Protection of Hamsters Challenged with SARS-CoV-2 Variants of Concern by Two Doses of MVC-COV1901 Vaccine Followed by a Single Dose of Beta Variant Version of MVC-COV1901

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Abstract

The current fight against COVID-19 is compounded by the Variants of Concern (VoCs), which can diminish the effectiveness of vaccines and potentially increase viral transmission and severity of disease. MVC-COV1901 is a protein subunit vaccine based on the prefusion SARS-CoV-2 spike protein (S-2P) and is adjuvanted with CpG 1018 and aluminum hydroxide. In this study, we used the Delta variant to challenge hamsters inoculated with S-2P from the Wuhan wildtype and the Beta variant in two-dose or three-dose regimens. Two doses of wildtype S-2P followed by the third dose of Beta variant was shown to induce the highest neutralizing antibody titer against live SARS-CoV-2 of the wildtype as well as all current VoCs. All regimens of vaccination were able to protect hamsters from SARS-CoV-2 Delta variant challenge and resulted in reduced lung live virus titer and pathology. Three doses of vaccination also significantly reduced lung viral RNA titer, regardless of whether the wildtype or Beta variant S-2P was used as the third dose. Based on the immunogenicity and viral challenge data, two doses of wildtype S-2P followed by the third dose of Beta variant S-2P induced a broad and potent immune response against the Alpha, Beta, Gamma, and Delta variants.
Introduction

As of September 2021, the COVID-19 pandemic shows no sign of abating despite the fact that over five billion doses of vaccines have been administered worldwide. The emergence of variants has undoubtedly played an important role in facilitating the global spread of COVID-19. Thus far, WHO has listed four VoCs: Alpha (B.1.1.7), Beta (B.1.351), Gamma (P.1), and Delta (B.1.617.2), and five Variants of Interest (VoIs): Eta (B.1.525), Iota (B.1.526), Kappa (B.1.617.1), Lambda (C.37), and Mu (B.1.621) [1]. In addition to increased transmission, the VoCs are known to reduce the in vitro neutralizing capability of currently available vaccines through mutations on the spike protein, especially in the receptor binding domain (RBD) [2-4]. These in vitro data have been shown to have a tangible impact on public health with reports of diminished vaccine efficacy, particularly among those infected with the Beta and Delta variants [5-8]. Rather than developing new vaccines for each variant or trying to create a universal SARS-CoV-2 vaccine, booster vaccines may be the most effective way to protect against these variants [9-11]

Medigen’s MVC-COV1901 is a subunit vaccine based on a stabilized pre-fusion S-2P protein adjuvanted with CpG 1018 and aluminum hydroxide [12]. This vaccine has been shown to be safe and highly immunogenic in both hamster challenge studies and clinical trials [13-15], and has been approved for emergency use in Taiwan. The vaccine is given intramuscularly as two doses separated by four weeks [16]. We have previously shown that two doses induce neutralizing antibodies against SARS-CoV-2 variants with a tendency of higher immunogenicity at higher dose levels [17]. We have also found that a third dose of this vaccine administered to rats increased neutralizing antibody titers against the Beta variant compared to just two doses [17]. For the current study we expanded on our previous findings to investigate the immunogenicity of third dose booster against VoCs.
Results

Induction of neutralizing antibodies against VoCs in hamsters immunized with two doses of S-2P derived from original MVC-COV1901, Beta variant, or a bivalent combination of both.

