the contrary, S-2P and RBD vaccinated mice remained positive (Fig. 3a, \( p < 0.05 \) Peto & Peto Left-censored k sample test). Similar results were observed in nasal turbinates (Fig. 3b), where gRNA was lower in S-2P and S-V987H groups compared to control-infected mice or the RBD group on days 6–7 after viral challenge, and the S-V987H group showed the lowest values by day 7. Interestingly, low levels of gRNA were detected in lung and brain from S-V987H immunized mice (lung \( p = 0.055 \), brain \( p < 0.05 \), Peto & Peto Left-censored k sample test) (Fig. 3c and d), whereas a progressive increase was observed in brains from the infected-control and RBD groups, and in the only S-2P vaccinated mouse that developed disease (Fig. 3c and 3d). Conversely, subgenomic RNA (sgRNA), which indicates active viral replication, was not detected at any time point in lung or brain of mice immunized with any of the S trimers, or in oropharyngeal swabs on day 4 and 7, except for the single S-2P mouse that developed illness (Supplementary Fig. 1e).

To confirm active viral replication, we analyzed Nucleoprotein (NP) levels in tissues by immunohistochemistry (IHC). NP was detected in lung and brain of both control and RBD groups; and in one animal from S-2P group that developed the disease, but not in S-V987H or disease-free S-2P-vaccinated mice after challenge (Fig. 3e, \( p < 0.05 \) asymptotic generalized Pearson Chi-Squared test corrected for multiple comparison using FDR). Low IHC scores were observed in nasal turbinates on days 2 and 4 with no major differences among study groups (Fig. 3e). Tissue damage was in line with the levels of viral antigens detected by IHC (Fig. 3f). No tissue damage was observed in lung or brain of mice...
vaccinated with S-2P and S-V987H, except for the S-2P mouse that became sick (Fig. 3f). A low lesion score was observed at early time points after challenge in nasal turbinate of all infected mice (Fig. 3f).

Overall, the immunogenicity of both S-2P and S-V987H trimers was equivalent in K18-hACE2 mice, and greater than the produced by the monomeric RBD immunogen. However, S-V987H vaccination improved mice protection against SARS-CoV-2 D614G variant over the S-2P immunogen, since all mice in S-V987H group were disease free and showed a faster viral clearance in tissues.

**S-V987H trimer vaccination protects golden Syrian hamsters from SARS-CoV-2 infection-associated disease.**

To confirm the results obtained in the transgenic mouse model, we performed a second experiment using golden Syrian hamster (GSH). Similar to K18-hACE2 mice, GSH were immunized using a prime-boost strategy, and intranasally challenged with SARS-CoV-2 D614G (Fig. 4a). Animals were monitored until day 7 post-inoculation, since it has been described that animals start spontaneously recovering around a week after viral infection\(^{17,18}\).

The magnitude of the humoral responses elicited against the S and the RBD by both S-2P and S-V987H trimers was similar and greater than those elicited by the RBD immunogen (Fig. 4b and Supplementary Fig. 2a). The levels of anti-RBD and anti-S IgG antibodies increased after each immunization and after viral challenge \((p < 0.05, \text{Friedman test})\) (Fig. 4b and Supplementary Fig. 2a), confirming the results obtained in K18-hACE2 mice. However, unlike mice, infected-control GSHs rapidly developed an anti-S humoral response after challenge, showing similar levels of anti-S and anti-RBD antibodies on day 7 to those observed in animals immunized with the RBD protein (Fig. 4b and Supplementary Fig. 2b). When the neutralizing activity of serum samples was analyzed, we observed that GSH immunized with S-2P or S-V987H proteins neutralized the WH1 variant and, to a lesser extent, the Beta VoC (Fig. 4c and d). The neutralizing activity against WH1 increased overtime after challenge in all study groups \((p < 0.05, \text{Conover-Iman test})\). Neutralization of WH1 was also detected in sera from infected control animals by day 4 after challenge, and their titers rapidly increased, becoming similar to the ones observed in S-V987H and RBD groups, and higher than those observed in S-2P vaccinated animals by day 7 \((p < 0.05, \text{Conover-Iman test})\). Intriguingly, despite all groups showed similar titers of NAb on day 7 after challenge, the levels of anti-RBD and anti-S binding antibodies (Fig. 4b and Supplementary Fig. 2a and b) were higher in the S-2P and S-V987H immunized groups than in infected-controls GSH. These results support that SARS-CoV-2 infection induced a rapid humoral response against SARS-CoV-2 in GSH that may be qualitatively different to the one elicited by immunization.

We then evaluated the clinical course after challenge. Animals in both control and RBD groups showed a progressive weight reduction until day 7 (end of the experiment) indicative of disease progression \((%\text{ of weight in infected controls} = 87.3 \pm 3.1; \text{RBD group} = 84.4 \pm 1.4)\) (Fig. 4e). Such weight loss was not observed in S-2P \((98.9 \pm 1.3)\) or S-V987H \((98.76 \pm 2.4)\) vaccinated GSH \((p < 0.05 \text{ Kruskal-Wallis corrected})\).
by Dunn’s test). Thus, both S trimers generated equivalent protection from disease development in vaccinated GSH (Fig. 4e).

The presence of SARS-CoV-2 was determined by RT-qPCR in oropharyngeal swabs and respiratory tissue samples (nasal turbinate and lung). Brain was not evaluated in GSHs since SARS-CoV-2 does not affect the brain in this animal model. Despite the levels of gRNA decreased over time in all analyzed samples, we detected lower gRNA levels in nasal turbinate of both S-2P and S-V987H groups on day 4 compared to the RBD and infected-control groups (Fig. 5; $p < 0.05$, Peto & Peto Left-censored k sample test). Remarkably, contrary to the RBD or infected-control groups, gRNA was undetectable on day 7 post-infection in S-2P and S-V987H immunized GSHs (Fig. 5a). No major differences were detected in the levels of gRNA in oropharyngeal swabs among the study groups (Fig. 5a). Interestingly, lower levels of gRNA were detected in lungs of S-2P and S-V987H vaccinated animals than in the RBD and infected-control groups on days 2 and 4 (Fig. 5a). While we did not observe significant differences among groups when sgRNA was analyzed in nasal turbinate or oropharyngeal swabs, we detected lower sgRNA levels in the lungs of S-V987H and S-2P groups compared to RBD and infected-control groups on days 2 and 4 after challenge (Supplementary Fig. 2c).

