An intranasal vaccine durably protects against SARS-CoV-2 variants in mice

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Recommended Citation
Hassan, Ahmed O; Shrihari, Swathi; Gorman, Matthew J; Ying, Baoling; Yaun, Dansu; Raju, Saravanan; Chen, Rita E; Dmitriev, Igor P; Kashentseva, Elena; Adams, Lucas J; Mann, Colin; Davis-Gardner, Meredith E; Suthar, Mehul S; Shi, Pei-Yong; Saphire, Erica Ollmann; Fremont, Daved H; Curiel, David T; Alter, Galit; and Diamond, Michael S, "An intranasal vaccine durably protects against SARS-CoV-2 variants in mice." Cell Reports. 36, 4. 109452 (2021).
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An intranasal vaccine durably protects against SARS-CoV-2 variants in mice

Highlights

- Immunization with ChAd-SARS-CoV-2 S induces durable immunity

- Intranasal ChAd-SARS-CoV-2 S induces inhibitory IgG and IgA Abs

- Abs induced by intranasal ChAd-SARS-CoV-2 S have robust Fc effector functions

- Intranasal ChAd-SARS-CoV-2 S confers cross-protection against variants of concern

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In brief
Hassan et al. show that immunization with ChAd-SARS-CoV-2-S is durably immunogenic and protects against SARS-CoV-2 challenge in a dose-dependent manner. Many months after single-dose intranasal immunization, ChAd-SARS-CoV-2 confers protection against variants of concerns of SARS-CoV-2 in both the upper and lower respiratory tracts of mice.
An intranasal vaccine durably protects against SARS-CoV-2 variants in mice


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SUMMARY

SARS-CoV-2 variants that attenuate antibody neutralization could jeopardize vaccine efficacy. We recently reported the protective activity of an intranasally administered spike protein-based chimpanzee adenovirus-vectored vaccine (ChAd-SARS-CoV-2-S) in animals, which has advanced to human trials. Here, we assessed its durability, dose response, and cross-protective activity in mice. A single intranasal dose of ChAd-SARS-CoV-2-S induced durably high neutralizing and Fc effector antibody responses in serum and S-specific IgG and IgA secreting long-lived plasma cells in the bone marrow. Protection against a historical SARS-CoV-2 strain was observed across a 100-fold vaccine dose range and over a 200-day period. At 6 weeks or 9 months after vaccination, serum antibodies neutralized SARS-CoV-2 strains with B.1.351, B.1.1.28, and B.1.617.1 spike proteins and conferred almost complete protection in the upper and lower respiratory tracts after challenge with variant viruses. Thus, in mice, intranasal immunization with ChAd-SARS-CoV-2-S provides durable protection against historical and emerging SARS-CoV-2 strains.

INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the etiologic agent of the coronavirus disease 2019 (COVID-19) syndrome, which can rapidly progress to pneumonia, respiratory failure, and systemic inflammatory disease (Cheung et al., 2020; Mao et al., 2020; Wichmann et al., 2020). The elderly, immunocompromised, and those with certain co-morbidities (e.g., obesity, diabetes, and hypertension) are at greatest risk of severe disease, requirement of mechanical ventilation, and death (Zhou et al., 2020). To date, approximately 184 million infections and 4 million deaths have been recorded worldwide (https://covid19.who.int) since the start of the pandemic. The extensive morbidity and mortality associated with COVID-19 pandemic have made the development and deployment of SARS-CoV-2 vaccines an urgent global health priority.

The spike (S) protein of the SARS-CoV-2 virion is the principal target for antibody-based and vaccine countermeasures. The S protein serves as the primary viral attachment and entry factor and engages the cell-surface receptor angiotensin-converting enzyme 2 (ACE2) to promote SARS-CoV-2 entry into human cells (Letko et al., 2020). SARS-CoV-2 S proteins are cleaved to yield S1 and S2 fragments (Hoffmann et al., 2020), with the S1 protein containing the receptor binding domain (RBD) and the S2 protein promoting membrane fusion and virus penetration into the cytoplasm. The prefusion form of the SARS-CoV-2 S protein (Wrapp et al., 2020) is recognized by potently neutralizing monoclonal antibodies (Barnes et al., 2020; Cao et al., 2020b; Pinto et al.,...
Many vaccine candidates targeting the SARS-CoV-2 S protein have been developed (Burton and Walker, 2020) using DNA plasmid, lipid nanoparticle encapsulated mRNA, inactivated virion, protein subunit, or viral-vectorized vaccine platforms (Graham, 2020). Several vaccines administered by intramuscular (IM) injection (e.g., Pfizer/BioNTech BNT162b2 and Moderna-1273 mRNA [Baden et al., 2021; Polack et al., 2020] and Johnson & Johnson Ad26.COV2 and AstraZeneca ChAdOx1 nCoV-19 adenoviral [Barrett et al., 2021; Sadoff et al., 2021]) platforms have been granted emergency-use authorization in many countries with hundreds of millions of doses given worldwide (https://covid19.who.int).