We have previously shown that there was approximately a 7-fold reduction in the neutralizing antibody titer against the Beta variant in the sera of individuals who received two doses of MVC-COV1901 during a phase I trial [20]. We subsequently developed a Beta variant version of S-2P produced by a stable CHO cell clone and adjuvanted with 750 μg of CpG 1018 and 375 μg of aluminum hydroxide. We have previously established that two doses using one-fifth the amount of either low dose or high dose of MVC-COV1901 were sufficient to protect hamsters from SARS-CoV-2 infection [13]. Since the Delta variant has become prevalent worldwide, we investigated the protective effect of MVC-COV1901 derived from Wuhan wildtype (W), its Beta variant version (B) of S-2P, and the wildtype/Beta S-2P bivalent vaccine in hamsters challenged with the Delta variant. We first examined the neutralizing antibody titers from hamsters immunized with two doses of 1 μg wildtype S-2P adjuvanted with 150 μg CpG 1018 and 75 μg aluminum hydroxide (Group A shown as W + W). As shown in Figure 2, at five weeks after the second injection, Group A hamsters showed a reciprocal 50% neutralizing antibody titer (NT₅₀) GMT of 2201, 581, 166, 193, and 742 against the wildtype, Alpha, Beta, Gamma, and Delta variants, respectively. Compared to the neutralizing titer against the wildtype, those against the Alpha, Beta, Gamma, and Delta variants showed a 3.79-, 13.30-, 11.39-, and 2.97-fold reduction, respectively. This demonstrated that two doses of S-2P derived from wildtype was relatively effective against the Alpha and Delta variants. However, the effectiveness was significantly reduced against the Beta and Gamma variants.

At the same time, we examined the neutralizing antibody titers from hamsters immunized with two doses of 1 μg of the Beta variant version of S-2P combined with 150 μg CpG 1018 and 75 μg aluminum hydroxide - Group B (B + B). Figure 2 shows that two doses of the adjuvanted Beta variant S-2P induced a satisfactory NT₅₀ GMT of 681 and 417 against the wildtype and Beta variant, respectively. However, the neutralizing titers of this regimen was less than desirable against the Alpha, Gamma, and Delta variants that were 181, 219 and 182, respectively.
We also explored the neutralizing antibody responses of bivalent vaccine (wildtype + Beta variant) in Group C hamsters [shown as (W + B) + (W + B)]. The bivalent vaccine induced a similar degree of neutralizing antibody titers against the wildtype, Alpha, and Delta variants to that of the W+W group. This combination fared better against the Beta and Gamma variants than that of the W+W group; however, neutralization titer against these variants in several individual hamsters were less than 200.

Induction of neutralizing antibodies against VoCs in hamsters immunized with two doses of MVC-COV19 combined with a third dose of MVC-COV1901 or its Beta variant version of S-2P.

We previously found that neutralizing antibody titers against the Beta variant increased substantially in rats that had received three rather than two doses of MVC-COV1901 [17]. We therefore immunized hamsters with a third dose of one-fifth amount of MVC-COV1901, referred to as Group D (W + W + W), and examined the antibody responses against VoCs. As shown in Figure 2, five weeks after the third dose in Group D hamsters, NT₅₀ GMTs were 4302, 1217, 281, 377, and 1368 against the wildtype, Alpha, Beta, Gamma, and Delta variants, respectively. The neutralizing titers against the Alpha, Beta, Gamma, and Delta variants had a 3.54-, 15.30-, 11.41- and 3.14-fold decrease, respectively, compared to that of the wildtype. Compared to Group A hamsters (which only received two doses), the neutralizing antibody titers in Group D hamsters against VoCs increased substantially with the additional third dose. The third dose not only increased the neutralizing antibody titers against the Delta variant but also boosted neutralizing antibody titers against the Beta and Gamma variants compared to the W+W group.

We also explored the possibility of using the Beta variant version of S-2P adjuvanted with CpG 1018 and aluminum hydroxide as the third dose in Group E (W + W + B). As shown in Figure 2, at five weeks after the third injection of Group E hamsters, NT₅₀ GMTs were 6643, 1889, 1034, 1306, and 3595 against the wildtype, Alpha, Beta, Gamma, and Delta variants, respectively. Compared to the neutralizing titer against the wildtype, that against the Alpha, Beta, Gamma, and Delta variants had a 3.52-, 6.42-, 5.09- and 1.85-fold reduction, respectively. Two doses of wildtype S-2P combined with CpG and aluminum hydroxide followed by third dose of the adjuvanted Beta variant S-2P induced the best neutralization effect against the wildtype.
and all of the VoCs tested when compared to the other groups, especially against the Delta variant. The dosing
regimens in Groups A to E resulted in a 1.9-3.8-fold lower NT$_{50}$ GMT against the Delta variant than against
the wildtype; however, the NT$_{50}$ titers against the wildtype were different in each group. Unexpectedly, the
Beta variant of S-2P appears to be the most suitable candidate for the third dose booster.

