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Highlights
- Inactivated vaccine based on scalable bioreactor and steric exclusion chromatography
- Persisting neutralizing antibodies after three immunizations in mice
- Variant cross-neutralizing antibodies after two immunizations in hamsters
- Prevention of disease and lung pathology in vaccinated hamsters after challenge
An inactivated SARS-CoV-2 vaccine induced cross-neutralizing persisting antibodies and protected against challenge in small animals

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SUMMARY
Vaccines have relieved the public health burden of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), and globally inactivated vaccines are most widely used. However, poor vaccination accessibility and waning immunity maintain the pandemic, driving emergence of variants. We developed an inactivated SARS-CoV-2 (I-SARS-CoV-2) vaccine based on a viral isolate with the Spike mutation D614G, produced in Vero cells in a scalable bioreactor, inactivated with β-propiolactone, purified by membrane-based steric exclusion chromatography, and adjuvanted with MF59-like adjuvant AddaVax. I-SARS-CoV-2 and a derived split vaccine induced persisting neutralizing antibodies in mice; moreover, lyophilized antigen was immunogenic. Following homologous challenge, I-SARS-CoV-2 immunized hamsters were protected against disease and lung pathology. In contrast with reports for widely used vaccines, hamster plasma similarly neutralized the homologous and the Delta (B.1.617.2) variant viruses, whereas the Omicron (B.1.1.529) variant was neutralized less efficiently. Applied bioprocessing approaches offer advantages regarding scalability and production, potentially benefiting worldwide vaccine coverage.

INTRODUCTION
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the etiological agent of coronavirus disease 2019 (COVID-19), was first detected in China in 2019 but quickly became a global concern.1–3 The ongoing pandemic has had substantial societal and economic impact,1 with more than 580 million confirmed infections and more than 6.4 million deaths as of early August 2022.5 The course of infection may be asymptomatic, or symptoms may develop ranging from mild to severe respiratory or systemic disease and death.6–8 SARS-CoV-2 is an enveloped positive sense single stranded RNA virus with the structural Spike (S), Membrane (M), and Envelope (E) proteins embedded in the virus envelope, as well as the Nucleocapsid (N) protein encapsulating the virus genome.9 Different strategies were pursued for development of COVID-19 vaccines, including new technologies not previously used in vaccines licensed for human use, particularly focusing on eliciting anti-S immune responses.10 With accelerated emergency approval schemes from regulatory agencies, several vaccines have been licensed in different countries including mRNA, inactivated virus, viral vector, and protein-based products.10,11 Although the mRNA vaccines are highly effective and the BNT162b2 vaccine (Pfizer/BioNTech) was reported to induce higher antibody titers and protection levels than the ChAdOx1 nCoV-19 viral vector vaccine (AstraZeneca) and the CoronaVac inactivated vaccine (Sinovac), they all confer significant protection from severe disease and death.12–14 However, the use of the ChAdOx1 nCoV-19 vaccine was stopped in several countries because of reported cases of vaccine-induced immune thrombocytopenia.15 In general, waning SARS-CoV-2 immunity and protection levels have been described and constitute a concern for all currently licensed vaccines.16–20

Worldwide vaccine supply remains a major concern.21,22 To cover the global need of vaccine doses, it is highly relevant to employ vaccines based on different technologies, and different types of vaccines may
be suitable for different target groups.\textsuperscript{21,23,24} Inactivated vaccines represent a well-known technology and are relatively cheap and easy to produce. Furthermore, these vaccines can be stored at 2–8°C, as opposed to mRNA vaccines which generally require frozen storage, thus complicating vaccine storage and distribution.\textsuperscript{10} The inactivated SARS-CoV-2 vaccines CoronaVac (Sinovac)\textsuperscript{25,26} and BBIBP-CorV (Sinopharm)\textsuperscript{27} have been widely distributed globally. \textsuperscript{28} Among other inactivated vaccines, BBV152 (Bharat Biotech) is the most widely used,\textsuperscript{29} and the inactivated vaccine candidate VLA2001 (Valneva Austria Gmb H) recently received authorization by the European Medical Agency.\textsuperscript{30}

Many developing countries still have low vaccination coverage largely because of poor vaccine supply, high costs, and logistic challenges.\textsuperscript{22,31} Although vaccine inequity is an ethical concern, continued high transmission rates additionally promote emergence of new variants, which may have global impact.\textsuperscript{22,32,33} Since late 2020, the World Health Organization (WHO) has listed several variants of concern.\textsuperscript{34} Some of these variants display enhanced transmissibility, such as the initially emerging Alpha (B.1.1.7) variant,\textsuperscript{35} and subsequently the Delta (B.1.617.2) variant,\textsuperscript{36} which spread to become the dominant variant worldwide in 2021.\textsuperscript{37} The Omicron (B.1.1.529) variant was declared a variant of concern by the end of November 2021,\textsuperscript{38} and quickly spread globally, followed by emergence of Omicron subvariants.\textsuperscript{39} These variants with relatively high numbers of mutations show further increased transmission rates and challenges immune responses induced by vaccines and previous infections by neutralization escape.\textsuperscript{40,41}

Our work aimed at developing an inactivated SARS-CoV-2 vaccine and was initiated at the beginning of the pandemic. As a first step, virus production was established in the WHO Vero cell line in a scalable packed-bed bioreactor to facilitate production of large vaccine quantities.\textsuperscript{42} The current study focused on inactivation of bioreactor-derived SARS-CoV-2 and capture purification by membrane-based steric exclusion chromatography (SXC), a fast, single-use, low cost, and scalable purification technology. Successful purification with this technology has previously been reported for a range of other viruses, including influenza A virus, hepatitis C virus, and adeno-associated virus.\textsuperscript{43-46} Moreover, using an analogue of a licensed adjuvant, we investigated vaccine immunogenicity in mice and hamsters and its capacity to protect hamsters from disease and lung pathology. Furthermore, we evaluated vaccine immunogenicity following virus particle lyophilization and splitting, approaches which could have implications for vaccine stability and safety. Finally, we evaluated longevity of induced neutralizing antibodies (NAb) in mice in a follow up of seven months after the final immunization, and the capacity of vaccine induced NAb from hamsters to neutralize Delta and Omicron variants.

RESULTS
The inactivated SARS-CoV-2 vaccine
To prepare the inactivated SARS-CoV-2 (i-SARS-CoV-2) vaccine candidate, an original D614G virus\textsuperscript{47} produced in a single-use bioreactor\textsuperscript{42} was inactivated with β-propiolactone (BPL), filtered, subjected to DNA digestion, and purified by membrane-based SXC (Figure 1A). SARS-CoV-2 particles were eluted in two separate elution steps with phosphate buffered saline (PBS) and PBS with 0.5 M NaCl, respectively, with subsequent product dialysis and characterization by several analytical techniques (Figures 1B–1F). Impurities were efficiently removed by the SXC step; the total protein and host cell DNA clearance in the SXC eluates were 94.6% ± 0.3 and 93.4% ± 0.4, respectively, and host cell DNA clearance was enhanced to
Neutralizing antibody titers in mice were enhanced by an increased I-SARS-CoV-2 dose

To improve antibody responses, a schedule of three immunizations was evaluated with two different I-SARS-CoV-2 doses as well as a split vaccine approach. Moreover, immunizations were administered with three-week intervals to enhance induction of NAb. 50 Groups of mice were immunized with I-SARS-CoV-2 using a low (0.1 µg S1) or a high (0.5 µg S1) dose, or with 100 µg OVA, all adjuvanted with AddaVax (Figure 3A). A split vaccine approach was attempted using both the low and high dose, where the antigen was incubated with detergent to solubilize the viral particle. Antigen splitting is commonly used for influenza vaccines and may pose advantages regarding vaccine safety. 58,49 S1S2 specific antibodies were detected already after the first immunization with lyophilized I-SARS-CoV-2 and were considerably increased by the second immunization reaching endpoint titers of 3.9 and 3.1 log₁₀, respectively (Figure 2B). N specific antibodies were also robustly induced after the second immunization reaching a titer of 3.2 log₁₀. NAb were induced by two immunizations with lyophilized I-SARS-CoV-2 with a mean 50% neutralization titer (NT50) of 78 (Figure 2C). Although the lyophlized I-SARS-CoV-2 antigen was immunogenic, further ex-NAb were induced by two immunizations with lyophilized I-SARS-CoV-2 with a mean 50% neutralization titer of N specific antibodies required at least two immunizations (Figure 3B). Overall, the highest NT50 values were reached in all I-SARS-CoV-2 groups. Similarly, after three immunizations all I-SARS-CoV-2 groups except the low dose group had RBD specific antibody titers of 3.2 log₁₀ (Figure 3B). Induction of N specific antibodies required at least two immunizations (Figure 3B). In all I-SARS-CoV-2 groups robust NAb responses were induced following two immunizations, whereas the third immunization only slightly increased NT50 values (Figure 3C). Overall, the highest NT50 values were