Viral RNA detection results were in line with the levels of NP detection by IHC (Fig. 5b). No major differences in NP levels were observed among study groups at any time points in nasal turbinate, becoming undetectable by day 7 (Fig. 5b) ($p < 0.05$, Asymptotic Generalized Pearson Chi-Squared Test). However, lower NP levels were detected in lungs of both S-2P and S-V987H vaccinated groups when compared with RBD and infected controls on days 2, 4 and 7. Interestingly, NP was not detected in lungs on day 7 in S-2P and S-V987H groups (Fig. 5b). All study groups showed a similar lesion degree in nasal turbinate, which decreased by day 7 after challenge ($p < 0.05$). By contrast, a lower tissue damage was observed in lung from S-V987H (on days 4 and 7) and in S-2P (on day 7) groups compared to RBD and infected control groups ($p < 0.05$) (Fig. 5c).

Overall, our results showed that the immunogenicity and protective efficacy of both S-2P and S-V987H trimers are equivalent in GSHs, and higher than the one conferred by RBD vaccination.

**S-V987H trimer vaccination protects K18-hACE2 mice from the SARS-CoV-2 Beta-variant challenge.**

From the beginning of the COVID-19 pandemic, several SARS-CoV-2 VoC have emerged. These VoC have shown different transmissibility, pathogenic potential and resistance to antibodies previously elicited by vaccination or natural infection. The results described above have shown that S-V987H-vaccinated animals were protected from COVID-19 development after SARS-CoV-2 D614G strain challenge. Additionally, vaccinated animals showed low sera neutralizing activity against the SARS-CoV-2 Beta variant. Since the Beta VoC is one of the most resistant to antibodies elicited by natural infection and the currently available vaccines, and also induces severe disease in K18-hACE2 mice, we evaluated whether the immune responses induced by S-V987H could protect against disease development after challenge with the SARS-CoV-2 Beta variant. Thus, we immunized twenty-one K18-hACE2 mice with S-
V987H or S-2P, using AddVax as adjuvant in this homologous prime-boost experiment (Fig. 6a). Unvaccinated mice were used as negative (n = 10) and positive (n = 16) controls of infection. Two weeks after receiving the protein boost, mice were challenged with the SARS-CoV-2 Beta variant (Fig. 6a). Six mice from each challenged group were euthanized on days 3 (n = 6) and 6 (n = 6) after infection. The remaining animals were euthanized on day 14 after challenge, excepting those mice that developed severe disease after day 3 (10 in the infected-control group and one in the S-2P group) that were euthanized before day 14 following the humane endpoints of the protocol and analyzed separately. Of note, both S-2P and S-V987H recombinant proteins induced similar levels of IgG antibodies against the S and the RBD, which increased after each boost and after viral challenge ($p < 0.05$, Conover-Iman test) (Fig. 6b and Supplementary Fig. 3a and b). Interestingly, three days after challenge, S-V987H immunized mice showed higher sera neutralizing activity against the WH1 (n = 6; 15376±9203) (Fig. 6c), and the Delta VoC (n = 6; 7750±8403) (Fig. 6d) than mice immunized with the S-2P (n = 6; WH1: 2913±3524; Delta: 1505±4773) (WH1: $p < 0.01$; Delta: $p = 0.055$; Conover-Iman test). Neutralizing activity against the Beta VoC increased after challenge ($p < 0.05$, Conover-Iman test) (Fig. 6e). In addition, we identified an increasing trend in sera neutralizing activity against Omicron over time ($p = 0.055$, Conover-Iman test) (Fig. 6f). These differences suggest that the humoral responses elicited after S-2P or S-V987H immunization evolved after challenge with the SARS-CoV-2 Beta variant, increasing neutralizing activity against Beta and Omicron VoC, as well as against Delta in the case of the S-2P group. Interestingly, neutralizing activity against the Beta VoC was detected in control-infected mice at clinical endpoint (Fig. 6e) with little or no cross-neutralization activity with other variants (Fig. 6c, d and f). No statistical differences in neutralizing activity were observed on days 6 and 14 between S-V987H and S-2P groups for any of four SARS-CoV-2 variants evaluated (Fig. 6c-f).

A reduction of body weight associated with disease progression was observed in mice from the infected control group starting on day 2 after challenge compared to mice vaccinated with S-2P and S-V987H (Fig. 6g) ($p < 0.05$, Kruskal-Wallis corrected by Dunn’s test). Mice in both Spike-vaccinated groups maintained their weight until day 14 [percentage of weight: S-2P = 99±4 (n = 9); S-V987H = 98±5 (n = 9)]. Contrarily to the S-2P and infected control groups, no mice from the S-V987H group (n = 9) showed any clinical signs of disease (Fig. 6g and 6h) during the experiment (day 14) ($p < 0.001$, Long rank test).

The analysis of viral load in tissues by RT-qPCR showed that both S-2P and S-V987H vaccinated groups had a progressive decrease in gRNA levels in oropharyngeal swabs and lung over time (Fig. 7a) ($p < 0.05$; Peto & Peto Left-censored k sample test). Interestingly, the S-V987H group displayed lower viral loads in nasal turbinate than S-2P and infected control animals on day 3, and also in oropharyngeal swab compared to the infected controls (Fig. 7a) ($p < 0.05$). However, these differences were not maintained over time and both S-trimer immunized groups showed low but equivalent values of gRNA on day 14 in all analyzed tissues (Fig. 7a). In addition, these groups displayed lower viral load in lung and brain compared to the infected control group at day 3 after challenge (Fig. 7a, $p < 0.05$).