Pseudovirus neutralization assays were performed with lentivirus pseudo-typed with spike protein from
the wildtype, Alpha, Beta, Delta (B.1.617.2), Delta plus (AY.1), Lambda, and Mu variants. Similar to the
results of live neutralization assay, the highest levels of neutralizing antibody titers against the wildtype and
variants were all found in the group receiving two doses of wildtype S-2P followed by one dose of the Beta
variant S-2P (Group E - Figure 3). All groups immunized with S-2P produced high levels of neutralizing
antibody against pseudoviruses of wildtype, Alpha, and Beta variants (Groups A to E). However, only
hamsters receiving two doses of wildtype S-2P followed by one dose of Beta variant S-2P (Group E) produced
high levels of neutralizing antibody against Beta, Delta, Delta plus, and Mu variant pseudoviruses. In contrast,
hamsters received two doses of S-2P (Groups A to C) had lower neutralizing antibody levels against the Beta,
Delta, and Mu variant pseudoviruses, whereas Group B produced the lowest level of antibodies against the
Delta variant pseudovirus (Figure 3). The pseudovirus neutralization assays were consistent with the live virus
neutralization assays that showed the administration of two doses of wildtype S-2P followed by one dose of
Beta variant S-2P provided the broadest spectrum of protection against wild-type SARS-CoV-2 and different
variants, Alpha, Beta, Delta, Lambda, and Mu variants.

Protection from the Delta variant challenge in hamsters immunized with two doses of MVC-COV1901
or combined with a third dose of MVC-COV1901 or its Beta variant version of S-2P.

Eight weeks after completion of the last immunization, hamsters were challenged with 10$^4$ PFU of the
Delta variant and body weights were monitored up to six days post infection (d.p.i.). All the vaccinated groups
showed no significant weight loss six days after virus challenge in contrast to the adjuvant control group that
showed a steady decline in weight loss during this period (Figure 4). The protection in all vaccinated hamster
groups was significant (p < 0.0001) at 6 d.p.i. as compared to the weight loss seen in the adjuvant control
group.
Lung viral load was measured by viral RNA and 50% tissue culture infectious dose (TCID\textsubscript{50}) assays. Figure 5A shows that at 3 d.p.i., lung viral RNA in Groups A to E hamsters were lower than in that of the adjuvant control group, but only in Group E was the lung viral RNA significantly lower than that in the adjuvant control group (p < 0.01), while by 6 d.p.i., the viral RNA in all groups were significantly (p < 0.05) lower than that of the control. In contrast, the viral titers in all of the vaccinated hamsters measured by TCID\textsubscript{50} were significantly lower (p < 0.05) than that of the adjuvant control group at 3 d.p.i. (Figure 5B). Note that the lung viral load in hamsters, both viral RNA and especially viral titer as measured by TCID\textsubscript{50}, dropped considerably at 6 d.p.i. in the negative control and adjuvant-only control groups likely due to hamsters’ natural immune response against the virus (Figure 5B). We also found a strong negative correlation (Spearman $r_s = -0.8227$) between NT\textsubscript{50} titer against the Delta variant from serum sampled five weeks after the final immunization and the number of viral genome at 3 d.p.i. (Figure 6). To examine the lung histopathology of the hamsters, lung necropsy sections were analyzed, and pathology scoring was tabulated at 3 d.p.i. or 6 d.p.i. (Figure 7). There were no differences at 3 d.p.i. between control and experimental groups; however, at 6 d.p.i., the adjuvant control group had significantly (p < 0.01) increased lung pathology including extensive and severe immune cell infiltration, hemorrhage, and diffuse alveolar damage, compared to groups receiving three doses of S-2P (Groups D and E).