Immunogenicity of lyophilized antigen in mice

Lyophilization of vaccine antigens may enhance product stability. 58,49 Thus, a first small batch of inactivated and purified SARS-CoV-2 was subjected to lyophilization. In an initial immunogenicity experiment, mice were immunized with lyophilized I-SARS-CoV-2 equivalent to 0.1 µg S1 or 100 µg Endofit Ovalbumin (OVA), formulated with the squalene-based emulsion adjuvant AddaVax, an analogue of MF59, which is approved for human use (Figure 2A). No adverse effects were observed following immunizations, as evaluated by clinical inspection. Serum antibodies binding S1S2 and the Spike-receptor binding domain (RBD) of S1 were detected already after one immunization with lyophilized I-SARS-CoV-2 and were considerably increased by the second immunization reaching endpoint titers of 3.9 and 3.1 log₁₀, respectively (Figure 2B). N specific antibodies were also robustly induced after the second immunization reaching a titer of 3.2 log₁₀. NAb were induced by two immunizations with lyophilized I-SARS-CoV-2 with a mean 50% neutralization titer (NT50) of 78 (Figure 2C). Although the lyophlized I-SARS-CoV-2 antigen was immunogenic, further experiments were carried out with a different batch of non-lyophlized antigen.

In analytical size exclusion chromatography (SEC) of the viral harvest before SXC, the SARS-CoV-2 particles elute from the column void first (Figure 1B, left orange peak) and other impurities elute progressively in order of decreasing hydrodynamic size (gray peaks). SEC fingerprints appeared identical before and after DNA digestion (Figure 1B). In contrast, SEC fingerprints of the SXC eluates demonstrated higher purity and virus particle concentration compared to the unpurified harvest (Figure 1C). The SEC fingerprints of SXC eluates solubilized with CHAPS showed three major unresolved peaks for eluate 1, whereas a single peak was observed for eluate 2 (Figure 1D). The two additional proteinaceous peaks detected for SXC eluate 1 which do not give rise to light scattering signals might represent or contain oligomers of S, as indicated by the detection of S in all collected SEC fractions by dot blots (Figure 1D). When analyzed by differential centrifugal sedimentation (DCS) in a sucrose gradient, both SXC eluates showed a dominant population of monodisperse virus particles of around 60 nm in hydrodynamic size without any major particles detected beyond 80 nm (Figure 1E). In analysis by negative staining transmission electron microscopy (TEM), SARS-CoV-2 particles were shown to be pleomorphic in shape (i.e., spherical, elongated, or dysmorphic) with an approximate size of 100 nm (Figure 1F). SXC eluates were pooled before use in animal experiments, and concentrations of protein and host cell DNA in the final sample were 61.2 ± 1.9 µg/mL and 153.5 ± 6.5 ng/mL, respectively. The S1 concentration of the final sample was 2.3 µg/mL. Thus, sample analysis confirmed that virus capture and purification by SXC was successful.
observed in the high dose group (mean NT50 of 508 and 583 after two and three immunizations, respectively), compared to lower NT50 values in the low dose group (NT50 of 195 and 150 after two and three immunizations, respectively).

Furthermore, in ELISpot assays, we found that splenocytes isolated from I-SARS-CoV-2 groups secreted IL-4 and IFN-γ when stimulated with the vaccine antigen (Figure S2), suggesting a Th1/Th2 type cellular response. These results were corroborated by detection of secreted IL-5, IL-10, and IFN-γ in supernatants from splenocytes stimulated with the vaccine antigen in meso scale discovery (MSD) assays (Figure S3).

Overall, increasing the antigen dose appeared to be important for enhancing NAb titers, with similar titers induced by the split and non-split antigen. Although the third immunization increased RBD and N specific antibody titers, it did not have a strong effect on NAb titers.

**Induction of a persistent neutralizing antibody response in mice**

Four of nine mice in the high and high-split dose groups (Figure 3A) were followed for seven months after the third immunization with periodical blood sampling (Figure 4A). In these mice, NAb titers persisted throughout this time with slightly higher titers in the high-split group (Figure 4B).

**One immunization prevented clinical disease in hamsters**

Hamsters are susceptible to SARS-CoV-2 and develop disease on infection. We used this animal model to evaluate the protective effect of I-SARS-CoV-2 formulated with AddaVax. Given that similar NAb responses were observed for I-SARS-CoV-2, split I-SARS-CoV-2, and lyophilized I-SARS-CoV-2, protection studies were carried out with I-SARS-CoV-2, thus avoiding an additional step in the purification and antigen preparation process. In an initial experiment, a group of hamsters was immunized once with I-SARS-CoV-2 at a dose of 1.1 μg S1 (Figure 5A). The immunization was well-tolerated, and no adverse effects were observed according to body weight and clinical inspection. On day 21 post immunization (dpi), the I-SARS-CoV-2 immunized animals and a non-immunized challenge control group were inoculated with...
100 50% tissue culture infectious dose (TCID₅₀) of homologous SARS-CoV-2 to evaluate protection from disease.

Following challenge, the non-immunized animals developed clinical disease with a progressive body weight reduction until day 5 post challenge (dpc) when they started to recover (Figure 5B). Conversely, the I-SARS-CoV-2 immunized animals were protected from clinical disease and body weight loss. Moreover, I-SARS-CoV-2 immunization reduced virus infectious titers in the upper airways. Although oral swab titers were similar on dpc 1 in I-SARS-CoV-2 immunized and non-immunized animals, with medians of 3.2 and 3.8 log₁₀ TCID₅₀/mL, respectively (Figure 5C), titers declined faster in immunized animals, with a median of 2.5 compared to 3.9 log₁₀ TCID₅₀/mL in non-immunized animals on dpc 2. Infectious virus was undetectable in both groups on dpc 6. Similarly, the decline of virus RNA titers in nasal lavage samples was accelerated in the immunized compared to the non-immunized animals (Figure 5D). The immunized animals had median titers of 4.1 log₁₀ copies/mL on dpc 6, compared to 5.3 log₁₀ copies/mL in the non-immunized animals. The virus RNA titers were undetectable in the immunized group already on dpc 7 and were undetectable in both groups on dpc 12.

To evaluate the immune response induced by immunization as well as infection, neutralizing NAb titers were determined. Before challenge, on dpc 0, plasma from the immunized animals had neutralizing activity with a mean 50% inhibitory dose (ID₅₀) of 331 (Figure 5E). In these animals, infection boosted NAb titers to an ID₅₀ of 4187 on dpc 20, whereas the non-immunized animals had an ID₅₀ of 1772 at this last timepoint. Thus, one immunization with I-SARS-CoV-2 induced moderate NAb titers, which were 5.4-fold lower than titers recorded after infection alone. Nevertheless, the induced immune responses
appeared to confer protection against disease and body weight loss on challenge, potentially mediated by a strong boosting effect of the challenge, resulting in a 12.6-fold increase in NAb titers following infection.

Protection from lung pathology and enhanced neutralizing antibody titers following two immunizations in hamsters

To evaluate if NAb titers could be enhanced and to more thoroughly evaluate protection conferred by I-SARS-CoV-2 immunization, groups of hamsters were immunized two times with either I-SARS-CoV-2 containing 1.1 μg S1 or 100 μg OVA (Figure 6A). Eight of 12 animals in each group were challenged after the second immunization on dpi 43, and half of these were euthanized on dpc 5 with the four non-challenged animals to evaluate lung pathology. The remaining challenged animals were followed until dpc 12 to monitor the course of disease.

Following challenge, the OVA immunized animals developed disease with progressive body weight reduction until dpc 7, whereas the I-SARS-CoV-2 immunized animals were protected from disease and body weight loss (Figure 6B).