Remarkably, NP was hardly detected in lung and brain from S-2P and S-V987H groups by IHC (Fig. 7b), which was in line with the low levels of gRNA detected in these animals. Despite that, S-2P vaccinated
mice showed a higher lesion score in lung at day 14 than the S-V987H group ($p < 0.01$; Asymptotic Generalized Pearson Chi-Squared test) (Fig. 7c), indicating that these mice presented a severe lung damage. Interestingly, both Spike-based immunogens protected from viral dissemination to the brain (Fig. 7).

To summarize, the immunogenicity of both S-2P and S-V987H trimers was similar in K18-hACE2 SARS-CoV-2 Beta-infected mice, although S-V987H promoted the development of higher serum neutralization, which might explain the increase in protection observed in S-V987H vaccinated animals, compared to the S-2P group.

**Discussion**

SARS-CoV-2 uses the S glycoprotein to infect susceptible cells through a complex process that involves binding to the ACE2 receptor and subsequent structural reorganization. Hence, antibodies that target this protein and block its interaction with the ACE2 receptor or hamper its structural rearrangement could prevent infections\(^5\). The S is also a major target of both CD4 + and CD8 + T cell responses\(^{23}\). Therefore, most of the available SARS-CoV-2 vaccines use this protein as immunogen. However, the structural plasticity of the S may limit its capability to induce NAbs, similar to what has been shown for other functional equivalent proteins in other viruses (e.g., respiratory syncytial virus and human immunodeficiency virus 1)\(^{11}\). To date, several strategies have been used to stabilize the S glycoprotein in its prefusion conformation. Among them, the incorporation of two proline mutations between the HR1 and the central helix within the S2 subdomain stabilized the S glycoprotein of several coronaviruses, including MERS-CoV and SARS-CoV-2\(^{13,14}\). Such modifications result in increased production yields and improved immunogenicity\(^{13,15}\). In fact, some SARS-CoV-2 vaccines are mainly based on the 2P-stabilized S glycoproteins (i.e. mRNA-1273, BNT162b2 or Ad26.COV2.S)\(^3\). Unfortunately, the yields of the S-2P remains low under culture conditions (about 0.5 mg/L\(^{14}\)), hampering its production and expression as a recombinant protein. To evaluate alternatives to the 2P strategy, we performed an *in silico* screening of a set of non-proline mutations based on their capability for maintaining a closed prefusion conformation of the Spike. In line with Xiong et al.\(^{24}\), we observed that mutations that close the Spike glycoprotein had a detrimental impact on protein yield, suggesting that the open conformation may contribute to the expression of the protein. We also observed that the V987H mutation increased the production of the recombinant protein by two-fold and improved the ACE2 receptor recognition, suggesting a better RBD exposure. Next, we evaluated the immunogenicity and prophylactic capability of S-V987H, S-2P and recombinant RBD proteins in two animal models (K18-hACE2 mice and GSH). While K18-hACE2 usually develop a severe form of viral-induced disease after challenge, and succumb to infection, GSHs progress to a moderate disease and spontaneously recover\(^{17,25}\). In addition to SARS-CoV-2 D614G variant, we also evaluated the protective efficacy of the S-V987H immunogen in K18-hACE2 mice challenged with the Beta variant. We chose this VoC since it showed higher resistance against antibodies generated after vaccination or infection\(^{21}\), and had increased virulence in K18-hACE2 mice\(^{22}\). Of note, we did not select the Omicron VoC because it showed reduced virulence in K18-hACE2 mice\(^{22}\). Since adjuvants and the
immunization strategy may impact on immunogenicity and efficacy of vaccines\textsuperscript{26}, we performed a heterologous prime/boost immunization strategy combining DNA (prime) and recombinant protein formulated with aluminum as adjuvant (boost), and two homologous administrations of the according recombinant protein formulated with AddaVax (MF59-like).

Even though S-V987H showed higher yields than S-2P, its immunogenicity was equivalent in both animal models. Vaccination with the S trimers elicited higher humoral responses than the recombinant RBD protein, showing enhanced protective capability against severe disease. However, while all K18-hACE2 mice immunized with S-V987H were disease free, two mice in the S-2P group (one in each experiment) developed severe disease. Thus, it is possible that S-V987H protective efficacy might be higher than the one observed with S-2P. Accordingly, mice immunized with S-V987H showed a faster viral clearance in respiratory tissues. In addition, we also evaluated the immunogenicity and prophylactic capability of these trimers in GSH, confirming the results obtained in the transgenic mouse model. Thus, our broad \textit{in vivo} immunogenicity analysis of the S-V987H has shown that this mutation may be a good alternative to S-2P, since the incorporation of this mutation increased protein yields and improved protection in animal models.

Since the 2P strategy stabilized the S glycoprotein in its prefusion state by preventing conformational rearrangement\textsuperscript{12}, we hypothesized that the V987H mutation might alter the equilibrium of the open/close dynamics of the trimer protein via steric effects, as we did not observe in our \textit{in silico} models any stabilizing interaction caused by the incorporation of this mutation in the open conformation that could directly explain the preference of this variant for such state.

In addition to the 2P strategy, other approaches have been implemented to increase the stability and production of the S glycoprotein. In this sense, the inclusion of four-additional proline mutations into the S2 subdomain of the S-2P protein (HexaPro Spike) increased protein yields by 10-fold and improved its thermal stability\textsuperscript{27}. Following a different approach, Juraszek and colleagues\textsuperscript{28} described a set of four mutations that stabilized the Spike protein in a closed conformation, increasing its expression by 6.4-fold. Remarkably, this protein did not require a heterologous trimerization domain. In addition, Riley and coworkers cross-linked the S2 subunit with different regions of the S1 subunit by introducing disulphide bridges, which also limited the motility of the RBD\textsuperscript{29}. Besides these structure-guided mutation selection strategies, Tan et al. developed a high-throughput methods for the identification of pre-fusion stabilizing mutations within the heptad repeat 1 and central helix regions of the S2 subunit\textsuperscript{30}. However, whether all these S protein variants show an improved immunogenicity or protective capabilities compared to the S-2P protein remains unknown. Nevertheless, Sun \textit{et al.} showed that Newcastle disease virus expressing the HexaPro Spike protects GSH from SARS-CoV-2 induced disease\textsuperscript{31}.