**Discussion**

This study is our second hamster SARS-CoV-2 vaccination and challenge study. In the first study we showed that vaccination with both low and high dose of S-2P were effective against live SARS-CoV-2 virus challenge [13]. In the current study we extended our concept to examine the effect of a variant-based booster vaccinations followed by challenge with the Delta variant. Since Delta has emerged as one of the more infectious and dominant variant globally, we chose it as our model virus for challenge infection [18]. The results of the immunogenicity studies showed that immunization with either two doses of wildtype S-2P, or two doses of the Beta variant S-2P could not confer broad immunogenicity against all the variants tested. Wildtype S-2P was ineffective against the Beta and Gamma variants, whereas Beta variant S-2P induced
higher neutralizing titers against only the Beta variant (Figure 2). A bivalent mixture of both wildtype and
Beta variant S-2Ps showed results similar to immunization with two doses of wildtype S-2P, but with slightly
increased immunogenicity against the Beta and Gamma variants. Three doses of wildtype S-2P was able to
boost the titers against both Beta and Gamma more than the bivalent vaccine and also increased the
neutralization titers against the Alpha and Delta variants as well. Unexpectedly, our study showed that using
the Beta variant S-2P as a third dose booster, induced the highest and broadest spectrum of neutralizing titers
against all variants as well as the wildtype. Pseudovirus neutralization assay also confirmed that the above
combination could also induce high levels of neutralizing titer to the Lambda and Mu variants as well (Figure
3).

A recent study investigated the neutralization ability of convalescent and BNT162b2 vaccinated sera
against pseudoviruses bearing variant spike proteins [19]. The Mu variant pseudovirus was the most refractory
to neutralization by either types of sera, even more resistant to neutralization than that of the Beta variant [19].
We found similar results with two doses of wildtype S-P (Group A) where the Mu variant pseudovirus had the
lowest GMT compared to other variants in the group, and the GMTs of other groups all remained low except
in Group E, where the Beta variant S-P was used as the third dose (Figure 3). It is of interest to note the ratio
between the NT50 of the wildtype and Alpha variant remain relatively constant ranging from 3.52 to 3.79, and
similar trend was observed for pseudovirus neutralization assay as well (Figures 2 and 3).

Comparing neutralization titers against different viruses is complicated, as the assays used are inherently
dissimilar. Nevertheless, our immunogenicity results demonstrated a clear correlation between neutralization
titers and lung viral clearance in the hamsters (Figure 5).

All five regimens of vaccination protected hamsters from weight loss induced by infection with the Delta
variant (Figure 4). Notably, while group B had a relatively poor antibody response against the Delta variant,
this group did not experience any weight loss or increase in lung pathology (Figures 2, 5, and 7). In addition,
the viral titers for the Delta variant in Group B were significantly lower than those of the adjuvant control,
suggesting that the amount of anti-Delta antibodies and/or T cell immune responses induced by two doses of
the Beta variant S-2P could have reduced viral replication in the lungs and protected the hamsters from weight
loss and lung pathology (Figures 5 and 7). T cell immunity could also have played a role in providing protection against SARS-CoV-2 infection and viral clearance, in concert with humoral immunity in both vaccine- or infection-induced immunity [20-23]. Previous studies have shown that the memory T cell pool from prior infection or vaccination can be activated upon encountering heterologous virus if cross-reactive epitopes are shared between the two viruses [24]. The broad neutralizing ability of immunizing with the wildtype followed by Beta variant S-2P booster could also have been induced by cross-reactivity of memory B cells and T cells. This is similar to the concept of the original antigenic sin, in which previous exposure to a virus can cause memory cell responses to preferably secrete antibodies against the first virus after exposure to a similar virus strain due to shared epitopes [24]. Cross-reactivity of T cells have also been noted for rapid induction of immunity following infection or immunization with SARS-CoV-2 [19]. Since neutralizing antibodies induced by vaccines are polyclonal, they could also be cross-reactive with shared epitopes between different variants. Polyclonal antibodies induced by SARS-CoV-2 spike mRNA vaccine were profiled and were found to consist of a mixture of antibodies targeting the N-terminal domain (NTD) and the RBD, and they differ in their binding and neutralizing abilities [25, 26]. The re-stimulation of immunity may explain the low viral RNA titer in hamsters immunized in our study with either of the three-dose regimens. Further, the TCID$_{50}$ live virus titers in all groups were very low and almost undetectable in most instances (Figure 5). This may be due to the sensitivity of the TCID$_{50}$ assay itself, or the viral RNA assay may be detecting fragments of viral RNA from dead viruses as opposed to live replicating viruses. In future studies, subgenomic RNA detection should also be used to detect replicating viruses to corroborate the TCID$_{50}$ results. The establishment of correlates of protection using the relationship between NT$_{50}$ titer and viral RNA in a given hamster challenge model will help facilitate the expedited evaluation of vaccine combinations in future studies.

