INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), emerged in Wuhan, China around December 2019. It has since caused a global pandemic that has to date, resulted in over 160 million confirmed infections and 3.5 million deaths (WHO COVID-19 Weekly Epidemiological Update; 1 June 2021), although the numbers are likely underestimated. Developing safe and effective vaccines and testing new vaccine platforms to control the pandemic are paramount.

The simplicity and stability of plasmid DNA vaccines make it an attractive immunization platform for emerging viral threats. The DNA vaccines can be designed and produced quickly once the genetic sequence is known and adapted rapidly to new emerging viral variants of concern. Clinically, the DNA vaccine modality is generally regarded as safe and is immunogenic in many different mammalian species including man. Inducing both broad antibody and cellular immune responses, DNA vaccines have the potential to reduce both infection and disease. Intrinsically, the DNA vaccines are stable and can be freeze-dried, allowing for long-term storage at ambient temperature. The plasmid DNA does not induce vector-specific antibodies, thus permitting multiple booster vaccinations including mixed modality prime-boost strategies.

Historically, first-generation DNA vaccines performed poorly in primates. This was compounded by the application of the platform to complex pathogens where the correlates of protection are undefined and where other traditional vaccines have similarly failed, such as human immunodeficiency virus (HIV). However, continued platform optimization has seen the improved performance of DNA vaccines in nonhuman primates and man. For example, a candidate Zika virus DNA vaccine protected rhesus macaques against viremia following Zika virus challenge and induced neutralizing antibody titers >300 when delivered intramuscularly with the needle-free Stratis Device (PharmaJet). A different flavivirus DNA vaccine, targeting West Nile virus, is FDA approved for horses but also induced T cell and neutralizing antibody responses in humans. Furthermore, an influenza trivalent DNA vaccine conferred protection against influenza challenge in a phase 1b clinical trial and, through cell-mediated immunity, a human papillomavirus DNA vaccine aided the regression of lesions and viral clearance in cervical intraepithelial neoplasia-3 patients.

Considering the success of mRNA vaccines and DNA delivered by recombinant viruses and the intrinsic advantages and recent improved performances of plasmid DNA vaccines, an evaluation of the plasmid DNA platform is warranted in the ongoing SARS-CoV-2 pandemic. Here we describe the pre-clinical evaluation of a candidate DNA vaccine that targets the spike protein of SARS-CoV-2. Using platform optimization strategies to improve safety, antigen expression, potency, and immunogenicity, we address shortcomings associated with first-generation DNA vaccines. These optimization strategies include using: (i) a vector that lacks any antibiotic resistance genes; (ii) an optimally reduced size vector; (iii) vaccine antigen codon optimization; (iv) co-expression of an immune stimulatory Retinoic-acid-inducible gene I (RIG-I) agonist that facilitates a type I interferon response; (v) high yield antibiotic-free production in a current Good Manufacturing Practice process, and (vi) needle-free jet administration to the skin or muscle.

Multiple SARS-CoV-2 vaccines are being developed at an unprecedented speed. To ensure thorough evaluation of the safety risks, potential autoimmune or hyper-immune reactions, and enhanced infection and/or disease, for as many different
platforms as possible, all vaccines need to be thoroughly assessed for safety and immunogenicity and protection from viral challenge in animal models prior to clinical evaluation. Here we describe the evaluation of the immunogenicity of an optimized DNA plasmid vaccine candidate in mice, rabbits, and nonhuman primates, as well as an assessment of the protective effect in rhesus macaques, the most extensively used model for evaluation of SARS-CoV-2/COVID-19 vaccine protection.

RESULTS

The DNA vaccine candidate

The DNA vaccine candidate hereafter referred to as pNTC-Spike, expresses an unmodified, wild-type full-length SARS-CoV-2 spike protein derived from the Wuhan-hu-1 reference strain. The human codon optimized nucleotide sequence was subcloned into the NTC8685-eRNA41H (Fig. 1a), a nano-plasmid eukaryotic expression vector approved for clinical use17. Considerations for the vector design are described in detail elsewhere12. Notable features of this vector backbone include: (i) the lack of any antibiotic resistance genes to improve DNA vaccine safety15; (ii) improved transgene expression over vectors containing antibiotic resistance genes18; (iii) co-expression of a RIG-I agonist that facilitates a type 1 interferon response and increases DNA vaccine-induced antibody and cellular responses14; and (iv) a reduced size (3.6 kb). NTC8685-eRNA41H has an established toxicity and bio-distribution profile and a documented history of testing in humans with other vaccine-gene inserts17. In order to demonstrate expression of the SARS-CoV-2 spike protein encoded by this optimized vector, we generated highly specific monoclonal antibodies (mAb) targeting the S1- or S2-domain of SARS-CoV-2 using hybridoma technology. Based on a stringent selection procedure, mAbs S1-1047 and S2-1254 were obtained that showed an excellent specificity against their respective target domain and no cross-reactivity against the related spike proteins of SARS-CoV, MERS-CoV or any of the four Coronavirus pathogenic to humans (Supplementary Fig. 1).