Overall, here we described a new S2-mutation (V987H) that improves the expression of the recombinant Spike glycoprotein. This protein was able to induce SARS-CoV-2 neutralizing antibodies and protect
animals against both SARS-CoV-2 D614G and the highly pathogenic Beta variant.

**Methods**

1) **Recombinant trimeric Spike glycoprotein design and modelling.**

Unsolved secondary structures of the trimer in closed (PDB: 6VXX) and open (PDB: 6VYB) conformations\(^8\) were reconstructed using SwissModel\(^32\). Then, all possible single mutations in both conformations were modelled using FoldX\(^33\). Comparison of the Gibbs free energy changes upon mutation between the open (\(\Delta\Delta G_{\text{open}}\)) and closed (\(\Delta\Delta G_{\text{closed}}\)) conformations (\(\Delta\Delta G\)) revealed a set of mutations predicted to strengthen the closed conformation. All single mutations predicted with \(\Delta\Delta G < -1\) kcal/mol were rationally addressed by inspecting the three-dimensional models. In this regard, the final selection was based in two steps: i) selection of mutations predicted to increase the stability of the closed-conformation over the open-conformation using FoldX, ii) selection of mutations creating well-defined intermolecular interactions between the RBDs (including hydrophobic bonds, π-π interactions and cation-π interactions, ionic bonds, hydrophobic contacts or cavity filling mutations) that would exert a negative impact on the opening motion of the trimer.

2) **Recombinant protein production and purification.**

The design of recombinant Spike glycoprotein is based on the one described by Wrapp\(^14\). Briefly, the C-terminal end of the extracellular portion of the S glycoprotein was fused to a T4-foldon trimerization domain followed by an 8xHis tag and a strep tag II. Plasmids for the expression of the recombinant S glycoproteins were obtained from GeneART (ThermoFisher scientific) in a pcDNA3.4 backbone. Proteins were produced by transient transfection using the ExpiFectamine 293 transfection kit (ThermoFisher Scientific) and following manufacturer specifications. Cell culture supernatants were harvested five days after transfection, clarified by centrifugation (3000xg for 20 minutes) or using Sartoclear Dynamics® Lab V (Sartorius) and filtered at 0.2 μm using Nalgene Rapid-Flow sterile single use vacuum filter units (ThermoFisher Scientific). Equivalent transfection efficiency was obtained for all the tested S variants. Proteins were purified by Immobilized Metal Affinity Chromatography using the Ni-Sepharose Excel histidine-tagged protein purification resin (Cytiva). Purified proteins where concentrated and buffer exchanged to phosphate buffer saline by ultrafiltration (Merck Millipore) and stored at -80°C until use.

Purified proteins were quantified by ELISA. Briefly, Nunc MaxiSorp ELISA plates were coated overnight at 4°C with 100 ng/well of HIS.H8, an anti-6xHis monoclonal antibody, in PBS (ThermoFisher Scientific). The following day, plates were blocked with PBS/1% of bovine serum albumin (BSA, Miltenyi BiotecBiotec) for two hours at room temperature. Commercial His-Spike (Sino Biological) was used as standard, starting at 1μg/ml and eight 1/3 serial dilutions. S variants were prepared in blocking buffer at 1/10 serial dilution for quantification. Samples were incubated overnight at 4°C. After that, plates were washed and incubated for 2 hours at room temperature with rabbit anti-SARS-CoV-2 Spike S2 IgG (SinoBiological) at 1/1000 dilution. Then, the HRP conjugated donkey anti-rabbit IgG (H + L) antibody (Jackson ImmunoResearch) at 1/10000 dilution was used as detection antibody. Plates were revealed
with o-Phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich) and stopped using 4N of H$_2$SO$_4$ (Sigma-Aldrich). The signal was analyzed as the optical density (OD) at 492 nm with noise correction at 620 nm.

Integrity and purity of purified proteins were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie G-250 staining (ThermoFisher Scientific).

3) Cell culture, viral isolation and titration.

Vero E6 cells (ATCC CRL-1586) were cultured in Dulbecco’s modified Eagle medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS; Invitrogen), 100 U/mL penicillin, 100 µg/mL streptomycin (all from Invitrogen).

Both SARS-CoV-2 isolates used in the present work (Cat 01 and Cat 24) have been described previously$^{34,35}$ and were isolated from a clinical nasopharyngeal swab, as previously described in $^{35}$. Viral isolates were subsequently grown in Vero E6 cells. Cat 01 is a SARS-CoV-2 D614G variant (ID EPI_ISL_510689) whereas Cat 24 is a SARS-CoV-2 B.1.351 variant (originally detected in South Africa; EPI_ISL_1663571). Genomic sequencing was performed from viral supernatant by using standard ARTIC v3 or v4 based protocols followed by Illumina sequencing (dx.doi.org/10.17504/protocols.io.bhjgj4jw). Raw data analysis was performed by viralrecon pipeline (https://github.com/nf-core/viralrecon) while consensus sequence was called using samtools/ivar at the 75% frequency threshold. SARS-CoV-2 D614G variant (ID EPI_ISL_510689) and SARS-CoV-2 B.1.351 variant (originally detected in South Africa; EPI_ISL_1663571) sequences were deposit at GISAID Viral stock was titrated in 10-fold serial dilutions on Vero E6 cells to calculate the TCID50 per mL.