One limitation of this study is that we have not tested the vaccine’s protection in vivo with other VoCs besides the Delta variant; the vaccine efficacy against other VoCs is inferred from the neutralizing antibody titers. The natural course of infection among the hamsters includes a convalescent state, so the model does not allow for evaluating mortality or severe disease as endpoints. T-cell functions were also not evaluated in the hamsters in this study, limiting our ability to assess the role of cellular immunity in providing protection. Two
recent studies investigated the effects of a booster dose of ChAdOx1 and mRNA-1273 [9, 27]. Administration of a third dose using the Beta variant version of mRNA-1273 (mRNA-1273.351) following two doses of mRNA-1273 increased immunogenicity against the Beta variant more than did three doses of mRNA-1273. The administration of either mRNA-1273 or mRNA1273-351 as third dose exponentially boosted immunogenicity against all variants tested compared to two doses of mRNA1273 [9]. Concerning similar studies with ChAdOx1 vaccine, the third dose boosted neutralization titers against the Beta and Delta variants as well as gamma-interferon levels [27]. These findings are similar to our results that showed a third dose of vaccination could boost an immune response against the virus as well as its variants. Our study also showed that a 3rd booster dose with both homologous (and especially with a heterologous Beta variant S-2P), increased immunogenicity against all the VoCs tested. Findings from this study provide evidence to support the further evaluation of both the original and a Beta variant S-2P vaccine as a booster dose for individuals fully vaccinated with MVC-COV1901 as well as other approved vaccines.

**Methods**

**Animals and ethical statements**

Female golden Syrian hamsters aged 8-10 weeks at study initiation were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Animal immunizations were conducted in the Testing Facility for Biological Safety, TFBS Bioscience Inc., Taiwan. At three weeks following the final immunization, the animals were transferred to Academia Sinica, Taiwan, for SARS-CoV-2 challenge. All procedures in this study involving animals were conducted in a manner to avoid or minimize discomfort, distress, or pain to the animals and were carried out in compliance with the ARRIVE guidelines (https://arriveguidelines.org/). All animal work in the current study was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) with animal study protocol approval number TFBS2020-019 and Academia Sinica (approval number: 20-06-1483).

**Immunization and challenge of hamsters**
The study design is outlined in Figure 1. The hamsters were split into the following six groups with $n =$ 10 for each group (Table 1):