Tissues from the nasal turbinates and lungs were evaluated by histopathological investigation. The tissues of the non-challenged OVA immunized animals served as controls representing healthy animals. On dpc 5, nasal turbinate sections from animals challenged by infection showed more pronounced pathological changes in OVA compared to I-SARS-CoV-2 immunized animals (Table S1). On dpc 12, this tissue of the upper airways had largely recovered in both of these groups (Table S1). Evaluating the lung tissue of dpc 5 and 12 from animals challenged by infection, there were clear lesions in sections from the OVA...
Figure 5. Body weight loss, virus titers in the upper airways, and neutralizing antibodies following one immunization with I-SARS-CoV-2

(A) Experiment outline. Hamsters were immunized once with 1.1 μg S1 of I-SARS-CoV-2 on dpi 0 (n = 4) or remained non-immunized (n = 4). All animals were challenged on dpi 21 and blood samples were obtained on the specified days. Animals were euthanized on day 20 post challenge (dpc).

(B) Body weight relative to dpc 1, symbols indicate means and error bars represent SD, the solid line shows 100%.

(C) Infectious titers given as 50% tissue culture infectious dose (TCID₅₀)/mL of oral swabs and (D) virus RNA titers of nasal lavages from individual challenged animals on the specified dpc. <, one or more values were below the LLOQ, these were given the value of the LLOQ for calculation of the graph medians. In (C) and (D) medians are shown by horizontal bars with interquartile range (IQR). The LLOQ is indicated by the dotted line.

(E) In vitro neutralization of the original D614G virus by plasma from dpc 12, 22, and 20. 50% inhibitory dilution (ID₅₀) values were calculated from the curves in Figure S1A, group means are shown by horizontal bars with SD. The LLOQ is indicated by the dotted line. A Mann-Whitney test (line with brackets) was used to compare individual groups in (C), (D), and (E); statistical analysis was only carried out where all values were above the LLOQ, only statistically significant differences are indicated, *, p<0.05.
Figure 6. Body weight loss, virus titers in the upper and lower airways, and lung pathology following two immunizations with I-SARS-CoV-2.

(A) Experiment outline. Hamsters were immunized two times with either 1.1 μg S1 of I-SARS-CoV-2 \((n = 12)\) or 100 μg OVA \((n = 12)\) on dpi 0 and 21. Blood samples were collected on the specified days. OVA \((n = 8)\) and I-SARS-CoV-2 \((n = 8)\) immunized animals were challenged (C) on dpi 43. Animals were euthanized; four animals of each group were euthanized on dpc 5 and 12, respectively. The remaining OVA \((n = 4)\) and I-SARS-CoV-2 \((n = 4)\) immunized animals were not challenged (NC) and were euthanized on the day corresponding to dpc 5. Group sizes were \(n = 4\) for all samples collected after dpc 5.

(B) Body weight relative to dpc 0, symbols indicate means and error bars represent SD, the solid line shows 100%.

(C) Histopathological changes in lungs collected from all animals on the day of euthanasia were evaluated blinded with respect to group allocation. Antigen is indicated above the graph; challenge status and day of euthanasia are indicated.
munization, were protected against disease and body weight loss. Compared to one immunization preceded the infection. We demonstrated that animals immunized two titers following both I-SARS-CoV-2 immunization and challenge were only slightly higher when two as titers by up to 2.6-fold and moreover appeared to boost antigen specific Th1-type cellular responses. NAb after SARS-CoV-2 infection alone. Challenge further boosted immunization leading to an increase in NAb (Figure S5).

Analyzing the induction of NAb, for the I-SARS-CoV-2 animals, mean ID50 values of plasma samples were 282 and 2074 after the first and second immunization, respectively (Figure 6C). These titers were boosted after challenge to a mean ID50 of 5510 on dpc 5 and 4467 on dpc 12. For OVA animals following challenge, mean ID50 of plasma samples were 1566 on dpc 5 and 2632 on dpc 12. The mean ID50 following two immunizations with I-SARS-CoV-2 was thus comparable to the ID50 induced by infection in OVA animals. Endpoint titers of virus-specific antibodies binding S1S2 were determined for samples from non-challenged I-SARS-CoV-2 animals (Figure 8B), with lung morphology resembling that of non-challenged OVA control animals (Figures 6C and 7C, Table S2). The lung morphology of this group strongly resembled that of non-challenged OVA control animals, and thus I-SARS-CoV-2 immunization did not induce lung pathology. Histopathological findings listed for the non-challenged I-SARS-CoV-2 immunized group were observed in one small, confined area of the tissue section from one animal (Figure 6C, Table S2).

Thus, two immunizations with I-SARS-CoV-2 resulted in high titers of NAb, which resembled titers observed after SARS-CoV-2 infection alone. Challenge further boosted immunization leading to an increase in NAb titers by up to 2.6-fold and moreover appeared to boost antigen specific Th1-type cellular responses. NAb titers following both I-SARS-CoV-2 immunization and challenge were only slightly higher when two as compared to one immunization preceded the infection. We demonstrated that animals immunized two times with I-SARS-CoV-2 were protected against lung pathology and, as also observed following one immunization, were protected against disease and body weight loss.

I-SARS-CoV-2 immunization elicited potent Delta variant neutralizing antibodies with reduced Omicron variant neutralizing efficacy

While the I-SARS-CoV-2 vaccine candidate is based on an original D614G virus, the Delta and the Omicron variants contain several additional mutations in S, including in the RBD, potentially affecting susceptibility to neutralization by vaccine induced antibody responses. For all groups of hamsters from the experiment outlined in Figure 6, plasma neutralized the Delta variant and the homologous SARS-CoV-2 virus (original D614G virus) with similar efficacy (Figure 9). Thus, when tested against the Delta variant,
challenged OVA animals had a mean ID50 of 2005 on dpc 5, similar to the mean ID50 of 2292 of non-challenged I-SARS-CoV-2 animals, whereas the mean ID50 was 7888 in challenged I-SARS-CoV-2 animals (Figure 9). When tested against the Omicron variant, plasma from challenged and non-challenged I-SARS-CoV-2 animals from dpc 5 neutralized the virus with mean ID50 values of 260 and 82, respectively, signifying a >21 and >32-fold reduction compared to NAb titers against the original D614G virus. However, only one OVA immunized and subsequently challenged animal had a detectable Omicron neutralizing response (Figure 9).

Thus, I-SARS-CoV-2 immunization- or infection-induced antibodies neutralized the Delta variant and the original D614G virus used as vaccine antigen to similar extend. Although I-SARS-CoV-2 immunization-induced antibodies retained low neutralization efficacy against the Omicron variant, SARS-CoV-2 infection-induced antibodies showed very limited to no neutralization efficacy against this variant.

DISCUSSION

In this study we demonstrate immunogenicity and protection conferred by an inactivated SARS-CoV-2 vaccine based on the original D614G virus, which was produced using an attractive scalable GMP-compliant, single-use, packed-bed bioreactor and purified by membrane-based chromatography technology for a fast and efficient capture purification step. To our knowledge, this is the first report of SARS-CoV-2 particle purification using membrane-based SXC. Importantly, this vaccine candidate, which is similar to widely used vaccines, induced persisting NAb in mice and protected hamsters from disease and lung pathology. Furthermore, vaccine induced antibodies retained neutralizing activity against the Delta variant, which was comparable to neutralization of the original D614G virus. Although neutralization of the Omicron variant was observed, it was at reduced levels.

A vast proportion of the world’s population does not have adequate access to vaccines against COVID-19.22 Thus, many communities are not appropriately protected from its devastating impact with a continued high risk of emergence and spread of new variants.22,32,33 Further SARS-CoV-2 vaccine candidate and vaccine manufacturing development should focus on closing this vaccination gap. Virus production and purification technologies applied in this study could facilitate quick deployment and affordable production at manufacturing scale.