4) In vivo immunization and challenge experiments. All animal procedures were performed under the approval of the Committee on the Ethics of Animal Experimentation of the IGTP and the authorization of Generalitat de Catalunya (Code: 10965 and 11094).

Prophylactic activity of the recombinant S-2P, S-V987H trimeric proteins and a recombinant monomeric RBD against SARS-CoV-2 D614G isolate (Cat01 isolate) was assessed in B6. Cg-Tg(K18-ACE2)2Prlmn/J (K18-hACE2) mice (stock #034860, Jackson Laboratories) and Golden Syrian hamsters (GHS) (Envigo). In addition, protection against the SARS-CoV-2 B.1.351 (Beta) isolate (Cat024 isolate) was evaluated in K18-hACE2 mice. The colony of these mice was maintained by breeding K18-hACE2 hemizygotes with C57BL/6J mice following the instructions of Jackson Laboratory (https://www.jax.org/strain/034860). Mice genotyping was performed according to the protocol 38170: Probe Assay - Tg(K18-ACE2)2Prlmn QPCR version 2.0 (https://www.jax.org/Protocol?stockNumber=034860&protocolID=38170). The GSH colony was maintained by brother/sister mating. Both mice and GSH colonies were stablished at the Centre for Comparative Medicine and Bioimage (CMCiB).

For the SARS-CoV-2 D614G challenge, 68 K18-hACE2 mice (50%male/50% female, 7–9 weeks old) were distributed in five experimental groups: S-2P (n = 16), V987H (n = 14), RBD (n = 15), infected positive controls (n = 19) and unvaccinated/uninfected negative control (n = 4). Sixty-eight GSH (were distributed
in S-2P, S-V987H, RBD and infected positive controls (n = 16/group) and unvaccinated/uninfected negative control (n = 4). Mice and GSH from the S-2P, S-V987H and RBD groups were DNA-immunized by electroporation in the quadricep posterior. Forty microgram of plasmid coding for the corresponding immunogens were used. Animals were electroporated using a NEPA21 electroporator and tweezer electrodes (Nepagene). Two (for mice) or four (for GSH) weeks later, DNA-immunized animals received a boosting dose consisting of 15µg of recombinant protein adjuvanted with adjust-Phos (Invivogen) in the hock. Control animals were primed with an empty vector and boosted with PBS + Adjust-Phos. Two weeks (mice) or ten days (GSH) after boosting, animals were intranasally challenged with 1000 (mice) or 10000 (GSH) TCID\textsubscript{50} of SARS-CoV-2 (Cat01 isolate) and followed up for 7 days. Weight and clinical signs were monitored daily after infection. Four animals for each experimental group, except for uninfected controls, were euthanized before challenge and on days 2, 4 or 7 post infection. Uninfected controls were euthanized on day 7 post-infection and only two mice from groups S-V987H and RBD were euthanized before challenge. However, any animal that showed a reduction of weight higher than 20%, a drastic reduction of mobility or a significant reduction of the response to stimuli were euthanized according to the humane endpoints defined in the supervision protocol. After euthanasia, oropharyngeal swab, nasal turbinate, lung and brain were collected for viral load determination and histopathological analysis. Blood samples were collected before each immunization and viral challenge, and at euthanasia. Blood was left at room temperature for two hours for clotting and serum was collected after centrifugation (10 minutes at 5000xg) and stored at -80°C until use.

For the SARS-CoV-2 B.1.351 (Beta) VoC challenge, 70 K18-hACE2 mice (50% male) were distributed as follows: S-2P (n = 21), S-V987H (n = 21), infected positive controls (n = 16) and unchallenged controls (n = 10). Mice from S-2P and S-V987H groups were immunized twice (spaced three weeks between both doses) with 15µg of recombinant protein adjuvanted with AddaVax (Invivogen) in the hock. Control mice received only PBS + AddaVax. Two weeks after the booster, mice from groups S-2P, S-V987H and challenged-controls were inoculated intranasally with 1000 TCID\textsubscript{50} of SARS-CoV-2 Beta VoC (Cat24 isolate).

5) Quantification of anti-Spike antibodies by ELISA.

IgG antibodies elicited against the Spike and RBD glycoproteins were determined using and in-house ELISA in serum samples obtained from animals before each immunization and before viral challenge. In addition, humoral response was also evaluated in serum samples obtained from animals euthanized on days 2, 4, 7 after viral challenge or after humane endpoint. One half of a Nunc MaxiSorp ELISA plate was coated overnight at 4°C with 50 ng/well of antigen in PBS (Spike or RBD, Sino Biologicals). The other half-plate was incubated only with PBS. Then, the whole plate was blocked using PBS/1% of bovine serum albumin (BSA, Miltenyi Biotech) for two hours at room temperature. Mouse standards were prepared as seven 1/3 dilution of the anti-6xHis antibody HIS.H8 (ThermoFisher Scientific), starting at 1 µg/mL. GSH standard was prepared similarly but using a positive GSH serum with the initial dilution at 1/100. All standards and samples were diluted in blocking buffer. After blocking, 50 µL of each standard or diluted samples were added to the antigen coated and antigen free wells in duplicate and incubated...
overnight at 4ºC. Each plate contained samples from all experimental groups. Plates were run in parallel to reduce inter-assay variability. After sample addition, plates were incubated overnight at 4ºC. The HRP conjugated (Fab)2 Goat anti-mouse IgG (Fc specific) (1/20,000), or Goat anti-hamster IgG (H + L) (1/20,000) (all from Jackson ImmunoResearch) were used as detection antibodies for mouse and GSH IgG determination, respectively. Plates were revealed with o-Phenylenediamine dihydrochloride (OPD) (Sigma-Aldrich) and stopped using 4N of H$_2$SO$_4$ (Sigma-Aldrich). The signal was analyzed as the optical density (OD) at 492 nm with noise correction at 620 nm.