<table>
<thead>
<tr>
<th>Groups</th>
<th>First immunization</th>
<th>Second immunization</th>
<th>Third immunization</th>
</tr>
</thead>
<tbody>
<tr>
<td>A: W + W</td>
<td>Wildtype (1 $\mu$g)</td>
<td>Wildtype (1 $\mu$g)</td>
<td>-</td>
</tr>
<tr>
<td>B: B + B</td>
<td>Beta variant (1 $\mu$g)</td>
<td>Beta variant (1 $\mu$g)</td>
<td>-</td>
</tr>
<tr>
<td>C: (W + B) + (W + B)</td>
<td>Wildtype (0.5$\mu$g) and Beta variant (0.5$\mu$g) bivalent</td>
<td>Wildtype (0.5$\mu$g) and Beta variant (0.5$\mu$g) bivalent</td>
<td>-</td>
</tr>
<tr>
<td>D: W + W + W</td>
<td>Wildtype (1 $\mu$g)</td>
<td>Wildtype (1 $\mu$g)</td>
<td>Wildtype (1 $\mu$g)</td>
</tr>
<tr>
<td>E: W + W + B</td>
<td>Wildtype (1 $\mu$g)</td>
<td>Wildtype (1 $\mu$g)</td>
<td>Beta variant (1 $\mu$g)</td>
</tr>
<tr>
<td>F: Adjuvant control</td>
<td>CpG 1018 (150 $\mu$g) and aluminum hydroxide (75 $\mu$g)</td>
<td>CpG 1018 (150 $\mu$g) and aluminum hydroxide (75 $\mu$g)</td>
<td>CpG 1018 (150 $\mu$g) and aluminum hydroxide (75 $\mu$g)</td>
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Hamsters in group A were vaccinated on days 22 and 43 with 1 $\mu$g of S-2P protein derived from the wildtype. Hamsters in group B were vaccinated on days 22 and 43 with 1 $\mu$g of S-2P protein derived from Beta variant. Hamsters in group C were vaccinated on days 22 and 43 with a mixture of the wildtype (0.5 $\mu$g) and Beta variant (0.5 $\mu$g) of S-2P protein (bivalent vaccine). Hamsters in group D were vaccinated on days 1, 22, and 43 with 1 $\mu$g of S-2P protein derived from the wildtype. Hamsters in group E were vaccinated on days 1 and 22 with 1 $\mu$g of wildtype S-2P protein, and on day 43 with 1 $\mu$g of S-2P protein derived from the Beta variant. Hamsters in group F served as an adjuvant control and were vaccinated with only 150 $\mu$g of CpG 1018 and 75 $\mu$g of aluminum hydroxide (alum) on days 1, 22 and 43. All immunization with S-2P were adjuvanted with 150 $\mu$g of CpG 1018 and 75 $\mu$g of alum. Serum samples were collected five weeks after the final immunization and immunogenicity was determined by neutralization assay with SARS-CoV-2 virus and the variants. Approximately three weeks after the serum sampling (53 days after the final immunization),
hamsters were challenged with the SARS-CoV-2 Delta variant (TCDC#1144) and then sacrificed at 3 d.p.i. (n = 5 per group) or 6 d.p.i. (n = 5 per group) for analyses of lung viral loads, lung TCID\textsubscript{50}. Body weight of individual hamsters were tracked daily up to the time of sacrifice. Necropsy were performed with lungs of euthanized hamster and histopathology sectioning, staining, and scoring were done as described previously [13].

**Live SARS\textsuperscript{2}CoV\textsuperscript{2} and pseudovirus neutralization assay**

SARS-CoV-2 virus strains (Wuhan wildtype, Alpha, Beta, Gamma, and Delta variants) were used in live virus neutralization assay as described previously [18]. Pseudovirus with lentivirus pseudotyped with S proteins of the wildtype, Alpha (B.1.1.7), Beta (B.1.351), Delta (B.1.617.2), Delta plus (AY.1), Lambda (C.37), and Mu (B.1.621) variants were conducted as previously described [12].

**Viral RNA quantification and cell culture infectious assay (TCID\textsubscript{50})**

Quantification of lung viral load by real-time PCR and TCID\textsubscript{50} assay were performed as previously reported [13].

**Statistical analysis**

The analysis package in Prism 6.01 (GraphPad) was used for statistical analysis. Spearman’s rank correlation coefficient and linear regression were calculated for Figure 5. Kruskal-Wallis with corrected Dunn’s multiple comparisons test and two-way ANOVA with Dunnett test for multiple comparison were used to calculate significance in Figures 2 to 4 where appropriate. * = p < 0.05, ** = p < 0.01, *** = p < 0.001, **** = p < 0.0001

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Level 3 Facility and for providing the environment needed for handling and performing the SARS-CoV-2 hamster challenge and live SARS-CoV-2 neutralization assays. We also thank Michael D. Malison and Han van den Bosch for reviewing and providing helpful inputs with the manuscript.