Based on our previous experience from successful SXC-based purification of a variety of virus species, we established SXC-based purification of inactivated SARS-CoV-2 for studies in small animal models. In SXC, successful product capture strongly depends on the size of the target species and is achieved with a relatively narrow concentration range of PEG during product load (typically 8–10% PEG-6000). This characteristic greatly shortens process development and deployment time. In addition, SXC recovery and purification performance is generally robust to changes in process parameters related to cell cultivation and virus production. In contrast, other techniques such as ion-exchange chromatography or ultracentrifugation generally require considerable process development and may suffer from high sensitivity to changes in process parameters and low productivity. Analysis demonstrated successful purification of our inactivated SARS-CoV-2 sample with efficient depletion of protein and host cell DNA and further confirmed the physical integrity of the recovered particles. Immunogenicity testing demonstrated retained biological activity following SXC-based purification, as also observed for other viruses.43,45,46,55

According to the analytics, the quality of the two separate SXC elution fractions appeared similar and were thus pooled before testing of our I-SARS-CoV-2 vaccine candidate in animal models. In this study, carrying out a second elution step with a higher NaCl concentration increased product recovery. However, as also
described for other viruses, in subsequent purification batches independent of the presented study, it was indeed possible to recover all SARS-CoV-2 samples with a single PBS elution step; in these cases, no signal was detected during a high salt elution. While not within the scope of the presented study, the purification process of our I-SARS-CoV-2 vaccine candidate could be further improved by introduction of polishing steps.

Among the widely used inactivated SARS-CoV-2 vaccines, CoronaVac and BBIBP-CorV are based on the original virus, whereas BBV152 is based on an original D614G virus isolate like our I-SARS-CoV-2 vaccine candidate. All three vaccines use aluminum hydroxide adjuvants, and the BBV152 vaccine additionally contains a TLR7/8 agonist to promote induction of Th1 type responses, whereas an MF59 analogue was used in this study. This adjuvant was reported to enhance induction of antibodies compared to aluminum hydroxide. Indeed, we recorded favorable NAb titers of up to 583 and 2074 in immunized mice and hamsters, respectively. In hamsters, two immunizations resulted in NAb titers matching titers induced by challenge only. Although direct comparison of NAb titers between different studies is difficult because of methodological differences, mouse NAb titers induced by the I-SARS-CoV-2 candidate appeared to be in the range of those observed for CoronaVac, BBV152, and BBIBP-CorV. In this study, increasing the I-SARS-CoV-2 dose improved induction of NAb when tested in mice. Although a second immunization resulted in a strong boost of NAb titers compared to a single immunization in both mice and hamsters, in mice, a third immunization only had a minor effect on NAb titers and S-specific antibody endpoint titers as compared to two immunizations. However, it is possible that the third immunization improved the longevity of the antibody response. These observations agree with previous findings of a dose effect in preclinical studies with inactivated SARS-CoV-2 vaccine candidates including those highlighted above. For BBIBP-CorV and BBV152, a third immunization in mice was observed to increase NAb and S-specific antibody titers, respectively; this difference compared to I-SARS-CoV-2 in the present study may be because of differences in experimental conditions such as shorter immunization intervals.

The waning immunity following infection and vaccination is a general concern. In humans, both antibody titers and protection levels decrease within a few months after a second immunization with several different vaccines. Although a third dose of BNT162b2, ChAdOx1 nCoV-19, or inactivated vaccines boosted NAb titers against the original virus, these waned considerably within six months for CoronaVac and more slowly following a third dose of BNT162b2. Furthermore, neutralizing activity against Omicron waned a few months post a third dose of BNT162b2. For such widely used vaccines,
available information about longevity of NAb in animals is limited, however, a reduction in antibody titers was observed within a couple of months in mice immunized twice with the inactivated BBIBP-CorV vaccine. Importantly, following three immunizations with our I-SARS-CoV-2 candidate in mice, NAb persisted during a follow-up period of seven months, whereas NAb longevity following two immunizations was not evaluated. These results are strengthened by other recent reports on induction of long-lived NAb by inactivated vaccine candidates based on original SARS-CoV-2 or original D614G viruses and adjuvanted with aluminum hydroxide. Of interest, for one of the candidates, NAb levels were also sustained in non-human primates, and the other candidate further protected non-human primates from disease when challenged five months post immunization. It remains to be determined how such findings on persisting NAb responses translate to humans.

Detection of IFN-γ secreting splenocytes in mice and hamsters, and relatively high titers of S-specific IgG2/3 in hamsters suggested that in addition to a Th2 type response expected when using the AddaVax adjuvant, immunization aided induction of Th1 type cellular responses. Th1 type responses were also suggested in a previous study evaluating an inactivated SARS-CoV-2 vaccine in hamsters.

Small animal infection models are essential for vaccine research and development. Although SARS-CoV-2 generally does not readily infect wild type mice, Syrian hamsters are susceptible to infection and represent a useful model for evaluating development of disease with pneumonia and lung pathology. In hamsters, our I-SARS-CoV-2 vaccine candidate prevented development of clinical disease and body weight loss after one immunization. Importantly, two immunizations were additionally confirmed to protect from lung pathology and strongly reduced the viral load in the lungs. Both one and two I-SARS-CoV-2 immunizations reduced viral titers in the upper airways and the duration of virus shedding compared to control immunized or non-immunized animals, suggesting an accelerated clearance of infection. Challenge resulted in a significant boost of NAb titers, with ~2-fold higher titers in I-SARS-CoV-2 immunized versus control immunized animals, and only a minor effect of one versus two immunizations on post challenge NAb titers. Several preclinical
SARS-CoV-2 vaccines have been evaluated in this animal model, including various vectored and inactivated vaccine candidates. One, two, and three doses of inactivated SARS-CoV-2 vaccines were reported to confer protection from disease in hamsters, with reduced virus titers in the upper airways, as well as reduction or absence of histopathological changes in lungs. Among widely used vaccines with demonstrated clinical protective effects, three doses of the BBV152 vaccine protected hamsters from disease, whereas virus titers in upper airways and lungs were detectable in immunized animals after challenge. To our knowledge, no protection studies in hamsters were reported for the BBIBP-CorV and CoronaVac vaccines. As described for the BBV152 vaccine, these vaccines conferred protection in non-human primates, although virus could be detected in the upper airways following challenge.

S or RBD is the only antigen in most SARS-CoV-2 vaccines, including the licensed mRNA and viral vector vaccines, whereas inactivated vaccines contain all structural proteins of the virus; S, N, M, and E. Immune responses to these proteins may contribute to protection, and besides S, the N protein is another major immunogen of the virus. Although their role has not been defined, N-specific antibodies are induced in natural infection. Importantly, N-specific antibodies were induced by the I-SARS-CoV-2 antigen in this study as well as by other inactivated vaccine candidates. Of interest, an N-encoding viral vector vaccine was protective in hamsters, and a combination of S- and N-encoding viral vector vaccines was superior to vaccines encoding individual antigens in protection of transgenic mice. High mutational rates are characteristic of RNA viruses and a selective pressure drives acquisition of mutations in the viral proteins, particularly, S and RBD mutations in emerging variants pose a concern. Even though mutations were also described in the N protein, efficacy of immune responses targeting N or other more conserved virus components was proposed to be relatively robust across variants.

With the global ongoing SARS-CoV-2 pandemic new variants continuously emerge, challenging vaccine efficacy. BNT162b2, ChAdOx1 nCoV-19, and inactivated vaccines were reported to have reduced protective effects against the Delta variant, which was frequently detected in breakthrough infections. Furthermore, the NAb responses induced by these vaccines have reduced in vitro Delta-neutralizing activity when compared to the original virus with or without the D614G mutation. Importantly, NAb induced by I-SARS-CoV-2 in this study had similar neutralization efficacy against the Delta variant and the original D614G virus, which are both associated with severe disease. It is possible that antibodies targeting epitopes outside of S might contribute to the observed neutralizing activity. Although associated with a milder course of disease, the Omicron variant and subvariants are causing surges of COVID-19 cases, also in vaccinated populations. Accordingly, protection conferred by BNT162b2 or ChAdOx1 nCoV-19 in vaccinated individuals was reduced for Omicron compared to Delta. Furthermore, antibodies induced by these two vaccines and the inactivated CoronaVac, BBIBP-CoV, or BBV152 vaccines in humans had limited or no Omicron neutralizing activity. NAb induced in hamsters by the I-SARS-CoV-2 candidate measurably neutralized Omicron, however, considerably less efficiently than Delta and the original D614G virus. Notably, immunization-induced NAb had higher Omicron neutralizing activity than infection-induced NAb. To obtain a broader immune response against emerging SARS-CoV-2 variants, potentially causing more severe disease than Omicron, heterologous prime-boost regimens, multivalent vaccines including variant-based antigens, and vaccines targeting conserved epitopes could be explored.