Using these mAbs, Western blot analysis of Vero E6 cells transfected with pNTC-Spike confirmed the expression of a full-length spike protein of ~190 kDa with an intact cleavage site, as suggested by the concurrent detection of the S1 and S2 subunits at ~110 kDa and S2 at ~100 kDa (Fig. 1b). The spike protein fragment sizes correspond to that observed by others and reflect the glycosylation of the full-length spike protein and its S1 and S2 subunits with all fragments migrating higher than the predicted 141 kDa, 77 kDa/, and 65 kDa, respectively19. Additional fragments of <100 kDa may be cleavage products produced by other cellular proteases such as cathepsin B and L, elastase, and trypsin, which have been demonstrated to cleave coronavirus spike proteins20,21, although this warrants further characterization.

The cellular distribution of expressed proteins from pNTC-Spike was visualized with immunofluorescence staining of Vero E6 cells 48 h post-transfection (Fig. 1c). SARS-CoV-2 spike S1 and S2 labeling with mAbs S1-1047 and S2-1254, respectively, showed cytoplasmic distribution for both proteins as reported22.

Immunogenicity in mice

The immunogenicity of the candidate DNA vaccine, pNTC-Spike, was first assessed in CB6F1 mice. These filial generation hybrid mice are a cross between C57BL/6 males and BALB/c females. With the parental strains each biased towards Th1 and Th2 responses, respectively, the cross confers a balanced Th1/Th2 responsiveness in the CB6F1 mice. The animals were immunized with either pNTC-Spike, in 10 µg (N = 5) or 50 µg (N = 5) doses, or vector control in a 50 µg dose (N = 5) without adjuvant at weeks 0, 2, and 4 by intradermal injection (Fig. 2a). Both 10 µg and 50 µg doses of pNTC-Spike elicited IgG responses specific to the SARS-CoV-2 spike ectodomain and receptor-binding domain (RBD) after the second immunization (week 4), which were further boosted by a subsequent immunization in a dose-dependent manner (week 6) (Fig. 2b). At week 6, the median end-point titer of antibodies specific for the spike ectodomain were 1547 (range: 10–17906) and 9887 (range: 167–27922) for the 10 µg and 50 µg group, respectively. All animals that developed spike- and RBD-specific binding IgG by ELISA also developed neutralizing antibodies as determined at week 6 in a SARS-CoV-2 virus microneutralization assay (Fig. 2c). Neutralizing antibody titers directly correlated with spike binding antibody titers (Spearman r = 0.758, P = 0.015) and RBD binding antibody titers (Spearman r = 0.818, P = 0.006). The limited volume of serum obtained from animals after the first and second immunization precluded an evaluation of neutralizing antibody responses at week 2 and week 4.

For mice immunized with pNTC-Spike, restimulation of splenocytes with the spike and RBD protein induced high levels of interferon gamma (IFN-γ), indicative of a Th1 helper (Th) 1 response, and comparatively low levels of interleukin (IL)–5 and IL-17 that represent Th2 and Th17 responses, respectively (Fig. 2c). To confirm the observed Th1 dominance, we measured SARS-CoV-2 spike-specific IgG subclass responses since the proportions of the different subclasses are dictated by the prevailing cytokine environment. IgG2a and IgG2c, which increase in response to the Th1 cytokine IFN-γ, occurred at notably higher titers relative to IgG1, which increases in response to the Th2 cytokine IL-4 (Fig. 2e). Overall, the ratio of IgG2a/IgG1 was 21.3 and the IgG2c/IgG1 ratio 9.2; thus, confirming the Th1 dominant vaccine response induced by the pNTC-Spike.

Immunogenicity and safety in rabbits

The small size of mice precludes the evaluation of needle-free jet administration in this animal model. Therefore, to determine the effect of needle-free administration of the candidate DNA vaccine, we evaluated the immunogenicity and safety of pNTC-Spike in rabbits using either needle-and-syringe injection or needle-free jet-injection administrations to skin or muscle. New Zealand white rabbits (10 weeks old) received three immunizations of 125 µg DNA without adjuvant at weeks 0, 2, and 4 by the intradermal route using needle-and-syringe injection intradermal (N = 3) or the PharmaJet® Tropis ID device (N = 4) or via the intramuscular route using the PharmaJet® Stratis IM device (N = 5; Fig. 3a) in separate experiments. While calibrated for human use, the devices deliver liquid to the skin (Tropis ID) and muscle (Stratis IM) of rabbits as determined empirically using a liquid dye (Supplementary Fig. 3). Overall, needle-free administration of the vaccine via the intradermal and intramuscular route induced comparable binding IgG and neutralizing antibody responses that were higher and more consistent than intradermal injection using needle and syringe (Figs. 3b and 3c).

The pNTC-Spike vaccine-induced IgG responses specific to the spike ectodomain and RBD after the first immunization (week 2) and were boosted by subsequent immunizations (Fig. 3b). At week 6, the median spike ectodomain-specific end-point titer were 398 (range: 209–11349), 10975 (range: 4667–15880), and 11956 (range: 8349–15242) for the groups immunized using needle and syringe, needle-free, Tropis ID, and needle-free Stratis IM, respectively. Virus-specific neutralizing antibodies were detected after the second immunization and increased following the third immunization reaching median 50% neutralization titers of 88 (range: 13–165), 111 (21–176), and 125 (67–144) at week 6 in the aforementioned groups, respectively (Fig. 3c). Neutralizing antibody titers directly correlated with spike binding antibody titers (Spearman r = 0.886, P < 0.001; Fig. 3d) and RBD binding antibody titers (Spearman r = 0.874, P < 0.001). For animals immunized intramuscularly and by needle-and-syringe, T cell induced immunity was also evaluated. Data for the Tropis ID group are unavailable. Restimulation of splenocytes with the spike and RBD
protein induced IFN-γ responses as measured by both IFN-γ ELISPOT and IFN-γ cytokine ELISA (Fig. 3e). In these animals, the smaller RBD protein (234 amino acids) induced a lower IFN-γ response compared to the full-length spike protein (1209 amino acids). In contrast to that observed for antibody responses, the IFN-γ responses were comparable for animals immunized with intradermal needle-and-syringe administration and intramuscular needle-free jet administration.