While vaccines administered by IM injection induce robust systemic immunity that protects against severe disease and mortality, questions remain as to their ability to curtail SARS-CoV-2 transmission, especially if upper-airway infection is not reduced. Indeed, many of the IM-administered vaccines showed variable protection against upper-airway infection and transmission in pre-clinical studies and failed to induce substantive mucosal (immunoglobulin A [IgA]) immunity (Mercado et al., 2020; van Doremalen et al., 2020; Wang et al., 2020; Yu et al., 2020). This issue is important because of the emergence of more transmissible SARS-CoV-2 variants including B.1.1.7 (Alpha), B.1.351 (Beta), B.1.617.2 (Delta) with substitutions in the spike protein. Experiments with pseudoviruses and authentic SARS-CoV-2 strains also suggest that neutralization by vaccine-induced sera is diminished against variants expressing mutations in the spike gene at positions L452, E484, and elsewhere (Chen et al., 2021; McCallum et al., 2021; Tada et al., 2021; Wang et al., 2021a, 2021b; Wibmer et al., 2021). Beyond possible negative impacts on protection, the combination of diminished immunity against certain variants and naturally lower anti-S IgG levels in the respiratory mucosa could create conditions for further selection of resistance in the upper airway and transmission into the general population.

We recently described a single-dose, intranasally (IN)-delivered chimpanzee Adenovirus (simian Ad-36)-based SARS-CoV-2 vaccine (ChAd-SARS-CoV-2-S) encoding a pre-fusion stabilized S protein that induced robust humoral, cell-mediated, and mucosal immune responses and limited upper- and lower-airway infection in K18-hACE2 transgenic mice, hamsters, and non-human primates (Bricker et al., 2021; Hassan et al., 2020b, 2021). This vaccine, which has advanced to human clinical trials (BBV154, Clinical Trial NCT04751682), differs from ChAdOx1 nCoV-19, a chimpanzee Ad-23-based SARS-CoV-2 vaccine, currently granted emergency use in some countries. Here, as a further step to evaluating the potential utility of ChAd-SARS-CoV-2-S, we assessed its dose response, durability, and cross-protective activity in mice including effects on upper- and lower-airway infection. At approximately 9 months after IN immunization, neutralizing antibody and anti-S protein IgG and IgA levels in serum of ChAd-SARS-CoV-2-S-vaccinated animals remained high and inhibited infection with SARS-CoV-2 strains with B.1.351, and B.1.1.28 spike proteins. At this time point, susceptible K18-hACE2 transgenic mice were fully protected against upper and lower respiratory tract infection after challenge with a SARS-CoV-2 virus displaying B.1.351 spike proteins.

**RESULTS**

**A single ChAd-SARS-CoV-2-S immunization induces durable anti-spike and neutralizing responses at different doses**

We assessed the durability of humoral immune responses in BALB/c mice 100 or 200 days post-IM or IN immunization with escalating doses of ChAd-SARS-CoV-2-S (10^8, 10^9, and 10^10 viral particles [vp]) or 10^10 vp of a ChAd-Control vaccine (Figure 1A). First, we measured anti-S and anti-RBD IgG and IgA levels by ELISA. Consistent with prior results at a 1-month time point (Hassan et al., 2020b), at 100 or 200 days post-vaccination, IN immunization with ChAd-SARS-CoV-2-S induced superior antibody responses in serum than IM immunization or vaccination with ChAd-Control (Figures 1B–1M and S1). At 100 days after IN immunization with 10^10, 10^9, and 10^8 vp of ChAd-SARS-CoV-2-S, geometric mean titers (GMTs) of serum S-specific IgG responses were 1.1 x 10^6, 4.8 x 10^5, and 2.6 x 10^5, and RBD-specific IgG were 3.2 x 10^5, 1.8 x 10^5, and 8.7 x 10^4, respectively (Figure 1B). In comparison, S- and RBD-specific IgG responses 100 days after IM immunization with 10^10, 10^9, and 10^8 vp of ChAd-SARS-CoV-2-S were 4- to 6-fold lower (p < 0.0001) with S-specific IgG titers of 2.1 x 10^5, 1.1 x 10^5, and 4.5 x 10^4 and RBD-specific IgG titers of 5.1 x 10^4, 2.9 x 10^4, and 2.3 x 10^4, respectively (Figure 1E). A similar dose response was observed with S- and RBD-specific IgG titers in serum at 200 days after IN or IM immunization (Figures 1H and 1K). At 200 days after IN immunization with 10^10, 10^9, and 10^8 vp of ChAd-SARS-CoV-2-S, GMT of S-specific IgG were 2.8 x 10^6, 2.4 x 10^5, and 1.2 x 10^5, and RBD-specific IgG were 1.1 x 10^5, 6.1 x 10^4, and 3.2 x 10^4, respectively (Figure 1H). At 200 days after IM immunization with 10^10, 10^9, and 10^8 vp of ChAd-SARS-CoV-2-S, S-specific IgG GMT were 8.1 x 10^5, 6.9 x 10^5, and 2.6 x 10^5, and RBD-specific IgG GMT were 1.4 x 10^5, 1.3 x 10^5, and 8.0 x 10^4, respectively.
(Figure 1K). Thus, anti-S and anti-RBD IgG levels were higher after IN than IM immunization and continued to rise in serum even several months after single-dose vaccination.

We next assessed the induction and durability of serum IgA responses. Although IM immunization failed to induce S- or RBD-specific IgA (Figures 1F and 1L), substantial levels of anti-S- and RBD IgA were detected after IN immunization at 100 or 200 days post-immunization (Figures 1C and 1I). At 100 days after IN immunization with 10^{10}, 10^{9}, and 10^{8} vp of ChAd-SARS-CoV-2-S, the GMT of S-specific IgA were 4.8 × 10^{3}, 1.2 × 10^{3}, and 8.4 × 10^{2}, and RBD-specific IgA were 2.2 × 10^{3}, 4.6 × 10^{2}, and 2.9 × 10^{2}, respectively (Figure 1C). As seen with IgG, the IgA levels continued to increase over time such that at 200 days the GMT of S-specific IgA were 1.1 × 10^{4}, 7.4 × 10^{3}, and 5.4 × 10^{3}, and RBD-specific IgA were 5.2 × 10^{3}, 3.8 × 10^{3}, and 9.8 × 10^{2} after IN immunization with 10^{10}, 10^{9}, and 10^{8} vp of ChAd-SARS-CoV-2-S, respectively (Figure 1I).