Limitations of the study
A limitation of this study is its timeliness, as several SARS-CoV-2 vaccines have been approved since late 2020. Nevertheless, our vaccine candidate compares favourably to other inactivated SARS-CoV-2 vaccines employing different viral isolates, adjuvants, and bioprocessing conditions, and reported data might facilitate inactivated vaccine production and global vaccine coverage. Although animal group sizes in this study are in the range of those in other studies, increasing the group sizes would increase the power of the statistical analysis. A more detailed analysis could clarify the role and contributions of T cell responses and antibodies, respectively, in vaccine-conferred protection in hamsters; however, such analysis is currently hampered by limited availability of hamster specific reagents. Furthermore, vaccine protection experiments were initiated in hamsters 5–7 weeks of age. As the risk of severe COVID-19 increases with age, testing protection in hamsters of older age would be of relevance. To date, experiments in non-human primates or initiation of clinical trials have not been possible with available resources; however, presented data could aid ongoing vaccine development and production efforts, including in resource limited countries.
STAR METHODS
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SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.105949.

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AUTHOR CONTRIBUTIONS
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DECLARATION OF INTERESTS


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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Judith Margarete Gottwein (jgottwein@sund.ku.dk).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- All relevant data are included in the manuscript.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell lines
VeroE6 cells (RRID: CVCL_0574) (a gift from J Dubuisson) were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Thermo Fisher Scientific #31966021) supplemented with 100 U/mL penicillin and...
100 μg/mL streptomycin (Sigma #P4333) and 10% fetal bovine serum (FBS) (v/v) (Sigma-Aldrich #F7524). VeroE6 cells were sub-cultured every 2–4 days and maintained at 37°C and 5% CO₂ as described.

Mouse experiments
Female BALB/c mice 6–8 weeks of age (Taconic #BALB-F MPF; RRID: IMSR_TAC:balb) arrived at the animal facility at least one week prior to initiation of experiments and were maintained in a manner consistent with affirmative response to the ARRIVE 10 questionnaire. A total of 40 mice were used, animals were randomly distributed between groups, and handled in random order upon immunization and sampling. Staff responsible for daily animal care were blinded to group allocation. No animals were excluded from this study. The number of animals per group was determined based on statistical, ethical, practical, and financial considerations. Experiments were carried out at the Faculty of Health and Medical Sciences at the University of Copenhagen in agreement with national Danish guidelines (LBK nr 474 af 15/05/2014) and approved by the Animal Experiments Inspectorate, license 2020-15-0201-00586.

Hamster experiments
Male Syrian hamsters 5–7 weeks of age (Janvier #HAMSTER - RjHan:AURA, NCBI:txid10036 Mesocricetus auratus Waterhouse, 1839) arrived at the animal facility at least one week prior to initiation of experiments and were maintained in a manner consistent with affirmative response to the ARRIVE 10 questionnaire. A total of 32 Syrian hamsters were used, animals were randomly distributed between groups, and handled in random order upon immunization and sampling. Staff responsible for daily animal care were blinded with respect to immunization group but not to animal challenge status, as infected animals were housed in a BSL3 facility from day post challenge. No animals were excluded from the study. The number of animals per group was determined based on statistical, ethical, practical, and financial considerations. Experiments were carried out at Statens Serum Institut in agreement with national Danish guidelines (LBK nr 474 af 15/05/2014) and approved by the Animal Experiments Inspectorate, license 2020-15-0201-00718.

METHOD DETAILS

Virus stocks
The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) used for neutralization assays, an original D614G virus (SARS-CoV-2/human/DNK/DK-AHH1/2020; GenBank: MZ049597; Spike (S) sequence as described in early outbreak isolates such as Wuhan-Hu-1 with a D614G mutation), a Delta variant (B.1.617.2) (SARS-CoV-2/human/DNK/DK-AHH3/2021; GenBank: OP271297), and an Omicron variant (B.1.1.529) (BA.1) (SARS-CoV-2/human/DNK/DK-AHH4/2021; GenBank: OP271296) were derived from patient isolates and prepared as previously described. These second passage virus stocks were analyzed as previously described by deep sequencing of five overlapping reverse transcription (RT)-polymerase chain reaction (PCR) amplicons to confirm the identity of the variant. The second passage original D614G virus stock was additionally used as inoculum in hamster challenge experiments. A serum-free fourth passage stock of this virus was used for inoculation of bioreactor cell cultures for the vaccine virus production as described in a previous study, as well as for mouse serum neutralization assays.

Virus inactivation for vaccine preparation
Production of the virus used as vaccine antigen was described in a prior study (experiment 5 and 6 in42). In brief, the virus was propagated in VeroCCL81 cells (Nuvonis) cultivated in serum-free medium in a single-use packed-bed CelCradle bioreactor with a 0.5 L working volume (Esco Aster Pte. Ltd.). The pooled virus harvest was inactivated with β-propiolactone (BPL) (Ferak #57-57-8 1 mL) at a concentration of 1:2000 (v/v). HEPES buffer (Sigma-Aldrich #H0887-100 mL) was added to the virus harvest to a final concentration of 0.025 M and a pH of 7.5, and the sample was incubated with BPL at 4°C for 16 h followed by incubation at 37°C for 3 h. Inactivation was confirmed as follows after sample filtration and sucrose spiking as described below (“purification of inactivated virus”). Aliquots of inactivated virus were inoculated in VeroE6 cells seeded in T25 flasks (Thermo Fisher Scientific #136196). On days 3–4 post infection, cultures were evaluated for virus-induced cytopathic effect by light microscopy and all cells of each indicator culture were detached by trypsin treatment (Sigma-Aldrich #T3924) and transferred to T80 flasks. A chamber slide (Thermo Fisher Scientific #177402) was seeded and fixed with methanol the next day as described below (“virus infectious titers”). Immunostaining was carried out using primary anti-S antibody (Sino Biological #40150-D004; RRID: AB_2827983) and secondary Alexa Fluor 488 Conjugated antibody (Thermo Fisher Scientific #A-11013; RRID:AB_2534080) as described previously to visualise SARS-CoV-2 infected cells. T80 flask
cultures were sub-cultured every 4–5 days for at least two weeks, each time the cytopathic effect was evaluated, and a chamber slide was seeded for immunostaining to confirm inactivation.

**Purification of inactivated virus**

The inactivated virus was filtered with 5 μm and 0.65 μm Sartopure PP3 filter capsules (Sartorius #5051342P5–OO–B, 5051305P5–OO–B), spiked with sucrose (Merck #S1888-500G) for a final concentration of 5% (w/v) and stored at −80°C until further processing. For purification, virus (~4.5 L) was thawed and treated with an unspecific nuclease (DENERASE, hereafter called “Denerase”, c-LEcta #20804-100k) to digest host cell DNA. The filtered virus was incubated with 50 U/mL Denerase and supplemented with MgCl₂ (Sigma-Aldrich #M8266-1 KG) and NaN₃ (Sigma-Aldrich #08591) for final concentrations of 2 mM and 0.05% (w/v), respectively. The sample was incubated for 6 h at room temperature under mixing with a magnetic stirrer at 250 RPM. Following host cell DNA digestion, the sample was filtered using 0.2 μm membranes (Cytiva #10410314) fitted into a reusable bottle top filter holder (vwr #528199-325). This digested harvest was purified by 9 runs of membrane-based steric exclusion chromatography (SXC). All chromatography experiments were performed at room temperature using an AKTA Pure 25 (Cytiva) liquid chromatography system controlled by the software UNICORN v6.3. The presence of virus particles was monitored with a NICOMP 380 (Particle Sizing Systems) submicron particle analyzer at a wavelength of 632.8 nm. Virus capture was carried out with membrane-based SXC using 25 mm diameter capsules assembled in-house with a total surface of around 100 cm². The column volume (CV) was defined as 1 mL. SXC was performed in multiple runs loading 500 mL of digested harvest in bind-elute mode. Each run consisted of four main steps. Briefly, (i) equilibration: the SXC device was equilibrated by 1:1 in-line mixing of 16% (w/v) PEG-6000 (Sigma-Aldrich #81260) in 1 x phosphate buffered saline (PBS) and 1 x PBS to achieve a final PEG-6000 concentration of 8%. (ii) Sample loading: the digested harvest was mixed 1:1 in-line with the same PEG stock solution as in step (i). (iii) Washing: after the entire volume of PEG-conditioned digested harvest was loaded, a wash was done as in step (i) until baseline UV absorbance was achieved. (iv) Elution: the virus particles were recovered from the SXC device by flushing it with a step gradient of 1 x PBS for 20 CV (SXC elution 1), followed by 1 x PBS, 0.5 M NaCl for 20 CV (SXC elution 2). The flow rate was 5–15 mL/min. The eluates from the multiple SXC runs were pooled and dialyzed overnight at 4°C in PBS (sample to buffer ratio of 1:1000) with a 300 kDa molecular mass cut-off dialysis tubing made of cellulose ester (Spectra Por #GZ-02890-77). The dialyzed samples were spiked with sucrose as a cryoprotectant and stabilizer (Sigma-Aldrich #57903) to a final concentration of 5% (w/v) and stored at −80°C.