Vaccine safety was observed in the rabbits that were immunized intramuscularly by monitoring temperature, body
weight, clinical signs, and behavior. No cases of death, impending death, or obvious clinical signs were observed in any of the animals. Local site reactions were rare to absent. Only one animal developed slight swelling at the injection site 2 days after the second immunization that resolved 4 days later. All animals continued to gain weight throughout the study, and none suffered acute weight loss following any of the immunizations (Fig. 3f). None of the animals developed a fever (≥40°C) on the day of immunization or in the days thereafter (Fig. 3g). The studies that evaluated intradermal administration of pNTC-Spike were not specifically designed to monitor safety. Nonetheless, there were no cases of death or impending death. Based on observations during standard care of the animals, there were no obvious clinical signs, and immunizations were well tolerated.

**Immunogenicity in rhesus macaques**

We next evaluated pNTC-Spike immunogenicity and protection against SARS-CoV-2 infection in a nonhuman primate model. Rhesus macaques (2–8 years old) were immunized with pNTC-Spike (N = 6) or were untreated sham controls (N = 2). The animals received three immunizations of 2 mg DNA without adjuvant at weeks 0, 2, and 4 by the intradermal route using the needle-free Tropis ID device (Fig. 4a). The immunizations were well tolerated based on daily cage-side observations of the animals during the study period. No adverse reactions at the injection sites were observed.

The candidate vaccine induced an increase in spike-specific binding IgG antibodies from baseline in all vaccinated animals after the second immunization (week 4; median end-point titer = 7440, range: 1509–13371) that were boosted by a third immunization (week 6; median end-point titer = 10494, range: 5569–16252) (Fig. 4b). One of the vaccinated animals had binding antibodies by ELISA at week 0. We speculate this might reflect the cross-reactivity of other natural primate coronaviruses. The pNTC-Spike vaccine nonetheless increased SARS-CoV-2-specific binding antibodies by ELISA in this animal. The ability of vaccine elicted antibodies to neutralize virus infection was evaluated with a live virus plaque reduction neutralization test (PRNT). Neutralizing antibodies capable of reducing plaque-forming units (PFU) by more than 50% at a serum dilution ≥1:20, were observed in five of six vaccinated animals after the second immunization (median: PRNT50 = 34, range:10–60), and a 90% reduction (PRNT90) in three out of six vaccinated animals (Fig. 4c). Neutralizing antibody responses were boosted by the third immunization, with all vaccinated animals having developed neutralizing antibodies by week 6 measured as PRNT50 (median: PRNT50 = 92, range: 30–125) and the more stringent PRNT90 (median: PRNT90 = 46, range: 22–68), 2 weeks before virus challenge. Overall, the spike-specific binding IgG titers correlated with the PRNT50 titers (Spearman r = 0.790, P = 0.003; Fig. 4d).

**Protective effects of pNTC-Spike plasmid vaccine**

At week 8, 4 weeks after the final immunization, all animals were challenged with 1.0 × 10^6 TCID50 SARS-CoV-2 by the intranasal and intratracheal routes. The SARS-CoV-2 virus was measured in broncho-alveolar lavage (BAL) and nasal swabs using an RT-PCR specific for subgenomic messenger RNA (sgmRNA), which are viral RNA intermediates believed to represent replicating virus23–25. The sham controls had a median peak of 3.74 log10 sgmRNA copies/mL in BAL (range: 2.5–4.03; Fig. 5a), which is consistent with a 4–5 log10 sgmRNA copies/mL peak observed in other studies conducted at the same facility with the same challenge dose and virus strain26–28. The vaccinated animals had a 2.04 log10 reduction in viral RNA in BAL. In particular, five out of six animals had viral loads below the quantitation limit of the assay (1.69 log10 sgmRNA copies/mL), one animal had a detectable low positive measure of 1.91 log10 sgmRNA copies/mL on one time point, day 4 post-challenge. In nasal swabs, vaccinated animals had a median peak viral load of 3.10 log10 sgmRNA copies/mL (range < 1.69–4.71; Fig. 5b). Varied viral loads measured for the two sham controls (6.41 and <1.69 log10 sgmRNA copies/mL) preclude a direct comparison to vaccines. However, rhesus macaque sham controls challenged with the same dose and virus strain in the same facility had higher average peak viral loads (5.59–7.00 log10 sgmRNA copies/mL)26–28 in nasal swabs compared to the vaccines in the present study. All vaccinated animals were SARS-CoV-2 antibody positive on the day of challenge (week 8); the anti-spike IgG titers increased the following challenge and was significantly higher 10 days post-challenge (median = 84775, range: 12669–148518) compared to 2 weeks after the third immunization (week 6; median = 10494, range: 5569–16525; P = 0.009) and on the day of challenge (week 8; median = 8314, range: 4598–11950; P = 0.002) (Fig. 5c).