The specific signal for each sample was calculated after subtracting the background signal obtained in antigen-free wells. Data is shown as arbitrary units (AU) according to the standard used.

6) **Neutralizing activity of serum samples.**

The neutralizing activity of serum samples was determined using HIV reporter pseudoviruses expressing SARS-CoV-2 S protein and Luciferase as described by Pradenas et al.$^{37}$ In brief, pseudoviruses were produced by co-transfecting Expi293F cells (ThermoFisher Scientific) with the pNL4-3.Luc.R.E- (NIH AIDS Reagent Program$^{38}$) and several SARS-CoV-2.SctΔ19 plasmids that code for the Spike glycoprotein of the WH1, Beta, Delta or Omicron variants. A VSV-G plasmid was used for the generation of VSV-G-pseudoviruses that were used as negative control. Transfections were performed using the ExpiFectamine293 Reagent kit (ThermoFisher Scientific). After 48h, supernatants were harvested, filtered at 0.45 µm and frozen at -80ºC until use. Pseudoviruses were titrated on HEK293T cells overexpressing human ACE-2 (HEK293T/hACE2) (Integral Molecular).

Serum samples were inactivated at 56ºC for 60 minutes before use. Inactivated samples were 1/3 serially diluted in cell culture medium (DMEN, 10% fetal bovine serum) (range 1/100–1/24300) before mixing with 200 TCID50 of SARS-CoV-2 derived pseudoviruses and incubated for 1 hour at 37ºC. Then, 2x10^4 HEK293T/hACE2 cells treated with DEAE-Dextran (Sigma-Aldrich) were added. After 48 hours BriteLite Plus Luciferase reagent (PerkinElmer) was added and the results read in an EnSight Multimode Plate Reader. Data were calculated using a 4-parameters logistic equation in Prism 8.4.3 (GraphPad Software) and showed as normalized ID50 (reciprocal dilution inhibiting 50% of the infection). This assay has been previously validated with a replicative viral inhibition assay$^{39}$.

7) **Viral load quantification in oropharyngeal swab and tissue samples.**

Oropharyngeal swabs and samples from nasal turbinate, lung and brain (only mice) were collected immediately after euthanasia in 1.5 mL Sarstedt tubes containing DMEM media supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL). Tissue samples were homogenized twice at 25 Hz for 30 sec using a TissueLyser II and a 1.5 mm Tungsten bead (QIAGEN). After centrifugation for 2 min at 2,000xg, supernatants were collected and stored at -80ºC until use.

RNA was isolated using the Viral RNA/Pathogen Nucleic Acid Isolation kit and a KingFisher instrument (ThermoFisher Scientific), or an IndiMag pathogen kit (Indical Bioscience) on a Biosprint 96 workstation.
(QIAGEN) following manufacturer's instructions.

PCR amplification in mice was based on the 2019-Novel Coronavirus Real-Time RT-PCR Diagnostic Panel guidelines and protocol developed by the American Center for Disease Control and Prevention. Briefly, a 20 µL PCR reaction was set up containing 5 µL of RNA, 1.5 µL of N2 primers and probe (2019-nCov CDC EUA Kit, Integrated DNA Technologies) and 10 µl of GoTaq 1-Step RT-qPCR (Promega, Madison). Thermal cycling was performed at 50°C for 15 minutes (min) for reverse transcription, followed by 95°C for 2 min and then 45 cycles of 95°C for 10 seconds (s), 56°C for 15s and 72°C for 30s in the Applied Biosystems 7500 or QuantStudio5 Real-Time PCR instruments (ThermoFisher Scientific). For absolute quantification, a standard curve was built using 1/5 serial dilutions of a SARS-CoV2 plasmid (2019-nCoV_N_Positive Control, 200 copies/µL, Integrated DNA Technologies) and run in parallel in all PCR determinations. The viral load of each sample was determined in triplicate and mean viral load (in copies/mL) was extrapolated from the standard curve and corrected by the corresponding dilution factor. Alternatively, results are shown as cycle threshold (Ct) or 2^-ΔCt.

SARS-CoV-2 subgenomic RNA (sgRNA) was performed as previously described with the following primers (Forward; 5-GCATCTCTTGTAGATCTTTCTC-3’; Reverse, 5′-ATATTGCAGCAGTACGCACACA-3’) and probe (5′- FAM-ACACTAGCCATCCTTTACTGCGCTTC-G-TAMRA-3’). Mouse gapdh gene expression was measured in duplicate for each sample using TaqMan gene expression assay (ThermoFisher Scientific) as amplification control.

SARS-CoV-2 genomic RNA (gRNA) detection in GSH was performed based on RT-PCR described by Corman et al., which was adapted to the AgPath-ID One-Step RT-PCR kit (Life Technologies). This RT-PCR targets a fragment of the envelope protein gene using the following primers (Forward: 5′-ACAGGTACGTATAGTTATAGCGT-3’; Reverse: 5′-ATATTGCAGCAGTACGCACACA-3’) and probe (5′-FAM-ACACTAGCCATCCTTTACTGCGCTTCG-TAMRA-3’). Thermal cycling was performed at 55°C for 10 min for reverse transcription, followed by 95°C for 3 min and then 45 cycles of 94°C for 15s, 58°C for 30s. SARS-CoV-2 subgenomic RNA detection in GHS was done as it is described in Wölfel et al. The primers and probes are the same as in gRNA determination except for forward primer: 5′-CGATCTCTTGTAGATCTTTCTC-3’. Thermal cycling for sgRNA was performed at 55°C for 10 min for reverse transcription, followed by 95°C for 3 min and the 45 cycles of 95°C for 15 s, 56 ºC for 30s. Results are shown as Ct or 2^-ΔCt.