We next evaluated a functional correlate of the serological response by assaying neutralizing activity (Figures 1D, 1G, 1J, 1M, and S1) using a focus-reduction neutralization test (FRNT) (Case et al., 2020). As expected, neutralizing activity was not detected in sera from ChAd-control-treated mice. At 100 days post-IN immunization with 10^{10}, 10^{9}, and 10^{8} vp of ChAd-SARS-CoV-2-S, the mean effective half maximal inhibitory titers [EC_{50}] were 39,449, 9,989, and 7,270, respectively (Figure 1D). In comparison, at this time point after IM immunization with 10^{10}, 10^{9}, and 10^{8} vp of ChAd-SARS-CoV-2-S, the EC_{50} values were 8- to 20-fold lower (p < 0.0001) at 4,988, 2,017, and 391, respectively (Figure 1I). At 200 days after IN immunization with 10^{10}, 10^{9}, and 10^{8} vp of ChAd-SARS-CoV-2-S, and consistent with the higher anti-S and RBD titers seen, EC_{50} values were 45,591, 22,769, and 23,433, respectively (Figure 1J). In comparison, 200 days after IM immunization with 10^{10}, 10^{9}, and 10^{8} vp of ChAd-SARS-CoV-2-S, EC_{50} values were much lower at 2,524, 940, and 716, respectively (Figure 1M).

Long-lived plasma cells (LLPCs) reside in the bone marrow and constitutively secrete high levels of antibody that correlate with serum levels (Amanna and Silfka, 2010). To assess the levels of antigen-specific LLPCs at 200 days after IN or IM immunization with 10^{10} vp of ChAd-SARS-CoV-2-S, CD138+ cells were isolated from the bone marrow and assayed for S-specific IgG or IgA production using an ELISPOT assay (Purtha et al., 2011). We observed a ∼4-fold higher frequency of LLPCs secreting S-specific IgG after IN immunization than IM immunization (Figure 1N). Additionally, after IN immunization, we detected LLPCs producing S-specific IgA, which were absent after IM immunization (Figure 1N).

**IN inoculation of ChAd-SARS-CoV-2-S induces broad antibody responses with Fc effector function capacity**

To characterize the humoral response further, we analyzed antibody binding to SARS-CoV-2 variant proteins and Fc effector functions using serum derived from BALB/c mice at 90 days after IN or IM vaccination. Our panel of SARS-CoV-2 proteins included spike (D614G, E484K, N501Y, ΔE98-70, K417N) and RBD (E484K) antigens corresponding to WA1/2020, B.1.1.7, B.1.351, and B.1.1.28 strains. We first measured the anti-SARS-CoV-2 specific antibody response for several isotypes (IgG1, IgG2a, IgG2b, IgG3, IgM, and IgA) and their ability to bind Fcγ receptors (mouse FcγRIIB, FcγRIII, and FcγRII) using a luminex platform. Consistent with data obtained by ELISA (Figures 1B and 1E), IN vaccination of ChAd-SARS-CoV-2-S induced higher levels of IgG1 to D614G spike and WA1/2020 RBD proteins than IM immunization, and as expected, decreasing doses of the vaccine elicited lower antibody titers (Figure 2A). Anti-SARS-CoV-2 IgG1 titers after IN immunization also were higher against all spike and RBD variants than after IM immunization, and titers decreased with vaccine dose (Figure 2B). As shown in a heatmap, this trend was observed for all anti-SARS-CoV-2 specific antibody isotypes and correlated with FcγR binding patterns (Figure 2C). These data suggest that IN vaccination induces a higher magnitude and broader antibody subclass response to SARS-CoV-2 than IM vaccination.

Antibody effector functions, such as opsonization, are mediated in part by Fcγ receptor engagement (Bruhns and Jönsson, 2015). To determine whether the observed differences in antibody titers and FcγR binding titers resulted in differences in effector functions, we performed antibody-dependent neutrophil (ADNP) and cellular phagocytosis (ADCP) assays (Figures 2D and 2E). Sera from IN-vaccinated mice stimulated substantially more ADNP than those obtained from IM-vaccinated mice. However, minimal differences in ADCP were apparent from IN vaccination compared to IM vaccination.
antibodies derived after IN and IM vaccination (Figures 2D and 2E). These data demonstrate that IN vaccination with ChAd-SARS-CoV-2-S induces a greater and more functional antibody response than after IM vaccination.