**Breadth of neutralizing antibody responses**

Cross-neutralizing antibody responses against SARS-CoV-2 variants of concern were determined for pNTC-Spike immunized rabbits and rhesus macaques after three immunizations (Fig. 6). The variants tested depending on the viral isolates available at the facilities where the animal studies were conducted. In rabbits, for all vaccine administration methods, pNTC-spike induced cross-reactive neutralizing antibodies against the Alpha (B.1.1.7), Beta (B.1.351), and Delta (B.1.617.2) variants in 91.7%, 66.7%, and 91.7% of vaccinated animals, respectively. With the optimized needle-free intradermal and intramuscular administrations, cross-neutralizing antibody responses increased to 100%, 77.7%, and 100%, respectively. Overall, the neutralization titers for the Alpha variant (median = 139, range: 5–136) and Delta variant (median: 108, range: 5–688) did not differ significantly from the early pandemic strain (median: 166, range: 10–275; p > 0.05 for both comparisons). However, the neutralization titers against the Beta variant (median: 27, range: 5–136) were reduced 6.1-fold (p = 0.0001). In rhesus macaques, pNTC-spike induced neutralizing antibodies against the Beta variant in 66.7% of animals; the neutralization titers for the early pandemic strain (median: 92, range: 30–95) and Beta variant (median: 87, range: 10–302) did not differ significantly (p = 0.563).
Fig. 2  Humoral and cellular immune responses to pNTC-Spike in mice.  

(a) An overview of the immunization strategy in CB6F1 mice. Animals received three immunization of either 10 µg pNTC-Spike (N = 5), 50 µg pNTC-Spike (N = 5), or 50 µg vector control (N = 5) at 2 week intervals via the intradermal route using needle-and-syringe injection. 

(b) Temporal end-point titers of IgG antibodies specific for the SARS-CoV-2 spike ectodomain and receptor binding domain (RBD). Dotted lines indicate the assay limit of quantitation. 

(c) 50% virus-neutralizing antibody (NAb) titer against SARS-CoV-2 clinical isolate as determined in a live virus microneutralization assay. Dotted lines indicate the assay limit of quantitation. 

(d) Interferon gamma (IFN-γ), interleukin 5 (IL-5), and interleukin 17a (IL-17a) levels—representing T helper (Th) 1, Th2, and Th17 responses—measured by ELISA following restimulation of immunized rabbit splenocytes with the SARS-CoV-2 spike ectodomain and RBD. Cell culture medium and concanavalin A (ConA) served as antigen negative and positive controls, respectively. 

(e) End-point titers of SARS-CoV-2 spike-specific IgG subclasses in mice after the third immunization with either 10 µg or 50 µg pNTC-Spike (week 6). The Th1/Th2 dominance determined as the ratio of IgG2a/IgG1 and IgG2c/IgG1 end-point titers. Bar graphs indicate the median with interquartile range. Mouse silhouette created with BioRender.com.
**Fig. 3**  Humoral and cellular immune responses to pNTC-spike vaccination in rabbits.  

**a**  An overview of the immunization strategy in New Zealand white rabbits. Animals received three immunization of 125 µg pNTC-Spike at 2 week intervals delivered either as a 100 µL intradermal dose using a needle-and-syringe (N=3) or the needle-free PharmaJet® Tropis ID device (N=4) or as a 500 µL intramuscular dose using the needle-free PharmaJet® Stratis IM device (N=5). 

**b**  Temporal end-point titers of IgG antibodies specific for the SARS-CoV-2 spike ectodomain and receptor-binding domain (RBD).

**c**  50% virus-neutralizing antibody titer against a SARS-CoV-2 clinical isolate as determined in a live virus microneutralization assay.

**d**  Correlation analysis between the level of SARS-CoV-2 spike-specific binding IgG and virus neutralization.

**e**  Interferon gamma (IFNγ) responses measured by ELISA and ELISpot following restimulation of immunized rabbit splenocytes with the SARS-CoV-2 spike ectodomain and RBD. Cell culture medium and concanavalin A (ConA) served as antigen negative and positive controls, respectively.

**f**  Percentage body weight change from baseline (day 0) of animals measured daily for 4 days after each intramuscular immunization and every 2 day thereafter. A temperature above 40 °C constitutes a fever (dotted line). Bar graphs indicate the median with interquartile range. Rabbit silhouette created with BioRender.com.
**DISCUSSION**

We developed a SARS-CoV-2-specific plasmid DNA vaccine candidate using platform optimization strategies to improve vaccine safety, antigen expression, potency, and immunogenicity. The candidate DNA vaccine is immunogenic in three different animal species, with a relatively small difference between smaller animals and nonhuman primates. The increased potency in nonhuman primates is likely attributed to a combination of improved vector design, optimization of antigen expression, and vaccine delivery through needle-free jet injection. Of note, the vaccine was equally immunogenic following administration, either intradermal or intramuscular, using needle-free jet delivery and were both superior to needle-and-syringe injection intradermal.

The safety evaluation of pNTC-Spike in rabbits reiterated the known and established excellent safety profile of DNA vaccines in general.