**Author Contributions**

T.-Y. K., C.-C. W., and W.-H. T. produced the wildtype and Beta variant versions of S-2P antigens used in the study. T.-Y. K., C.-E. L., Y.-J. L., M.-Y. L., C.-C. W., W.-H. T., J. D. C., P. T., Y.-S. C., and C. C. designed the study and experiments. Y.-J. L. and Y.-S. C. supervised the experiments at TFBS Bioscience and Academia Sinica. Y.-J. L., M.-Y.-L., Y.-S. C., and L. T.-C. L. analyzed the results. M.-Y. L., Y.-S. C., and L. T.-C. L. drafted the manuscript. All authors reviewed and approved of the final version of the manuscript.

**Competing Interests**


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**Figures**

Figure 1. Study design of the hamster challenge study.

Hamsters (N=10 per group) were immunized twice (groups A, B, and C) or three times (groups D, E, and F) at three weeks apart and serum samples were taken for immunogenicity assays five weeks after the final immunization. Eight weeks after the final immunization, hamsters were challenged with $10^4$ PFU of SARS-CoV-2 Delta variant. The animals were euthanized on the third or sixth day after infection for necropsy and tissue sampling to determine viral load. Body weight of individual hamster were tracked daily up to the time of sacrifice.
Figure 2. Neutralizing antibody titers with live SARS-CoV-2 neutralization assay in hamsters five weeks after the final immunization. Hamsters were immunized as in Figure 1. Five weeks after the final immunization (second immunization for groups A, B, and C; third immunization for groups D, E, and F), serum samples were taken for neutralization assays against live SARS-CoV-2 ancestral strain and Alpha, Beta, Gamma, and Delta variants. Results are shown as bars indicating the NT_{50} GMT with individual values displayed as symbols and error bars showing the 95% confidence intervals. Average fold reduction in GMT of variants against the ancestral strain were calculated and shown above brackets above the corresponding bars. W: Wildtype S-2P; B: Beta variant S-2P; W + B: bivalent mixture of wildtype and Beta variant S-2Ps. Statistical significance was calculated with Kruskal-Wallis test with corrected Dunn’s multiple comparisons test.
Figure 3. Neutralizing antibody titers with pseudovirus neutralization assay in hamsters five weeks after the final immunization. Hamsters were immunized and serum samples taken as in Figure 2. The samples were tested against lentivirus pseudotyped with the spike proteins of SARS-CoV-2 wildtype, Alpha, Beta, Delta (B.1.617.2), Delta plus (AY.1), Lambda, and Mu variants. Results are shown as bars indicating the NT$_{50}$ GMT with individual values displayed as symbols and error bars showing the 95% confidence intervals. Average fold reduction in GMT of variants against the ancestral strain were calculated and shown above brackets above the corresponding bars. Statistical significance was calculated with Kruskal-Wallis with corrected Dunn’s multiple comparisons test.
Figure 4. Change in body weight in hamsters after infection with SARS-CoV-2 Delta variant.

Hamsters were challenged with SARS-CoV-2 Delta variant eight weeks after the final immunization. The body weights of individual hamsters were tracked daily up to the time of euthanizing at six days post infection (n = 5/group). Results are shown as percent of weight relative to the day of challenge (day 0). Statistical significance was calculated with two-way ANOVA with Dunnett multiple comparison test with adjuvant only as a control.

Figure 5. Viral load in hamsters three or six days post infection with SARS-CoV-2 Delta variant.

The hamsters were euthanized at three or six days (n = 5/group) after infection and lung tissue samples were collected for viral load determination by “A” quantitative PCR of viral genome RNA, and “B” TCID₅₀ assay for virus titer. Results are presented as geometric mean values with error bars representing 95% confidence intervals. Statistical significance was calculated with Kruskal-Wallis corrected Dunn’s multiple comparisons test.
Figure 6. Correlation between SARS-CoV-2 viral genome copy numbers and NT<sub>50</sub> titers against the Delta variant. Values of viral genome copy numbers 3 days post infection and NT50 titers against the Delta variant five days after the final immunization were tabulated (n = 29). Spearman’s rank correlation coefficient and linear regression were calculated with dotted bands and shaded area representing the 95% confidence bands of the linear regression line.