Intranasally administered ChAd-SARS-CoV-2-S induces durable protection against SARS-CoV-2 challenge in BALB/c mice

To assess the efficacy of the ChAd-SARS-CoV-2-S vaccine, immunized BALB/c mice given the dosing regimen described in Figure 1A were challenged with SARS-CoV-2. Virus challenge was preceded by intranasal introduction of Hu-Ad5-hACE2, which enables ectopic expression of hACE2 and productive infection of SARS-CoV-2 in BALB/c mice by historical SARS-CoV-2 strains (Hassan et al., 2020a; Sun et al., 2020). Animals were immunized once via IN or IM routes with $10^{10}$ vp of ChAd-Control or $10^8$, $10^9$, or $10^{10}$ vp of ChAd-SARS-CoV-2-S. At day 95 or 195 post-vaccination, mice were given $10^8$ plaque-forming units (PFUs) of Hu-Ad5-hACE2 and anti-Ifnar1 mAb; the latter attenuates innate immunity and enhances pathogenesis in this model (Hassan et al., 2020a). Five days later, BALB/c mice were challenged with $5 \times 10^4$ FFU of SARS-CoV-2 (strain WA1/2020) via IN route. At 4 days post-infection (dpi), lungs, spleen, and heart were harvested from mice challenged at 100 days post-immunization, and lungs, nasal turbinates, and nasal washes were collected from a second cohort challenged at 200 days post-immunization. Tissues were assessed for viral burden by quantitative reverse transcription PCR (qRT–PCR) using primers for the subgenomic RNA (N gene). IN immunization with all three doses induced remarkable protection at 100 days post-vaccination as evidenced by a virtual absence of viral RNA in lungs, spleen, and heart compared to animals receiving the ChAd-Control vaccine (Figures 3A–3C). At 200 days post-immunization, protection conferred by the IN delivered ChAd-SARS-CoV-2-S remained robust in the upper and lower respiratory tracts compared to ChAd-Control immunized mice. Nevertheless, we observed limited infection breakthrough in the lungs and nasal turbinates in animals immunized with the lowest $10^8$ vp dose of ChAd-SARS-CoV-2-S (Figures 3G and 3I). In comparison, protection at 100 days post-IM immunization was less than after IN immunization at the same challenge time point. Although viral RNA was not detected in the heart and spleen (Figure 3E–F), at least 1,000- to 30,000-fold (p < 0.0001) higher levels were measured in the lungs of mice immunized with ChAd-SARS-CoV-2-S by
the IM compared to IN route (Figures 3A and 3D). We also observed a greater impact of dosing by the IM route, as the reduction in viral RNA load in the lungs at 10^8 vp dose no longer was different than in the ChAd-control-vaccinated mice (Figure 3D). At 200 days post-IM immunization, we observed less protection against SARS-CoV-2 infection in the lungs, nasal washes, and nasal turbinates than after IN immunization (Figures 3G–3L).

**ChAd-SARS-CoV-2-S induces durable immunity in hACE2 transgenic mice**

We next assessed the immunogenicity of intranasally delivered ChAd-SARS-CoV-2-S in K18-hACE2 C57BL/6 mice, which are more vulnerable to SARS-CoV-2 infection than BALB/c mice (Golden et al., 2020; Winkler et al., 2020; Yinda et al., 2021). Five-week-old K18-hACE2 mice were inoculated via an IN route with 10^9 vp of ChAd control or ChAd-SARS-CoV-2-S. Serum samples were collected 6 weeks later, and humoral immune responses were evaluated. IN immunization of ChAd-SARS-CoV-2-S but not ChAd control induced high levels of S- and RBD-specific IgG and IgA (Figures 4A and 4B). Neutralizing antibody titers against WA1/2020 and three other SARS-CoV-2 strains with spike proteins from B.1.351 and B.1.1.28 variants or B.1.617.1 isolate were measured by FRNT assay (Figures 4C–4E and S2). High levels of neutralizing antibody against WA1/2020 were induced after a single IN dose of ChAd-SARS-CoV-2-S. As seen with vaccine-induced human sera against some variant viruses (Chen et al., 2021; McCallum et al., 2021; Tada et al., 2021; Wang et al., 2021a, 2021b; Wibmer et al., 2021), we observed decreases in neutralizing titers against Wash-B.1.351 (≈5-fold, p < 0.0001; Figure 4C), Wash-B.1.1.28 (≈3-fold, p < 0.0001; Figure 4D), and B.1.617.1 (≈7-fold, p < 0.01, Figure 4E) strains compared to WA1/2020. To assess the durability of humoral responses, a separate cohort of K18-hACE2 mice was immunized via the IN route, and serum samples were collected at 9 months. ChAd-SARS-CoV-2-S induced high levels of S- and RBD-specific IgG and IgA and neutralizing antibody against WA1/2020 (EC_{50} of 12,550) at this time point (Figures 4F–4J and S2). When tested against the Wash-B.1.351, Wash-B.1.1.28, and B.1.617.1 viruses, we also observed a diminished neutralizing titer (≈6- to 8-fold, p < 0.05; Figures 4H–4J) compared to WA1/2020, although they still remained high (EC_{50} of 1,627, 1,918, and 2,108, respectively).