We evaluated the protective effect of pNTC-Spike in a vaccination-challenge study of nonhuman primates. The macaque monkey is considered a suitable model for studying SARS-CoV-2 vaccine protection. The animal species are susceptible to SARS-CoV-2 and develops virus-specific humoral and cellular immunity that may confer protection against subsequent infection. Naïve animals develop a systemic infection with high levels of SARS-CoV-2 replication in the upper and lower airways. The disease course is generally mild and self-limiting, although pathological evidence of pneumonia is observed. Several, including now approved, SARS-CoV-2 vaccines have undergone evaluation in nonhuman primates. These include inactivated vaccines (Picovacc and BBIBP-CorV), viral vectored vaccines (ChAdOx1 and Ad26.COV2.S), mRNA vaccines (mRNA1273 and BNT162b2), and a subunit vaccine (NVX-CoV3273).
pNTC-Spike is one of three similar SARS-CoV-2 spike DNA vaccine candidates tested in rhesus macaques to date. The other includes a prototype vaccine expressing unmodified full-length wild-type spike (S) from within pcDNA3.1 + (a vector not approved for clinical use) and Inovio’s INO-4800 vaccine that comprises a clinical plasmid vector (pGX0001) encoding the full-length spike with an N-terminal IgE leader sequence and delivers intramuscularly by electroporation. These vaccines were independently evaluated at the same nonhuman primate facility using the same challenge strain and challenge dose. The three DNA vaccines, administered intramuscular or intradermal, as two or three doses of 1–5 mg (without adjuvant) 2–4 weeks apart, all induced SARS-CoV-2 spike-specific immune responses in rhesus macaques and conferred protection against lower respiratory disease. Protection was not sterilizing, but likely the result of rapid control of viremia following challenge, as suggested by anamnestic antibody responses in protected prototype S and INO-4800 vaccinated animals. In the present study, a 2 week interval between immunizations was deemed necessary to address the urgency for rapid vaccination during the pandemic; however, longer immunization intervals may likely increase immunogenicity and protection. The previously reported SARS-CoV-2 plasmid DNA vaccines tested in nonhuman primates were well tolerated and none resulted in vaccine-associated enhanced respiratory disease (VAERD). Moreover, pNTC-Spike induced comparable antibody responses in small animals and nonhuman primates. These data support the known excellent safety profile of DNA vaccines and demonstrate that different SARS-CoV-2 spike DNA vaccines are immunogenic under various conditions without any adjuvants.

Fig. 5  Viral load and anamnestic responses in rhesus macaques following challenge with SARS-CoV-2 by the intranasal and intratracheal route. a Peak and temporal SARS-CoV-2 viral loads, measured as SARS-CoV-2 E gene subgenomic mRNA, in BAL of pNTC-Spike vaccinated animals (N = 6) and untreated sham controls (N = 2). b Peak and temporal viral loads in nasal swabs of pNTC-Spike vaccinated animals and untreated sham controls. c End-point titers of binding IgG antibodies specific for the SARS-CoV-2 spike ectodomain on the day of virus challenge and days 2, 4, 7, and 10 following inoculation. Bar graphs indicate the median with interquartile range; Line graphs present each animal as a separate black line and the red line the median for each time point. Dotted lines indicate the assay limit of quantitation (1.69 log_{10} sgmRNA copies/mL).
for injectable or needle-free delivery, has been used in clinical trials testing nucleic acid vaccines, including subunit vaccines. A DNA prime with a viral vector boost can mediate by rapid immunological control of viral replication. To complete or near-complete protection in BAL samples likely demonstrated enhanced viral replication or clinical disease but further optimized a vaccine regimen may include increasing the dose amount to reduce the number of doses required to induce sufficient quality immunity, although in affluent countries three immunizations may be feasible.

METHODS

DNA vaccine

The study vaccine, pNTC-Spike, contains a DNA plasmid encoding an unmodified SARS-CoV-2 spike protein derived from the Wuhan-Hu1 strain (MN908947). The human codon optimized SARS-CoV-2 spike sequence was synthesized by GeneArt (Thermo Fisher Scientific, Germany) and subcloned using EcoRI and XhoI into the NTC8685-eRNA41H vector backbone (Nature Technology Corporation, Lincoln, NE, USA). pNTC-Spike was produced by Nature Technology Corporation using an antibiotic-free selection procedure in NTC4862 E.coli cells (DHSa attP5/6 6/6-RNA IN-SacI, Cm") at 10 mg/mL in phosphate buffered saline (PBS). The plasmid preparation contained <2.0 EU/mg endotoxin, as determined by a Limulus Amoebo- cytose test using the Endosafe nexgen-PTS LAL assay (Charles River, Wilmington, MA, USA). The construct was sequenced and tested for expression prior to use.