**Characterization of purified virus**

SXC eluates 1 and 2 collected from each run were pooled into two fractions and characterized separately for quality control. Analytical size exclusion chromatography (SEC) was carried out using a packed-bed Superdex 200 Increase 10/300 GL column (Cytiva #17517501) as described previously at a flow rate of 0.8 mL/min. Inactivated, purified SARS-CoV-2 particles in SEC fractions were detected by dot blot using anti-SARS-CoV-2 Spike glycoprotein rabbit IgG and HRP-conjugated goat anti-rabbit IgG (H&L) antibodies (Abcam #ab272504; RRID:AB_2847845 and #ab205718; RRID:AB_2819160). Particle size distribution was determined hydrodynamically using differential centrifugal sedimentation (DCS) as described previously. Virus particle size and morphology were assessed with negative staining transmission electron microscopy (TEM). Briefly, to obtain TEM images virus particles were spotted onto copper palladium 400 mesh hexagonal pattern grids coated with a 4 nm layer of carbon (Plano GmbH). The grids were incubated with the samples for 1 min, then blotted quickly with Whatman 50 filter paper and placed directly into a drop of 1% phosphotungstic acid (EM) in water (pH 7.0). The grids were quickly blotted and placed onto a second drop of stain for 1 min followed by blotting. The grids were subsequently imaged on a Bio-twin 120 kV electron microscope (Philips) and images were taken on a SIS Keenview camera (Olympus). Host cell DNA and total protein were quantified with PicoGreen reagent kit (Thermo Fisher Scientific #P7581) and Bradford assay (Bio-Rad, #5000205), respectively, as previously reported. Alternatively, total protein was quantified with a Pierce BCA Protein Assay Kit (Thermo Fisher Scientific #23225). Prior to use in immunizations, fractions of SXC elution 1 and 2 were pooled, and the Spike protein subunit 1 (S1) concentration was determined by ELISA, as described below (“S1 quantification by ELISA”). For one immunization experiment, sample from a separate round of purification was additionally lyophilized overnight using an Alpha 1–2 LD plus table-top freeze dryer (Margin Christ Gefriertrocknungsanlagen GmbH). Prior to use in immunization experiments, lyophilized samples were reconstituted in sterile water at one-fifth of their original volume.
**Immunization and sampling of BALB/c mice**

In one experiment, our inactivated SARS-CoV-2 preparation (I-SARS-CoV-2) was lyophilized and mice were immunized subcutaneously with I-SARS-CoV-2 corresponding to 0.1 μg S1 (n = 4) (6.4 μg total protein) or 100 μg Ovalbumin (n = 4) (OVA, Invivogen #vac-pova), both mixed 50:50 with AddaVax (Invivogen #vac-adx-10). Immunizations were carried out on day 0 and 14 post immunization (dpi). Blood samples were collected on dpi 7 and upon euthanasia on dpi 35. Serum was prepared by centrifugation and stored at −80°C. In another experiment, mice were immunized with a different batch of I-SARS-CoV-2 corresponding to 0.1 μg S1 (n = 5), 0.5 μg S1 (n = 9) (2.9 and 14.5 μg total protein, respectively) or 100 μg OVA (n = 4) mixed 50:50 with AddaVax. For a split vaccine approach, CHAPS (Merck #C5070) was added to I-SARS-CoV-2 containing either 0.1 μg S1 (n = 5) or 0.5 μg S1 (n = 9) for a final concentration of 0.5% (w/v) and incubated for 15 min at room temperature before mixing with adjuvant. Immunizations were carried out on dpi 0, 20, and 42. Blood samples were collected on dpi 14, 35, and 56. The low dose groups and five animals of the high-dose groups were euthanised upon euthanasia on dpc 7 and upon euthanasia on dpi 35. Serum was prepared by centrifugation and stored at −80°C. In another experiment, all animals were challenged by nasal inoculation with 100 50% tissue culture infectious dose (TCID₅₀) of the virus stock as described above (“virus stocks”). Nasal lavages were collected on dpi 0, prior to immunization. Until challenge, nasal lavages or oropharynx swabs were collected every 1–3 days. From day 0 until day 9 post challenge (dpc), nasal lavages or oropharynx swabs were collected every day, and from dpc 9 to dpc 20 samples were collected every 1–3 days. These samples were stored in transport medium (DMEM supplemented with 10% FBS and Antibiotic-Antimycotic 100X (Thermo Fisher Scientific #15240062)). Blood samples were collected in EDTA tubes on dpc –22, –1, and 20; plasma was prepared by centrifugation and stored at −80°C. In another experiment animals were immunized twice with I-SARS-CoV-2 corresponding to 1.1 μg of S1 mixed 50:50 with AddaVax (n = 12) or 100 μg OVA (n = 12) on dpi 0 and 21, followed by challenge of 8 animals in each group on dpi 43. Nasal lavages or oropharynx swabs were collected every day from dpc –1. Blood samples were collected on dpc –43, –22, 0, 5, and 12. Spleens, nasal turbinates, and lungs were collected upon euthanasia on dpc 5 (8 animals from each group, 4 challenged and 4 non-challenged) and on dpc 12 (4 animals from each group, all challenged). Splenocytes were isolated and ELISpot assays were carried out as described below (“mouse and hamster ELISpot assays”). Nasal turbinates and lungs were processed as described below (“tissue pathology and lung virus titers”). Potential adverse effects of the immunizations and the general health/disease status of animals following infection challenge were monitored by body weight and clinical inspection. Saline was administered subcutaneously to alleviate clinical symptoms when relevant, no animals required euthanasia before scheduled according to the experimental outline.

**Immunization of syrian hamsters**

In one experiment one dose of I-SARS-CoV-2 corresponding to 1.1 μg of S1 (30.6 μg total protein) mixed 50:50 with AddaVax (n = 4) was administrated on dpi 0. Animals for the non-challenged control group (n = 4) arrived one week prior to challenge. On dpi 21, all animals were challenged by nasal inoculation with 100 50% tissue culture infectious dose (TCID₅₀) of the virus stock as described above (“virus stocks”). Nasal lavages were collected on dpi 0, prior to immunization. Until challenge, nasal lavages or oropharynx swabs were collected every 1–3 days. On dpi 21, animals were challenged by intranasal inoculation with 1×10⁶ TCID₅₀ (± AddaVax). Nasal lavages were collected on dpi 0, 21, and 28; serum was prepared by centrifugation and stored at −80°C. In another experiment animals were immunized twice with I-SARS-CoV-2 corresponding to 1.1 μg of S1 mixed 50:50 with AddaVax (n = 12) or 100 μg OVA (n = 12) on dpi 0 and 21, followed by challenge of 8 animals in each group on dpi 43. Nasal lavages or oropharynx swabs were collected every day from dpc –1. Blood samples were collected on dpc –43, –22, 0, 5, and 12. Spleens, nasal turbinates, and lungs were collected upon euthanasia on dpc 5 (8 animals from each group, 4 challenged and 4 non-challenged) and on dpc 12 (4 animals from each group, all challenged). Splenocytes were isolated and ELISpot assays were carried out as described below (“mouse and hamster ELISpot assays”). Nasal turbinates and lungs were processed as described below (“tissue pathology and lung virus titers”). Potential adverse effects of the immunizations and the general health/disease status of animals following infection challenge were monitored by body weight and clinical inspection. Saline was administered subcutaneously to alleviate clinical symptoms when relevant, no animals required euthanasia before scheduled according to the experimental outline.