**8) Pathology and immunohistochemistry**

SARS-CoV-2 NP was detected by IHC using the rabbit monoclonal antibody 40143-R019 (Sino Biological) at 1:15,000 dilution. For immunolabelling visualization, the EnVision®+ System linked to horseradish peroxidase (HRP, Agilent-Dako) and 3,3’-diaminobenzidine (DAB) were used. The amount of viral antigen in tissues was semi-quantitatively scored as indicated in . The following score was used: 0: No antigen detection, 1-low, 2-moderate and 3- high amount of antigen (Supplementary Fig. 4a). Nasal turbinate and lung from mice and GSH, and brain from mice were collected on days 0 (before viral
challenge), 2, 4, 7 or at clinical endpoint after viral challenge, fixed by immersion in 10% buffered formalin and embedded into paraffin blocks. The histopathological analysis was performed on slides stained with hematoxylin/eosin and examined by optical microscopy. A semi-quantitative scored based on the level of inflammation (0-No lesion; 1-Mild, 2-Moderate or 3-Severe lesion) was established (Supplementary Fig. 4b) based on previous classifications 16,18.

9) Statistical analysis.

ELISA binding data and neutralizing activity in each time point were analyzed using Conover Test with multiple comparison correction by false discovery rate (FDR). Differences among animals within a particular group along time were analyzed using the Friendman test corrected for multiple comparison using FDR. Weight variation in SARS-CoV-2 challenged mice over time was analyzed using Kruskal-Wallis corrected by Dunn's test. Severe disease incidence was represented by Kaplan-Meier plots. Statistical analysis was performed against unvaccinated group using Mantel-Cox test. Levels of SARS-CoV-2 gRNA in tissues were analyzed using Peto & Peto left-censored k sample test corrected by FDR. Histopathology analysis was carried out using Asymptotic Generalized Pearson Chi-Squared test with FDR correction. P values are indicated as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Whereas p values close to statistical significance are shown as number. Statistical analyses were conducted using the R (version 3.6) software environment and GraphPad Prism v8.0.

Declarations

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Author contributions

JC, BC, JS, JB, JVA, NIU, VG and AV: Study conception, design, and funding

JC, NPL, CAN, PAR: Manuscript draft preparation


Competing interests

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References


**Figures**

**Figure 1**

**Selection of mutations that stabilized the S glycoprotein in a closed conformation.** An *in silico* saturation mutagenesis study was performed for selecting mutations that could stabilize the S glycoprotein in a closed conformation. The production of selected variants and the exposure of the RBD was evaluated by ELISA. **a** Saturation mutagenesis of the SARS-CoV-2 S glycoprotein. Mutations selected for experimental characterization (with a favorable predicted \( \Delta \Delta G < -1 \) Kcal/mol and showing stabilizing interactions
between the RBDs in the closed conformation) are colored depending on the mutant residue. Labeled mutations were selected for experimental characterization. b Three-dimensional location of the selected mutations (displayed as dots and colored by their belonging monomer) in the closed state of the SARS-CoV-2 S glycoprotein (PDB:6VXX). Mutations are in multiple domains of the S glycoprotein, including NTD, CDT1, CDT2 and HR1-CH region. c Levels of recombinant proteins in a 5-day cell culture supernatant of transiently transfected Expi293F cells. Mean plus standard deviation of three experiments are shown. d RBD exposure index in selected recombinant proteins. Data are shown as ratio between RBD binding and total protein. Mean plus standard deviation of three experiments are shown.

Figure 2
Prophylactic activity of S-V987H immunization and analysis of the humoral response elicited in vaccinated K18-hACE2 mice challenged with the SARS-CoV-2 D614G variant. K18-hACE2 mice were immunized following a DNA/protein prime/boost strategy with S-V987H, S-2P or RBD, and challenged with SARS-CoV-2 D614G. The humoral response, weight changes and survival of mice were evaluated after immunization and/or viral challenge. a Purified S-V987H, S-2P and RBD were analyzed by SDS-PAGE and Coomassie G-250 staining. b Overview of vaccination strategy and infection timeline. Blood drops indicate collection of biological samples. c Kinetics of anti-RBD IgG in serum samples express as arbitrary units (AU) per mL. Red triangles: S-2P group (n= 16). Blue squares: S-V987H group (n= 14). Black diamonds: RBD (n=15). White circles: unvaccinated mice (n=19). Groups in each time point were analyzed using Conover Iman test with multiple comparison correction by FDR. Differences among animals within a particular group along time were analyzed using the Friedman test corrected for multiple comparison using FDR. Mean plus standard errors of the means (SEM) are shown. d Levels of NAbs against SARS-CoV-2 WH1 variant after viral challenge. e Levels of NAbs against SARS-CoV-2 B.1.351 (Beta) variant after viral challenge. Neutralization data were analyzed as indicated in b. f Percentage of weight variation in SARS-CoV-2 D614G-infected K18-hACE2 mice over time. Statistical analysis was performed comparing each vaccinated group with the unvaccinated group using Kruskal-Wallis corrected by Dunn’s test. g Kaplan-Meier plot showing the survival rate during the course of the experiment. Statistical analysis was performed against unvaccinated group using Mantel-Cox test. * p<0.05, ** p<0.01, *** p<0.001.

Figure 3

Viral load and histopathological analysis of tissue samples from SARS-CoV-2 D614G infected K18-hACE2 mice after vaccination. SARS-CoV-2 viral load was analyzed in oropharyngeal swabs, and samples from nasal turbinate, lung, and brain of K18-hACE2 mice upon challenge. Virus distribution and tissue damage were analyzed by histopathology. Levels of SARS-CoV-2 gRNA (expressed as logarithmic of copies/mL) in a oropharyngeal swabs, b nasal turbinate, c lung, and d brain during infection. Dot line indicates limit of detection (100 copies/mL). Differences between animals were analyzed using Peto & Peto left-censored k sample test, correcting by FDR. e Detection of SARS-CoV-2 nucleocapsid protein in brain, lung, and nasal turbinate by immunohistochemistry. Staining score: (0) no, (1) low, (2) moderate, and (3) high amount of viral antigen. f Histopathological analysis of nasal turbinate, lung and brain by hematoxylin and eosin staining. Lesion score: (0) no, (1) mild, (2) moderate, and (3) severe lesion. Differences between groups were analyzed using Asymptotic Generalized Pearson Chi-Squared test with FDR correction. * p<0.05, ** p<0.01. P values proximal to statistical significance are shown as numbers.
Figure 4

Prophylactic activity of S-V987H immunization and analysis of the humoral response elicited in vaccinated golden Syrian hamsters challenged with the SARS-CoV-2 D614G variant. GSH were immunized following a prime/boost strategy with S-V987H, S-2P or RBD, and challenged with SARS-CoV-2 D614G. The humoral response and weight changes of GSH were evaluated after immunization and/or viral challenge. 