Generation of antibodies specific for SARS-CoV-2 spike protein

Handling of laboratory animals for the production of monoclonal and polyclonal antibodies complied with the regulations of the German Animal Welfare Act and European legislation for the protection of animals used for scientific purposes (Directive 2010/63/EU). Immunizations of mice to generate monoclonal antibodies S1-1047 (IgG1) and S2-1254 (IgG1) received ethical approval by the State Office for Health and Social Affairs in Berlin (LAGeSo Berlin, Germany) under the registration number H129/19.
Supplementary Fig. 2. Fisher). SARS-CoV-2 spike protein expression was detected with a 1:5000 PageRuler™ Fisher, Waltham, MA, USA), and 25 µg cell lysate was separated on a Novex (0.125 M NaCl, 20 mM Tris [pH 8.0], 0.5% Igepal). The protein content was assayed by a modified Bradford assay (Bio-Rad). Samples were run on a gel in a volume of 200 µL per well of 96 well cell culture plates in RPMI 1640 media supplemented with 20% fetal calf serum, 50 U/mL recombinant murine IL-6, 1% glutamine, 5.7 µM azaserine and 100 µM hypoxanthine. Starting at day 10 after fusion, antibodies from hybridoma supernatants underwent a stringent screening procedure employing e.g. ELISA and surface plasmon resonance spectroscopy to identify hybridoma clones with superior specificity, affinity, and broad applicability in different assays; selected clones were subcloned twice to ensure clonality. A rabbit polyclonal antibody (KSpike) was generated by subcutaneous immunization of a New Zealand rabbit with 25 µg of recombinant SARS-CoV-2 spike S152 protein (Cat. # WP0889-V0881, Sino biological, Beijing, China) for two times with an interval of 4 weeks. The IgG fractions were affinity purified from hybridoma culture supernatants or rabbit serum using Protein A or G columns, respectively, on an ÄKTA LC-instrument (ÄKTA, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The monoclonal antibodies S1-1047 and S2-1254 showed high specificity for their respective target domain in the spike protein of SARS-CoV-2 as shown by indirect ELISA using the rabbit pAb KSpike as control reagent (Supplementary Fig. 1).

Western blot

The day before transfection, 1.2 × 10^5 Vero E6 cells were seeded per well in a six well tissue culture plate with glass coverslips and incubated overnight at 37°C, 5% CO2. Vero E6 cells were transfected with 2 µg of pNTC-Spike using Fugene HD Transfection Reagent (Cat. # H1825, Sigma-Aldrich), permeabilized at −20 °C for 10 min in 4% paraformaldehyde (Cat. # HTS01124-8L, Sigma-Aldrich), permeabilized at −20 °C for 10 min in 100% methanol. Cells were washed three times with DPBS and blocked for 1 h at room temperature in blocking buffer (5% BSA in DPBS). The blocking buffer was aspirated, and cells were incubated for 2 h at 4 °C with a 1:1000 dilution of primary antibody in blocking buffer (S1-1047 or S2-1254). Cells were washed three times with DPBS and incubated for 1 h at room temperature in the dark with an Alexa Fluor 488 conjugated goat-anti-mouse IgG (H + L) Trial Superclonal™ antibody (1:1000 dilution, Cat. # A28175, Thermo Fisher) in blocking buffer. Coverslips were washed three times with DPBS, dried on a paper towel, mounted with VectaSHIELD medium containing 4, 6-diamino-2-phenylindole hydrochloride (DAPI) (Cat. # H-1200-10, VECTOR Laboratories, Burlingame, CA, USA), and sealed with nail polish. Immunostained cells were observed with an Olympus BX61 fluorescence microscope using the Ultrafluor Entry software version 1.11 for image capture. Imaging software Image J, version 1.53j (NIH, USA) was used to merge images and visualize the results.

Animals and study design

Mice. Eight week old female CB6F1 mice (Envigo, Netherlands), offspring of a cross between BALB/c and C57BL/6 mice, were randomly assigned to receive either 10 µg pNTC-Spike (N = 5), 50 µg pNTC-Spike (N = 5) or 50 µg of a vector control (N = 5). The unadjuvanted vaccine doses were prepared in PBS in a final volume of 50 µl and administered in two 25 µl injections per immunization. The mice were immunized via the intradermal route with needle injection at the base of the tail at weeks 0, 2, and 4.

Rabbits. Nine to ten week old female New Zealand white rabbits (Charles River, France) were immunized with 125 µg pNTC-Spike in PBS without adjuvant at weeks 0, 2, and 4. Three administration routes were evaluated in the following studies in the following order: intradermal route using the Tropis ID Needle-free Injection System (PharmaJet, Inc.) (N = 4); intradermal route using needle-and-syringe (N = 3); intramuscular route in a single dose of 500 µL using the Stratsis Needle-free Injection System (PharmaJet, Inc.) (N = 5). In the latter study, vaccine safety was observed.

Nonhuman primates. Eight male and female adult rhesus macaques (Macaca mulatta), 2–8 years old (mean: 4 years), were randomly divided into two groups: pNTC-Spike vaccinates (N = 6) and sham controls (N = 2). Animals received three immunizations of 2 mg DNA each at weeks 0, 2, and 4. The unadjuvanted vaccine was administered via the intradermal route using the Tropis ID needle-free injection system (PharmaJet, Inc.) with four 100 µL doses per immunization, equally distributed over the left and right scapula region. The interval between last immunization to viral challenge varies for the different SARS-CoV-2 vaccine candidates (adenoviral vector, mRNA, live attenuated, protein and DNA) evaluated in nonhuman primates (median: 25 days; range: 14–77 days) to enable comparison with the latter, we selected an interval of 28 days between the last immunization and challenge. At week 8, all eight animals were challenged with 1.0 × 10^6 TCID_50 (1.2 × 10^8 RNA copies, 1.1 × 10^4 PFU) SARS-CoV-2 (strain nCoV-WAI-2020; MN985325.1; BEI Resources, Manassas, VA, USA). In similar studies conducted at the same facility, BIOQUAL, Inc., the 1.0 × 10^6 TCID_50 challenge dose consistently resulted in infection of SARS-CoV-2 immune naïve rhesus macaques (N = 28), total with detectable viral loads by 2 days post-challenge in BAL and nasal swabs.