**ChAd-SARS-CoV-2-S confers cross-protection against Wash B.1.351 and Wash-B.1.1.28 challenge in hACE2 transgenic mice**

We tested the protective efficacy of ChAd-SARS-CoV-2-S against WA1/2020 and two chimeric viruses (Wash-B.1.351 and Wash-B.1.1.28) with spike genes corresponding to variants.
of concern (Figure 5A). Five-week old K18-hACE2 mice were immunized via an IN route with a single 10^9 vp dose of ChAd control or ChAd-SARS-CoV-2-S. Six weeks later, mice were challenged by an IN route with 10^4 FFU of Wash-B.1.351, Wash-B.1.1.28, or WA1/2020. All mice immunized with ChAd-SARS-CoV-2 exhibited no weight loss, whereas most ChAd-Control-vaccinated mice experienced substantial weight loss at 3 to 6 dpi (Figures 5B, 5G, and 5L). Remarkably, vaccination with ChAd-SARS-CoV-2-S resulted in almost no detectable SARS-CoV-2 RNA in the upper and lower respiratory tracts, heart,
and brain at 6 dpi (Figures 5C–5F, 5H–5K, and 5M–5O). As a further test of the durability of the cross-protective response, 5-week-old K18-hACE2 mice were immunized via an IN route with a single 10^{10} vp dose of ChAd control or ChAd-SARS-CoV-2-S. Nine months later, mice were challenged via IN route with 10^6 FFU of Wash-B.1.351. ChAd-SARS-CoV-2-S-vaccinated mice maintained weight in contrast to ChAd-Control-treated mice (Figure 5P). Moreover, substantial virological protection was observed, as only very low amounts of Wash-B.1.351 SARS-CoV-2 RNA were detected in the upper and lower respiratory tracts, heart, and brain in some of the mice (Figures 5Q–5T).

**DISCUSSION**

The durability of vaccine-induced immune responses is a key for providing sustained protection against SARS-CoV-2 infection and curtailing the current pandemic. Here, we show that a single IN immunization with ChAd-SARS-CoV-2-S induced S- and RBD-specific binding and neutralizing antibodies that continued to rise for several months, suggestive of sustained germinal center reactions. LLPCs in the bone marrow were detected 6 months after IN vaccination, secreting SARS-CoV-2-specific IgG and IgA that likely contributed to the durably high antiviral antibody levels in circulation (Amanza and Siflíka, 2010). In comparison, IM immunization with ChAd-SARS-CoV-2-S induced lower levels of serum neutralizing antibodies, fewer spike-specific IgG secreting LLPCs, and virtually no serum or cellular IgA response. At least in mice, a single IN dose immunization with ChAd-SARS-CoV-2-S produced durable humoral immunity that was observed across a 100-fold dose range. These pre-clinical immunogenicity results compare favorably with studies in humans with mRNA vaccines against SARS-CoV-2, which show humoral immune responses lasting at least several months (Doria-Rose et al., 2021; Widge et al., 2021). In comparison, the durability of antibody responses after natural SARS-CoV-2 infection can vary considerably (Dan et al., 2021; Gudbjartsson et al., 2020).

A single immunization of ChAd-SARS-CoV-2-S conferred durable protection against SARS-CoV-2 (WA1/2020 strain) challenge in hACE2-tranduced BALB/c mice or K18-hACE2 transgenic C57BL/6 mice at multiple time points. IN immunization in particular provided virtually complete virological protection against upper and lower respiratory tract infection, with only a limited infection breakthrough seen at the 100-fold lower vaccine dose. The abrogation of infection in the upper respiratory tract suggests that IN vaccination could prevent transmission, although corroborating studies are needed in other rodent (e.g., hamsters or ferret) models better suited to studying this question (Muñoz-Fontela et al., 2020). In comparison, IM immunization reduced the viral RNA levels in the lungs but showed substantially less protection against the homologous WA1/2020 strain in samples from the upper respiratory tract. While many SARS-CoV-2 vaccine candidates from different platforms have demonstrated immunogenicity and protective efficacy in animals models (García-Arriaza et al., 2021; Henrich et al., 2021; Tostanoski et al., 2020; van Doremalen et al., 2020; Vogel et al., 2021; Yao et al., 2021; Yu et al., 2020), to our knowledge, none have established durability or protection against variant viruses. The long-term protection conferred by IN immunization even at 100-fold lower inoculating doses is promising but remains to be validated in human clinical trials with ChAd-SARS-CoV-2-S. If results in mice were recapitulated, dose sparing strategies could enable production of a large number of vaccine doses that could curtail infection and transmission of SARS-CoV-2.

The emergence of SARS-CoV-2 S variants with mutations of amino acids in the receptor binding motif (e.g., B.1.351, B.1.1.28, and B.1.617) is of concern because of their resistance to the inhibitory activity of many neutralizing antibodies (Chen et al., 2021; Wang et al., 2021a, 2021b). Indeed, human sera from subjects vaccinated with BNT162b2 mRNA or ChAdOx1 nCoV-19 (AZD1222) vaccines showed reduced neutralization against B.1.351 (Chen et al., 2021; Madhi et al., 2021; Zhou et al., 2021). Concerningly, IM-administered ChAdOx1 nCoV-19 (AZD1222) showed reduced protective efficacy against mild to moderate B.1.351 infection in humans (Madhi et al., 2021). In K18-hACE2 transgenic mice, when we compared the immunogenicity of IN-delivered ChAd-SARS-CoV-2-S against WA1/2020 and chimeric SARS-CoV-2 strains expressing B.1.1.28 or B.1.351 spike proteins or a B.1.617.1 isolate, we also observed reduced (3- to 8-fold) neutralization of the variant viruses although the titers remained >1,000. At 6 weeks post-IN immunization of ChAd-SARS-CoV-2-S, K18-hACE2 mice were fully protected against weight loss and infection in the upper and lower respiratory tracts and brain by WA1/2020, Wash-B.1.351, and Wash-B.1.1.28. Remarkably, in a separate cohort of K18-hACE2 mice challenged 9 months after single IN immunization, animals maintained high neutralizing titers against all of the variant strains and were fully protected against Wash-B.1.351 challenge. Although correlates of protection are not fully established for SARS-CoV-2 vaccines, the high levels of cross-neutralizing antibodies against the variant viruses combined with robust virus-specific systemic and mucosal CD8+ T cell responses described previously (Hassan et al., 2020b) likely contribute to protection. Beyond this, antibody effector functions also might contribute to prevent SARS-CoV-2 infection and disease (Bartsch et al., 2021; Schafer et al., 2021; Winkler et al., 2021). Indeed, we observed enhanced Fc effector functions against SARS-CoV-2 variant proteins in serum derived from IN-delivered ChAd-SARS-CoV-2-S including robust induction of ADNP and ADCP responses.