Figure 7. Lung histopathology scoring in hamsters at 3 or 6 days post infection with SARS-CoV-2 Delta variant. The hamsters were challenged with SARS-CoV-2 Delta variant and euthanized at three or six days after infection as in Figure 5. Lung sections were prepared and stained and histopathology scores were calculated. Results are presented as mean with error bars representing standard deviation. Statistical significance was calculated with Kruskal-Wallis corrected Dunn’s multiple comparisons test.
Groups A, B, and C (n=10/group)

**Prime** Day 1

**Boost** Day 22

**Bleed** Day 57

**Challenge** Day 75
SARS-CoV-2 Delta variant
1 x 10^4 PFU, 50 μl intranasally

**Sacrifice** 3 dpi (n=5/group) or 6 dpi (n=5/group)
Day 78 or 81

**Neutralizing antibody titer**

Viral load: 3 and 6 dpi
Body weight: 0 to 6 dpi

Groups D, E, and F (n=10/group)

**Prime** Day 1

**Boost** Day 22

**2nd Boost** Day 43

**Bleed** Day 78

**Challenge** Day 96
SARS-CoV-2 Delta variant
1 x 10^4 PFU, 50 μl intranasally

**Sacrifice** 3 dpi (n=5/group) or 6 dpi (n=5/group)
Day 99 or 102

**Neutralizing antibody titer**

Viral load: 3 and 6 dpi
Body weight: 0 to 6 dpi
<table>
<thead>
<tr>
<th>Comparison</th>
<th>Mu vs Wildtype</th>
<th>Lambda vs Wildtype</th>
<th>Delta plus vs Wildtype</th>
<th>Delta vs Wildtype</th>
<th>Beta vs Wildtype</th>
<th>Alpha vs Wildtype</th>
</tr>
</thead>
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<tr>
<td></td>
<td>2.21</td>
<td>2.48</td>
<td>6.52</td>
<td>9.83</td>
<td>3.84</td>
<td>1.22</td>
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<td>1.06</td>
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<td>1.90</td>
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<td>16.82</td>
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<td></td>
<td></td>
<td>4.00</td>
<td>1.51</td>
</tr>
<tr>
<td></td>
<td>6.50</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

### Average NT fold reduction over wildtype

<table>
<thead>
<tr>
<th>Comparison</th>
<th>A: W × W</th>
<th>B: B × B</th>
<th>C: (W × B) + (W × B)</th>
<th>D: W × W × W</th>
<th>E: W × W × B</th>
<th>F: Adjuvant control</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
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<td></td>
<td></td>
<td>14.23</td>
<td>9.48</td>
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<tr>
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<td></td>
<td>8.16</td>
<td>16.82</td>
<td>6.50</td>
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<td></td>
</tr>
</tbody>
</table>

### ID₅₀ (GMT with ±95% CI)

- **Wildtype**: Blue dots
- **Alpha**: Red squares
- **Beta**: Green triangles
- **Delta**: Orange diamonds
- **Delta plus (AY.1)**: Purple pentagons
- **Lambda**: Yellow circles
- **Mu**: Brown diamonds

Comparison details:

- **Mu vs Wildtype**: 2.21
- **Lambda vs Wildtype**: 8.87
- **Delta plus vs Wildtype**: 10.58
- **Delta vs Wildtype**: 7.79
- **Beta vs Wildtype**: 3.84
- **Alpha vs Wildtype**: 1.22

Significance:

- **Mu vs Wildtype**: *p < 0.05
- **Lambda vs Wildtype**: **p < 0.01
- **Delta plus vs Wildtype**: *p < 0.05
- **Delta vs Wildtype**: *p < 0.05
- **Beta vs Wildtype**: **p < 0.01
- **Alpha vs Wildtype**: *p < 0.05

Note: The table and graph provide a comprehensive view of the fold reduction over wildtype for different comparisons, with significant differences indicated by asterisks.
Spearman $r_s = -0.8227$

$P < 0.0001$

$y = -0.884x + 13.18$

$R^2 = 0.7226$