The challenge stock was propagated at BIOQUAL, Inc. in Vero E6 cells from a seed stock obtained by National Institutes of Health, National Institute of Allergy and Infectious Diseases, Biomedical Advanced Research and Development Authority (BARDA) and World Health Organization Center for Emerging Viruses and Arboviruses, UTMB, Galveston, TX (lot no. TV 23156). The stock was deep sequenced (SRA accession no. SR17249718) by Shelby O’Connor’s laboratory, Univ. Wisconsin-Madison, WI. Sequencing confirmed the expected sequence identity. The virus was diluted in PBS and administered as 1 mL by the intranasal (IN) route and 1 mL by the intratracheal (IT) route. The mice and rabbits were housed in a pathogen-free and climate-controlled animal facility at Statens Serum Institut, Denmark. All cages were provided with bedding material and environmental enrichment. Animals had access to water and a standard pelleted diet ad libitum. Animal husbandry and procedures comply with the Danish legislation, which is based on the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. The experiments received ethical approval by The Animal Experimentation Council, the National Competent Authority within this field (approval number 2017-15-0201-01322), and were supervised by the laboratory animal veterinarians at Statens Serum Institut. The nonhuman primates were housed at BIOQUAL Inc. (Rockville, MD) and associated animal studies conducted in compliance with relevant local, state, and federal regulations and received ethical approval by the Institutional Animal Care and Use Committee (IACUC). The assays used to quantitate vaccine-induced immune responses in the small animals and rhesus macaques were performed at Statens Serum Institut and BIOQUAL, Inc., respectively, and were depended on the assays available at each institute.
Mouse and rabbit antibody enzyme-linked immunosorbent assay (ELISA)

Spike- and RBD-specific binding immunoglobulin G (IgG) titers were determined by standard ELISA. In brief, Nunc™ MaxiSorp™ plates were coated with 100 µL of 4 µg/mL recombinant SARS-CoV-2 spike S152 protein (Cat. # 40589-V08B1; Sino biological) or 4 µg/mL SARS-CoV-2 spike RBD protein (Cat. # 40592-V08B; Sino biological) at 4°C overnight. In consecutive order, with wash steps in between, plates were incubated at room temperature on an orbital shaker with 150 µL of blocking buffer (S-SI Dilution Buffer [Cat. # 1322, SSI Diagnostica], 2% skim milk), 100 µL of 5-fold serial dilutions of a mouse or rabbit sera (1:20 to 1:1562500), 100 µL of horseradish peroxidase (HRP) conjugated goat anti-mouse IgG antibody (1:10000 dilution) or mouse-anti-rabbit IgG antibody (1:2000 dilution) (Cat. # A4416 and A1949, respectively; Sigma-Aldrich), and 3, 3’, 5, 5’-Tetramethylbenzidine (TMB) One Substrate (Cat. # 4380, KemEnTec, Denmark). TMB reaction was stopped with H2SO4, and absorbance read at 450 nm using 620 nm as a reference on a FLUOstar Microplate Reader (MBG LABTECH, Germany). Each wash step comprised three washes with 250 µL wash buffer (PBS with 0.05% Tween20) for 1 min.

Spike ectodomain-specific antibody ELISA

White Nunc™ MaxiSorp™ microtiter plates were coated with 100 µL of 1 µg/mL recombinant SARS-CoV-2 Spike His-tag protein (Cat. # 10549-CV-100; R&D Systems) at 4°C overnight. In consecutive order, with wash steps in between, plates were incubated with 150 µL of blocking buffer (Dilution Buffer pH 7.2 [Cat. # 1322, SSI Diagnostica], 2% bovine serum albumin, and 0.1% Tween20), 100 µL of 5-fold serial dilutions of rabbit sera (1:20 to 1:1562500), 100 µL of HRP conjugated mouse-anti-rabbit IgG antibody (1:2000 dilution) (Cat. # A1949 Sigma-Aldrich), and BM Chemiluminescent Substrate (Cat. # 11582950001, Sigma-Aldrich). Luminescence was read on a FLUOstar Microplate Reader (MBG LABTECH, Germany). Each wash step comprised three washes with 250 µL wash buffer (PBS with 0.05% Tween20) for 30 s. ELISA end-point titers were calculated from a four-parameter logistic regression curve in GraphPad Prism 8.3.0, using the reciprocal serum dilution that yielded an absorbance above a positive cut-off value calculated for each group of animals based on absorbances measured on day 0 at a serum dilution of 1:20 that is (mean absorbance for all animals) + (3 × standard deviation of the absorbance measured for all animals).

Virus microneutralization test

A 2-fold serial dilution of heat-inactivated serum/plasma samples were mixed with 300 × TCID50 SARS-CoV-2 virus, as determined from a virus titration 96 h post-inoculation. The solution was incubated for 1 h at 37°C, 5% CO2, and added to Vero E6 cells (kindly provided by Bjoern Meyer, Institut Pasteur, Paris, France) in a 96 well tissue culture plate seeded with 104 cells per well the day prior. The inoculated cells were incubated at 37°C with 5% CO2, and added to Vero E6 cells (Cat. # CRL-1586, ATCC, Manassas, VA, USA) per well. The PRNT was performed in six-well tissue culture plates seeded with 1.75 × 105 Vero E6 cells. All virus stocks were deep sequenced to confirm the absence of cell culture-derived mutations, and the presence of lineage-specific mutations in the spike protein. The comparison between strains was done on the same day using a single dilution for each serum sample.