**Limitations of study**

Although a single intranasal administration of ChAd-SARS-CoV-2-S durably protected against SARS-CoV-2 variant replication in the upper and lower respiratory tracts even ~9 months after immunization, we note several limitations in our study. (1) We performed challenge studies in BALB/c mice transduced with hACE2 or C57BL/6 mice expressing an hACE2 transgene. Durability and protection studies will need to be corroborated in hamsters, non-human primates, and ultimately in humans. (2) Although our studies suggest that the mucosal immunity induced by intranasal vaccination could limit SARS-CoV-2 transmission, the use of mice precluded formal respiratory transmission analysis, which is better studied in hamsters and ferrets (Muñoz-
and include the following:

Detailed methods are provided in the online version of this paper. STAR can be a promising platform for preventing SARS-CoV-2 infection, variants of concern, IN delivery of ChAd-SARS-CoV-2-S may provide a sufficient cushion to overcome this loss in potency. Studies in other animals or with even lower doses of vaccine where neutralizing titers might be lower are needed to determine whether the protective phenotype against variants of concern is maintained. (4) Finally, we did not establish the correlates of protection in these studies, as passive antibody transfer or T cell deletions were not performed. Such studies could be performed in follow-up experiments.

In summary, our study shows that IN immunization with ChAd-SARS-CoV-2-S induces robust and durable binding IgG and IgA antibody, neutralizing antibody, Fc effector functions, and LLPC responses against SARS-CoV-2. In mice, a single IN immunization with ChAd-SARS-CoV-2-S confers cross-protection against SARS-CoV-2 strains displaying spike proteins corresponding to B.1.351, B.1.1.28, and B.1.617.1 variants, even 9 months after vaccination. Given the efficacy of preclinical evaluation in multiple animal models (Bricker et al., 2021; Hassan et al., 2021; Hassan et al., 2020b) and the durable protective immunity against variants of concern, IN delivery of ChAd-SARS-CoV-2-S may be a promising platform for preventing SARS-CoV-2 infection, curtailing transmission, and, thus, warrants further clinical evaluation in humans.

STAR METHODS

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## STAR★METHODS

### KEY RESOURCES TABLE

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<tr>
<td>Anti-SARS-CoV-2 mAb cocktail</td>
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<td>Steptavidin-R-Phycoerythrin</td>
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<td>SARS-CoV-2 (Strain B.1.267.1)</td>
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<td>Adv5-hACE2</td>
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<td><strong>Experimental models: Cell lines</strong></td>
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<td>J774A.1</td>
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<td>Thermo Fisher Scientific</td>
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<td>SARS-CoV-2 Spike</td>
<td>Dr. Daved Fremont and Dr. Erica Saphire</td>
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<td>SARS-CoV-2 RBD</td>
<td>Dr. Daved Fremont and Dr. Florian Krammer</td>
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<td>Murine FcγRIib, RIII, RIV</td>
<td>Produced at the Duke Human Vaccine Institute</td>
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</table>

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Michael S. Diamond (diamond@wusm.wustl.edu).

Materials availability
All requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact author. This includes mice, antibodies, viruses, vaccines, and proteins. All reagents will be made available on request after completion of a Materials Transfer Agreement.

Data and code availability
All data supporting the findings of this study are available within the paper or from the corresponding author upon request. 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

Viruses and cells
Vero E6 (CRL-1586, American Type Culture Collection (ATCC), Vero-TMPRSS2 (Zang et al., 2020), Vero (CCL-81, ATCC) and HEK293 (CRL-1573, ATCC) cells were cultured at 37 °C in Dulbecco’s Modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES pH 7.3, 1 mM sodium pyruvate, 1X non-essential amino acids, and 100 U/ml of penicillin–streptomycin. Vero-TMPRSS2 cells also were supplemented with 5 μg/mL of blasticidin.

SARS-CoV-2 strain 2019n-CoV/USA_WA1/2020 (WA1/2020) was obtained from the Centers for Disease Control and Prevention. The virus was passaged once in Vero CCL-81 cells and titrated by focus-forming assay (FFA) on Vero E6 cells. The Wash-B.1.351 and Wash-B.1.1.28 chimeric viruses with variant spike genes were described previously (Chen et al., 2021; Xie et al., 2020), passaged...
once in Vero-TMPRSS2 cells, and subjected to next-generation sequencing to confirm the introduction and stability of substitutions. The B.1.617.1 variant was plaque purified from a midturbinate nasal swab, passed twice on Vero-TMPRSS2 cells, and next-generation sequenced (spike substitutions: G142D, E154K, L452R, E484Q, D614G, P681R, Q1071H, and H1101D). All virus experiments were performed in an approved Biosafety level 3 (BSL-3) facility.