**Virus RNA titers**

Virus RNA titers were determined by quantitative PCR (qPCR), as described. Briefly, nasal lavage samples were clarified by centrifugation, mixed 1:3 with Trizol LS (Thermo Fisher Scientific #15596018), and extracted with chloroform (Sigma-Aldrich #C2432) in SPRIIME Phase Lock Gel Heavy tubes (Quantabio #2302830). RNA purification was carried out with the RNA Clean and Concentrator-5 kit (Zymo Research #R1014) according to the manufacturer’s protocol and samples were eluted in nuclease-free water (Ambion #AM9930). qPCR probe and primers as described elsewhere were adapted to use with TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher #4444434). RNA standards ranging from 10⁵ to 10⁸ RNA copies/μL (Twist Bioscience #102024), a negative control, and diluted samples were included as two technical replicates in each analysis (LightCycler 96 System (Roche)). A standard curve was generated in the LightCycler 96 software version 1.1.0.1320 (Roche) and used to interpolate sample RNA titers. The lower limit of quantification (LLOQ) is defined as the mean value of medium only samples plus three times the standard deviation (SD) of medium only samples of all qPCR plates of the experiment.

**Virus infectious titers**

Infectious titers of SARS-CoV-2 were determined in a 96-well based TCID₅₀ assay in VeroE6 cells evaluated by immunostaining of S1, as previously described. Oropharynx samples were clarified by centrifugation at
500 g prior to analysis. Briefly, serially diluted samples were added to VeroE6 cells seeded the day before at 10⁴ cells per well in 96-well clear plates (Thermo Fisher Scientific #167008) followed by incubation at 37°C and 5% CO₂. After 48 h (+/- 1 h), plates were fixed by 20 min incubation in cold methanol and rinsed with PBS containing 0.1% (v/v) tween 20 (Sigma-Aldrich #P9416). Plates were incubated with 3% H₂O₂ for 10 min at room temperature and stained with primary anti-S antibody (Sino Biological #40150-D004; RRID:AB_2827983) diluted 1:5000 in PBS containing 1% (w/v) bovine serum albumin (BSA) (Roche #10735086001) and 0.2% (w/v) skimmed milk (Easisand stained with primary anti-S antibody (Sino Biological #40150-D004; RRID:AB_2827983) diluted 1:5000 in PBS containing 1% (w/v) bovine serum albumin (BSA) (Roche #10735086001) and 0.2% (w/v) skimmed milk (Easis containing 5% (w/v) skimmed milk powder for 2 h at room temperature. Plates were incubated with secondary antibody Fab(Picker) anti-Human IgG Fc Cross-Absorbed Secondary Antibody, horseradish peroxidase (HRP) (Thermo Fisher Scientific #A24476; RRID:AB_2535945) diluted 1:2000 in PBS-1% BSA-0.2% skimmed milk for 1 h at room temperature. S1-positive cells were visualized with the Bright-DAB solution kit (Immunologic #BS04-500). Each well of the 96-well plates was automatically imaged with an Immunospot series 5 UV analyzer (CTL Europe GmbH). The infectious titer was determined according to the Reed-Muench method. The LLOQ is defined by the lowest titer which could be determined with the applied starting dilution.

Low-volume neutralization assay for mouse serum

Mouse serum was heat treated at 56°C for 30 min and 50% neutralization (NT50) was determined in a low-volume neutralization assay as described with minor modifications. Briefly, five replicates of serially diluted sera in a total volume of 3 µL as well as 7 µL of virus master mix for an MOI of 0.01–0.05, with MOI selection based on input pilot experiments, were incubated in a pre-plate for 1 h at 37°C. Subsequently, 90 µL of pre-warmed DMEM was added to each well of the pre-plate and the total volume was transferred to VeroE6 cells seeded the day before at 10⁴ cells per well in a 96-well plate. An S neutralizing antibody (Sino Biological #40591-MM44; RRID:AB_2857934) was included as a positive neutralization control in each plate. Plates were fixed and stained as described above (“virus infectious titers”). Wells were imaged and S-positive cells were counted automatically with an Immunospot series 5 UV analyzer (CTL Europe GmbH). Eight virus-only wells and six negative control wells were included in each plate and across experiments, these yielded approximately 2000–5000 and 0–70 counts, respectively. The average count of the negative control wells was subtracted from all values. The percentage of neutralization was calculated relating counts of individual wells to the average count of the virus-only wells. The NT50 was determined as the reciprocal of the last serum dilution with ≥50% neutralization. The LLOQ is defined by the lowest dilution used in the assay, 1:12.5.

Neutralization assay for hamster plasma

Hamster plasma was heat treated at 56°C for 30 min, and neutralization was evaluated as described. Briefly, serially diluted plasma prepared with DMEM was mixed 50:50 with a virus master mix for an MOI of 0.02–0.06, with MOI selection based on input pilot experiments, and incubated for 1 h at room temperature. Plasma-virus mixtures were transferred to four replicate wells of VeroE6 cells seeded the day before at 10⁴ cells per well in a 96-well plate. An S neutralizing antibody (Sino Biological #40592-MM57; RRID:AB_2857935) was included as a positive control, except in B.1.1.529 neutralization assays, as this antibody did not neutralize the B.1.1.529 variant. Plates were fixed and stained, and S-positive cells were counted as described above (“virus infectious titers”). Eight virus-only wells and four negative control wells were included in each plate, and across experiments these yielded approximately 2000–5000 and 0–80 counts, respectively. The percentage of inhibition was calculated for each well as described above (“low-volume neutralization assay for mouse serum”). The 50% inhibitory dilution (ID50) was determined using GraphPad Prism version 9, equation y = bottom+(top-bottom)/(1 + 10^((logEC50-x)*HillSlope)), with bottom and top constrains set at 0 and 100, respectively. The LLOQ is defined by the lowest dilution used in the assay, 1:25.

Detection of virus specific IgG in ELISA

MaxiSorp plates were coated with 2 μg/mL of either S1S2 (Sino Biological #40589-V08B1), Spike-receptor binding domain (RBD) (Sino Biological #40592-V08B), or Nucleocapsid protein (N) (Sino Biological #40588-V08B) diluted in carbonate-bicarbonate buffer (Sigma-Aldrich #3041-50CAP) and incubated overnight at 4°C. Plates were rinsed in washing buffer (PBS-0.1% tween 20) and blocked with PBS-0.1% tween 20 containing 5% (w/v) skimmed milk powder for 2 h at room temperature. Plates were rinsed and mouse serum or hamster plasma diluted in PBS-0.1% tween 20 containing 1% (w/v) skimmed milk was added to replicate wells, with 3-fold dilutions starting at 1:100. After 2 h of incubation at room temperature plates were rinsed and incubated with HRP-conjugated anti-mouse IgG antibody (GE Healthcare #NA931; RRID:AB_772210), anti-hamster IgG antibody (Invitrogen #PA1-29626; RRID:AB_10985385), or anti-hamster IgG2/3 antibody.
the other lung was stored in transport medium at 4°C. Cell types was confirmed by immunohistochemical staining as described previously.119 To obtain virus titers, until processing for paraffin embedding. The tissues were sectioned at 4–5 μm onto slides, deparaffinized and stained with hematoxylin and eosin for histological evaluation, which was carried out blinded. Lesions identified in the nasal turbinates were scored as absent, focal, or extensive (−/−+/+); or as absent or present (−/+), scores are given in Table S1. Lesions identified in lung sections were scored as absent or present (−/−); or as absent, few, or numerous (−/++/+), scores are given in Table S2. The identity of identified cell types was confirmed by immunohistochemical staining as described previously.119 To obtain virus titers, the other lung was stored in transport medium at −80°C. It was subsequently thawed and dried with sterile adsorbent paper, and a piece comprising 40–60% was cut of the lung and transferred to a small volume of PBS, Invitrogen #15576028) for 14 days. Tissues were then stored in 70% ethanol until processing for paraffin embedding. The tissues were sectioned at 4–5 μm onto slides, deparaffinized and stained with hematoxylin and eosin for histological evaluation, which was carried out blinded. Lesions identified in the nasal turbinates were scored as absent, focal, or extensive (−/+/++); or as absent or present (−/+), scores are given in Table S1. Lesions identified in lung sections were scored as absent or present (−/−); or as absent, few, or numerous (−/+++), scores are given in Table S2. The identity of identified cell types was confirmed by immunohistochemical staining as described previously.119 To obtain virus titers, the other lung was stored in transport medium at −80°C. It was subsequently thawed and dried with sterile adsorbent paper, and a piece comprising 40–60% was cut of the lung and transferred to a small volume of PBS, Invitrogen #15576028) for 14 days. Tissues were then stored in 70% ethanol until further analysis by virus titration as described above (“virus RNA titers”). Virus titers per gram tissue pellet were normalized across samples taking obtained supernatant volumes into account, thus representing equivalent ratios of microliters supernatant per gram tissue pellet.