**a** Outline of vaccination strategy and infection timeline. Blood drops indicate collection of biological samples. 

**b** Kinetics of anti-RBD antibodies in serum samples. Red triangles: S-2P group (n=16). Blue squares: S-V987H group (n=16). Black diamonds: RBD (n=16). White circles: unvaccinated and challenged GSH (n=16). Grey circles: unvaccinated and uninfected GSH (n=4). Groups in each time point were analyzed using Conover-Iman test with multiple comparison correction by FDR. Differences among animals within a particular group along time were analyzed using the Friedman test with FDR correction.

**c** Sera neutralizing activity against SARS-CoV-2 WH1 and **d** SARS-CoV-2 B.1.351 (Beta) variants after viral challenge. Neutralization data were analyzed as indicated in **a**.

**e** Percentage of weight variation in SARS-CoV-2 D614G-infected GSH over time. Statistical analysis was performed against the unvaccinated group using Krustal-Wallis correcting by Duns test. *p<0.05, **p<0.01, ***p<0.001.
Viral load and histopathology analysis of tissue samples from SARS-CoV-2 D614G infected golden Syrian hamsters after vaccination. SARS-CoV-2 viral load was analyzed in oropharyngeal swabs, and samples from nasal turbinate and lung of infected GSHs. Virus distribution and tissue damage was analyzed by histopathology. 

**a** Levels of SARS-CoV-2 gRNA, expressed as cycles threshold (Cts), in oropharyngeal swabs, nasal turbinate and lung during infection. Dot line indicates limit of detection (Cts ≥ 40). Differences between groups were analyzed using Peto & Peto left-censored k sample test with FDR correction.

**b** Detection of SARS-CoV-2 nucleocapsid protein in lung and nasal turbinate by immunohistochemistry. Staining score: (0) no, (1) low, (2) moderate, and (3) high amount of viral antigen.

**c** Histopathologic analysis of nasal turbinate and lung by hematoxylin and eosin staining. Lesion score: (0) no, (1) mild, (2) moderate, and (3) severe lesion. Differences between groups were analyzed by the Asymptotic Generalized Pearson Chi-Squared test corrected using FDR. * p<0.05.
Figure 6

Prophylactic activity of S-V987H immunization and analysis of the humoral response elicited in vaccinated K18-hACE2 mice challenged with the SARS-CoV-2 B.1.351 (Beta) variant. K18-hACE2 mice were immunized twice with S-V987H or S-2P, adjuvanted with AddaVax. Then, mice were challenged with the SARS-CoV-2 B.1.351 VoC. The humoral response, weight changes and survival of mice were evaluated after immunization and/or viral challenge. a Overview of vaccine strategy and infection timeline. Blood drops indicate collection of biological samples. b Kinetics of anti-RBD antibodies in serum samples. Red triangles: S-2P group (n= 21). Blue squares: S-V987H group (n= 21). White circles: unvaccinated-challenge mice (n=16). Grey circles: uninfected and unvaccinated mice (n=10). Groups in each time point were analyzed using Conover-Iman test with multiple comparison correction by FDR. Differences among animals within a particular group along time were analyzed using Friedman test with FDR correction. Sera neutralizing activity against: c SARS-CoV-2 WH-1, d B.1.617.2 (Delta), e B.1.351 (Beta), and f B.1.1.529 (Omicron) variants after viral challenge. Neutralization data were analyzed as indicated in a. g Percentage of weight variation in SARS-CoV-2 B.1.351 infected K18-hACE2 mice over
time. Statistical analysis was performed against the unvaccinated and challenged group using Kruskal-Wallis with Dunn’s test correction. f Kaplan-Meier plot showing the percentage of SARS-CoV-2-infected animals that were survive at the end of the experiment. Statistical analysis was performed against unvaccinated group using Mantel-Cox test. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Mean plus standard errors of the means (SEM) are shown.

Figure 7

Viral load and histopathology analysis of tissue samples from SARS-CoV-2 B.1.351 infected K18-hACE2 mice after vaccination. SARS-CoV-2 viral load was analyzed in oropharyngeal swabs, and samples from nasal turbinate, lung and brain of infected K18-hACE2. Virus distribution and tissue damage was analyzed by immunohistochemistry and histopathology. a Levels of SARS-CoV-2 gRNA (expressed as logarithmic of copies/mL) in oropharyngeal swabs, nasal turbinate, lung, and brain during infection. Dot line indicates limit of positivity (100 copies/mL). Differences between groups were analyzed using Peto & Peto left-censored k sample test with FDR correction. b Detection of SARS-CoV-2 nucleocapsid protein in brain, lung, and nasal turbinate by immunohistochemistry. Staining score: (0) no, (1) low, (2) moderate, and (3) high amount of viral antigen. c Histopathological analysis of brain, lung, and nasal turbinate by hematoxylin and eosin staining. Lesion score: (0) no, (1) mild, (2) moderate, and (3) severe lesion. Differences between groups were analyzed using Asymptotic Generalized Pearson Chi-Squared test with FDR correction. * p<0.05, ** p<0.01.

Supplementary Files
This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFig1.pdf
- SupplementaryFig2.pdf
- SupplementaryFig3.pdf
- SupplementaryFig4.pdf