Mouse experiments
Animal studies were carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved by the Institutional Animal Care and Use Committee at the Washington University School of Medicine (Assurance number A3381-01). Virus inoculations were performed under anesthesia that was induced and maintained with ketamine hydrochloride and xylazine, and all efforts were made to minimize animal suffering.

Female BALB/c (catalog 000651) and K18-hACE2 C57BL/6 (catalog 034860) mice were purchased from The Jackson Laboratory. Four to five-week-old animals were immunized with 10¹⁰ vp of ChAdV-control or 10⁶, 10⁸, or 10¹⁰ vp of ChAd-SARS-CoV-2-S in 50 µl PBS via IM (hind leg) or IN injection. Vaccinated BALB/c mice (10 to 11-week-old) were given a single intraperitoneal injection of 2 mg of anti-Ifnar1 mAb (MAR1-5A3 (Sheehan et al., 2006) (Leinco) one day before IN administration of 10⁸ PFU of Hu-Ad5-hACE2 (Hassan et al., 2020a). Five days after Hu-Ad5-hACE2 transduction, mice were inoculated with 4 × 10⁵ PFU of WA1/2020 SARS-CoV-2 by the IN route. K18-hACE2 mice were challenged on indicated days after immunization with 10⁴ PFU of SARS-CoV-2 (WA1/2020, Wash-B.1.351, or Wash-B.1.1.28) via IN route. Animals were euthanized at 6 dpi, and tissues were harvested for virological analysis.

METHOD DETAILS

Chimpanzee and human adenovirus vectors
The ChAd-SARS-CoV-2 and ChAd-Control vaccine vectors were derived from simian Ad36 backbones (Roy et al., 2011), and the constructing and validation has been described in detail previously (Hassan et al., 2020b). The rescued replication-incompetent ChAd-SARS-CoV-2-S and ChAd-Control vectors were scaled up in HEK293 cells and purified by CsCl density-gradient ultracentrifugation. Viral particle concentration in each vector preparation was determined by spectrophotometry at 260 nm as described (Maizel et al., 1968). The Hu-AdV5-hACE2 vector also was described previously (Hassan et al., 2020b) and produced in HEK293 cells. The viral titer was determined by plaque assay in HEK293 cells.

SARS-CoV-2 neutralization assays
Heat-inactivated serum samples were diluted serially and incubated with 10⁵ FFU of different SARS-CoV-2 strains for 1 h at 37°C. The virus-serum mixtures were added to Vero cell monolayers in 96-well plates and incubated for 1 h at 37°C. Subsequently, cells were overlaid with 1% (w/v) methylcellulose in MEM supplemented with 2% FBS. Plates were incubated for 30 h before fixation using 4% PFA in PBS for 1 h at room temperature. Cells were washed and then sequentially incubated with an oligoclonal pool of SARS2-2, SARS2-11, SARS2-16, SARS2-31, SARS2-38, SARS2-57, and SARS2-71 (Liu et al., 2021) anti-S antibodies and HRP-conjugated goat anti-mouse IgG (Sigma, 12-349) in PBS supplemented with 0.1% saponin and 0.1% bovine serum albumin. TrueBlue peroxidase substrate (KPL) was used to develop the plates before counting the foci on a BioSpot analyzer (Cellular Technology Limited).

Protein expression and purification
The cloning and production of purified S and RBD proteins corresponding to the WA1/2020 SARS-CoV-2 strain have been described previously (Alsoussi et al., 2020; Hassan et al., 2020b). Briefly, prefusion-stabilized S (Hsieh et al., 2020) and RBD were cloned into a pCAGGS mammalian expression vector with a hexahistidine tag and transiently transfected into Expi293F cells. Proteins were purified by cobalt-charged resin chromatography (G-Biosciences).

ELISA
Purified antigens (S or RBD) were coated onto 96-well Maxisorp clear plates at 2 µg/mL in 50 mM Na₂CO₃ pH 9.6 (70 µL) overnight at 4°C. Coating buffers were aspirated, and wells were blocked with 200 µL of 1X PBS + 0.05% Tween-20 + 1% BSA + 0.02% NaN₃ (Blocking buffer, PBSTBA) overnight at 4°C. Heat-inactivated serum samples were diluted in PBSTBA in a separate 96-well polypropylene plate. The plates then were washed thrice with 1X PBS + 0.05% Tween-20 (PBST), followed by addition of 50 µL of respective serum dilutions. Sera were incubated in the blocked ELISA plates for at least 1 h at room temperature. The ELISA plates were again washed thrice in PBST, followed by addition of 50 µL of 1:1,000 anti-mouse IgG-HRP (Southern Biotech Cat. #1030-05) in PBST or 1:1000 of anti-mouse IgA-HRP in PBSTBA (SouthernBiotech). Plates were incubated at room temperature for 1 h, washed thrice in PBST, and then 100 µL of 1-Step Ultra TMB-ELISA was added (ThermoFisher Cat. #34028). Following a 10 to 12-min incubation, reactions were stopped with 50 µL of 2 M sulfuric acid. Optical density (450 nm) measurements were determined using a microplate reader (Bio-Rad).

ELISpot assay
To quantitate S-specific plasma cells in the bone marrow, femurs and tibias were crushed using a mortar and pestle in RPMI 1640, filtered through a 100 µm strainer and subjected to ACK lysis. CD138+ cells were enriched by positive selection and magnetic beads...
according to the manufacturer’s instructions (EasySep Mouse CD138 Positive Selection, STEMCELL). The enriched CD138+ cells were incubated overnight in RPMI 1640 supplemented with 10% FBS in MultiScreen-HA Filter Plates (Millipore) pre-coated with SARS-CoV-2 S protein. Foci were developed using TruBlue substrate (KPL) following sequential incubation with anti-mouse IgG-biotin or anti-mouse IgA-biotin and streptavidin-HRP. Plates were imaged using a BioSpot instrument, and foci enumerated manually.