**Tissue pathology and lung virus titers**

Upon euthanasia of hamsters on dpc 5 and 12, the turbinate from the left side of the nasal cavity and the left lung from each animal were fixed in 10% formalin for 24–48 h. The turbinates were decalcified in EDTA solution (EDTA 0.25M 1× PBS, Invitrogen #15576028) for 14 days. Tissues were then stored in 70% ethanol until processing for paraffin embedding. The tissues were sectioned at 4–5 μm onto slides, deparaffinized and stained with hematoxylin and eosin for histological evaluation, which was carried out blinded. Lesions identified in the nasal turbinates were scored as absent, focal, or extensive (−/+/++); or as absent or present (−/+), scores are given in Table S1. Lesions identified in lung sections were scored as absent or present (−/−); or as absent, few, or numerous (−/+++), scores are given in Table S2. The identity of identified cell types was confirmed by immunohistochemical staining as described previously.119 To obtain virus titers, the other lung was stored in transport medium at −80°C. It was subsequently thawed and dried with sterile adsorbent paper, and a piece comprising 40–60% was cut of the lung and transferred to a small volume of PBS, Invitrogen #15576028) for 14 days. Tissues were then stored in 70% ethanol until further analysis by virus titration as described above (“virus RNA titers”). Virus titers per gram tissue pellet were normalized across samples taking obtained supernatant volumes into account, thus representing equivalent ratios of microliters supernatant per gram tissue pellet.

**Isolation of splenocytes**

Spleens were obtained from animals upon euthanasia in experiments outlined in Figure 3 (dpi 56) and Figure 6 (dpi 5 and 12). Spleens were passed through a Falcon 100 μm sterile nylon cell strainer (Fisher Scientific #10282631) and red blood cells were lysed with RBC Lysis Buffer (Thermo Fisher Scientific #A1049201) or ACK Lysing Buffer (Thermo Fisher Scientific #A1049201) to isolate splenocytes, which were subsequently used in analysis.

**Mouse and hamster ELISPOT assays**

Mouse splenocytes were stimulated with I-SARS-CoV-2 antigen, S1 or S2 peptide pools (JPT Peptide Technologies #PM-SARS2-S-MUT-1 and JPT Peptide Technologies #PM-WCPV-S-2), an RBD peptide pool (JPT Peptide Technologies #PM-WCPV-S-RBD-2), or OVA peptide (323–339) (Sigma-Aldrich #O1641). IL-4 and IFN-γ were analyzed in an ELISPOT assay kit (ImmuNoSpot #Mouse IFN-γ/IL-4 Double-Color ELISPOT) according to the manufacturer’s instructions. Briefly, the membrane was activated with 70% ethanol and incubated with capture antibody overnight at 4°C. Peptides were dissolved in DMSO and diluted in complete medium supplied with the kit and added to wells at a concentration of 2.5 μg/mL, I-SARS-CoV-2 was used at 0.08 μg S1/mL. Positive control wells contained brefeldin A (Invitrogen #00-4506-51) at a concentration of 10 μg/mL, and unstimulated control wells contained medium only. Cells were seeded at 250,000 cells per well, with duplicate wells for each condition. Color development was carried out after 32 h incubation at 37°C and 5% CO2. Wells were imaged, and positively stained cells were counted automatically with an Immunospot series 5 UV analyzer (CTL Europe GmbH). For analysis, mean counts of unstimulated wells from each animal were subtracted from counts of wells containing stimulants from that same animal. The mean of duplicates was converted to spots per 10^6 cells per well.

Hamster splenocytes were stimulated with I-SARS-CoV-2 antigen, an S1S2 peptide pool, an RBD peptide pool, and an N peptide pool (JPT Peptide Technologies # PM-WCPV-NCAP-2) at concentrations of 2 μg/mL. IFN-γ was evaluated in an ELISPOT assay kit (Mabtech #3102-2H) according to the manufacturer’s instructions with minor modifications; after emptying and rinsing of plates, prior to addition of detection antibody, plates were inactivated by submersion in methanol for 20 min and subsequently washed three times with PBS containing 0.1% tween 20 (Sigma-Aldrich #P9416). Results were analyzed as outlined above. Cells were counted manually with an ImmuNoSpot series 5 UV analyzer (CTL Europe GmbH). For analysis, mean counts of unstimulated wells from each animal were subtracted from counts of wells containing stimulants from that same animal. The mean of duplicates was converted to spots per 10^6 splenocytes for each animal.
were seeded in duplicate wells for each condition at a density of 250,000 cells per well in RPMI medium, supplemented with 10% FBS (Sigma-Aldrich #F7524), 100 U/mL penicillin, 100 μg/mL streptomycin (Sigma #P4333), 1% HEPES (Thermo Fisher Scientific #15630056), and 0.1% β-mercaptoethanol (Sigma-Aldrich #M7522).

**MSD analysis**

Mouse splenocytes were stimulated as described for ELISpot analysis, and in addition OVA protein was used for stimulation. After 96 h incubation at 37°C and 5% CO₂, plates were centrifuged at 1000 RPM for 10 min, supernatants were transferred to clean 96-well plates, and stored at −80°C. The meso scale discovery assay (mouse U-plex assay for cytokines IFN-γ, IL-17, IL-5, IL-13, IL-2, and IL-10, Meso Scale Discovery #K15069L-2) was carried out according to the manufacturer’s instructions. The plates were analyzed on a Sector Imager 2400 system (Meso Scale Discovery) and cytokine concentrations were calculated based on the standard curve generated in the Discovery Workbench 4.0.12 software with a 4-parameter logistic non-linear regression analysis.

**S1 quantification by ELISA**

S1 of the inactivated and purified virus was quantified by ELISA as described. Briefly, 96-well Maxisorp plates (Thermo Fisher Scientific #439454) were coated with 2.5 μg/mL capture antibody (Sino Biological #40150-D003; RRID:AB_2827982) in PBS, shaken for 1 min at 500 RPM, and incubated overnight at 4°C. Plates were rinsed in washing buffer (PBS-0.1% tween 20) and blocked for 2 h at room temperature in PBS containing 2% (w/v) BSA. Serially diluted S1 standard (Sino Biological #40591-V08H), samples, and negative control were added in duplicate and incubated for 1.5 h at room temperature with shaking at 500 RPM. Plates were subsequently rinsed in washing buffer and incubated with HRP-conjugated detection antibody (Sino Biological #40150-D001-H; RRID: AB_2857930) diluted 1:5000 in PBS-0.1% tween 20 containing 2% (w/v) BSA for 1.5 h at room temperature with agitation. Plates were rinsed in washing buffer and incubated with TMB substrate (Thermo Scientific #34028) for 5–10 min, followed by addition of stop solution (Invitrogen #S504). OD450 was measured with a microplate reader (BIO-TEK Instruments). Mean OD values from negative control wells were subtracted from all values. S1 sample concentration was calculated based on the standard curve generated in GraphPad prism version 9 with a 4-parameter logistic non-linear regression analysis.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data analysis and statistical analysis were carried out in GraphPad prism version 9 as specified in figure legends. Group sizes (n = 4–9 with few exceptions) are indicated in figure panels or figure legends. In general, Mann-Whitney tests were used to compare individual groups and Kruskal-Wallis tests were carried out to compare more than two groups. A Wilcoxon test was used to compare different time points/conditions within the same group. For means and medians calculated and shown in graphs, values below LLOQ of an assay were given the value of the LLOQ; for means and medians higher than the LLOQ a “<” above a dataset in graphs indicates that at least one of the values used to calculate the mean or median in the graph was below the LLOQ. Statistical analysis was only carried out where all values were above the LLOQ. Only statistically significant differences are shown in the graphs by bold bars (Kruskal-Wallis test) and lines with brackets (Mann-Whitney test). Statistical analysis was not carried out for body weight, and similarly not for ELISpot data and MSD data presented in the supplemental information.