Measurement of viral burden
SARS-CoV-2 infected mice were euthanized using a ketamine and xylazine cocktail, and organs were collected. Tissues were weighed and homogenized with beads using a MagNA Lyser (Roche) in 1 mL of Dulbecco’s Modified Eagle’s Medium (DMEM) containing 2% fetal bovine serum (FBS). RNA was extracted from clarified tissue homogenates using MagMax mirVana Total RNA isolation kit (Thermo Scientific) and the KingFisher Flex extraction system (Thermo Scientific). SARS-CoV-2 RNA levels were measured by one-step quantitative reverse transcriptase PCR (qRT-PCR) TaqMan assay as described previously (Hassan et al., 2020a). SARS-CoV-2 nucleocapsid (N) specific primers and probe sets were used: (N: F Primer: ATGCTGCAATCGTGCTACAA; R primer: GACTGCCGCTCTGCTC; probe: /56-FAM/TCAAGGAAC/ZEN/AACATTGCCCCA/3IABkFQ) (Integrated DNA Technologies). Viral RNA was expressed as (N) gene copy numbers per milligram on a log10 scale.

Luminex analysis
Luminex analysis was conducted as described previously (Brown et al., 2017). Briefly, proteins (Spike: D614G, E484K, N501D69-70, K417N, B.1.1.7, B.1.351; RBD (ImmuneTech): WT, E484K, B.1.1.7, B.1.351, B.1.128) were carboxy-coupled to magnetic Luminex micropelx carboxylated beads (Luminex Corporation) using NHS-ester linkages with Sulfo-NHS and EDC (Thermo Fisher) and then incubated with serum (IgG1, FcγRIIb, FcγRIII 1:3000; IgG2a, G2b, G3, A, FcγRIV 1:1000, IgM 1:500) for 2 h at 37 °C. Isotype analysis was performed by incubating the immune complexes with secondary goat anti-mouse-PE antibody (IgG1 1070-09, IgG2a 1080-09S, IgG3 1100-09, IgM 1020-09, IgA 1040-09, Southern Biotech) for each isotype. FcγR binding was quantified by incubating immune complexes with biotinylated FcγRs (FcγRIIB, FcγRIII, and FcγRIV, courtesy of Duke Protein Production Facility) conjugated to Steptavidin-PE (Prozyme). Flow cytometry was performed with an IQue (Intellicyt), and analysis was performed on IntellCyt ForeCyt (v8.1).

Antibody-dependent neutrophil or cellular phagocytosis
Antibody-dependent neutrophil phagocytosis (ADNP) and cellular phagocytosis (ADCP) assays were conducted as described previously (Butler et al., 2019; Gunn et al., 2021; Wessel et al., 2020). Briefly, spike protein was carboxy coupled to blue, yellow-green, or red FluoSphere™ Carboxylate-modified microsphere, 0.2 μm (ThermoFisher). Spike-coated beads were incubated with diluted serum (1:150 ADNP, 1:100 ADCP) for 2 hours at 37 °C. For the ADNP assay, bone marrow cells were collected from BALB/c mice, and red blood cells were subjected to ACK lysis. The remaining cells were washed with PBS, and aliquoted into 96-well plates (5 × 10^4 cells per well). The bead-antibody complexes were added to cells and incubated for 1 h at 37 °C. After washing, cells were stained with the following antibodies: CD11b APC (BioLegend 101212), CD11c A700 (BioLegend 117320), Ly6G Pacific Blue (127628), Ly6C BV605 (BioLegend 128036), Fcblock (BD Bioscience 553142) and CD3 PE/Cy7 (BioLegend 100320). Cells were fixed with 4% PFA, processed on an BD LSRFortessa (BD Biosciences). Neutrophils were defined as CD3-, CD11b+, Ly6G+. The neutrophil phagocytosis score was calculated as (FITC+) x (geometric mean fluorescent intensity of FITC)/10000. For the ADCP assay, J774A.1 (ATCC TIB-67) murine monocytic cells were incubated with the Spike-coated bead-antibody complexes for 1 h at 37 °C. Cells were washed in 5 mM EDTA PBS, fixed with 4% PFA, and analyzed on an BD LSRFortessa (BD Biosciences). The cellular phagocytosis score was calculated as (FITC+) x (geometric mean fluorescent intensity of FITC)/10000.

QUANTIFICATION AND STATISTICAL ANALYSIS
Statistical significance was assigned when P values were < 0.05 using Prism Version 8 (GraphPad) or Jupyter Notebook 6.1.4. Tests, number of animals (n), median values, and statistical comparison groups are indicated in the Figure legends. Analysis of anti-S, anti-RBD, neutralization titers, and ELISPOT values after vaccination was performed using a one-way ANOVA with a Dunnett’s post-test or a Mann-Whitney test. Differences in viral titers after SARS-CoV-2 infection of immunized mice were determined using a Kruskal Wallis ANOVA with Dunn’s post-test or a Mann-Whitney test. Differences in neutralization titers for different variants were compared using two-tailed Wilcoxon matched-pairs signed rank test. Weight changes were analyzed using area under the curve analysis and a Student’s t test.