Mouse and rabbit splenocyte isolation, restimulation, and cytokine quantification

Following excision, the spleens were submerged in RPMI medium and processed aseptically within an hour. The spleens were homogenized through a 70 um cell strainer using a syringe plunger. The single-cell suspensions were washed twice with cold PBS followed by lysis of red blood cells using Red Blood Cell Lysis buffer (Cat. # R7757, Sigma-Aldrich). The splenocytes were resuspended in culture medium (RPMI containing 10% fetal bovine serum, 1% Penicillin and Streptomycin, 1 mM sodium pyruvate, 10 mM HEPES and 50 µM 2-Mercaptoethanol). A total of 8 × 105 splenocytes were stimulated in duplicate with 2 µg/mL SARS-CoV-2 spike S152 protein (Cat. # 40589-V08B1; Sino biological), 2 µg/mL SARS-CoV-2 spike RBD protein (Cat. # 40592-V08B; Sino biological), 5 µg/mL concanavalin A (Cat. # C0412, Sigma-Aldrich) as a positive control, or culture media as mock stimulated control. The re-stimulated splenocytes were kept under standard tissue culture conditions (37°C with 5% CO2) for 48 h. The level of secreted interferon-γ (IFN-γ), interleukin 5 (IL-5), or interleukin 17 (IL-17) in clarified cell culture supernatants were determined by cytokine ELISA (Cat. # 3321-1H-6, 3391-1H-6, 3521-1H-6, 3110-1H-6; MBTECH AB, Sweden) according to the manufacturer’s instructions. For the rabbit IFN-γ ELISPOT (Cat. # 3110-4HPW-10; MBTECH AB, Sweden), a total of 1 × 106 splenocytes were stimulated for 18 h using the aforementioned antigen stimulations and tissue culture conditions. The secretion of IFN-γ was determined according to the manufacturer’s instructions and spots were counted using CTL Immunospot® analyzer and Immunospot® Software (version 7.0.22.1).

Nonhuman primate antibody enzyme-linked immunosorbent assay (ELISA)

SARS-CoV-2 spike protein-specific IgG in serum was quantified in duplicate by ELISA. In brief, Nunc™ MaxiSorp™ microtiter plates were coated with 50 µL of 2 µg/mL recombinant SARS-CoV-2 spike S152 protein (Cat. # 40589-V08B1, Sino Biological) in PBS and incubated overnight at 4°C. Plates were washed five times with wash buffer (0.05% Tween20 in PBS) and blocked with 100 µL 1% bovine serum albumin in PBS for 2 h at room temperature. The block solution was discarded and 100 µL of serial 4-fold dilutions of serum starting at a 1:20 dilution were added to the wells, followed by a 1 h incubation at room temperature. Plates were washed three times with wash buffer and incubated for 1 h at room temperature with 50 µL of 1:10000 dilution of goat anti-mouse IgG (H + L) (Cat. # PA1-84631, Invitrogen); Plates were washed five times with wash buffer and once with PBS, followed by the addition of 100 µL of SureBlue TMB 1-Component Microwell Peroxidase Substrate (Cat. # 5120-0075, SeraCare, Milford, MA, USA). The reaction was stopped after 10 min with the addition of 100 µL TMB Stop solution per well. The absorbance was measured at 450 nm using 620 nm as a reference. ELISA end-point titers were defined as the highest reciprocal serum dilution that yielded an absorbance >0.300.

Plaque reduction neutralization test (PRNT)

The PRNT was performed in six-well tissue culture plates seeded with 1.75 × 106 Vero E6 cells (Cat. # CRL-1586, ATCC, Manassas, VA, USA) per well.
the day before. Serum samples were heat-inactivated at 56 °C for 30 min and tested in duplicate in a three-fold serial dilution ranging from 1:2 to 1:4860. Each serum dilution was pre-incubated with 30 PFU SARS-CoV-2 for 1 h at 37 °C before addition to the Vero76 monolayers. Following an incubation of 1 h at 37 °C, the supernatants containing the serum/virus mixture were removed and the monolayer washed once with PBS before overlaying with a semi-solid culture medium. Following a 3 day incubation at 37 °C with 5% CO2, the cells were fixed and stained with crystal violet. The reciprocal of the serum dilutions causing plaque reductions of 90% (PRNT90) and 50% (PRNT50) were recorded as titers. Virus neutralization was measured for the early pandemic SARS-CoV-2 strain nCoV-WAI-VAI-2020 (Cat. # NR-52281, BEI Resources, Manassas, VA, USA) and the Beta variant (lineage B.1.351) strain 2019-nCoV/South Africa/KRISP-K005325/2020 (Cat. # NR-54009, BEI Resources). All virus stocks were deep sequenced to confirm identity.

Subgenomic SARS-CoV-2 RNA assay

Replicating the SARS-CoV-2 virus was detected and measured using a real-time RT-PCR assay targeting viral replication intermediates not packaged into virions34. RNA was extracted using the QIAamp Viral RNA Mini Kit (Cat. # 52904, Qiagen, Hilden, Germany). The SARS-CoV-2 E gene subgenomic messenger RNA (sgmRNA) was detected using a leader-specific primer (5′-CGATCTGGATGTGTCTTCCCAAGAAC-3′) located upstream of SARS-CoV-2 ORF1a and a reverse primer (5′-ATATTGGCAAGATGACCCACACA-3′) and probe (FAM-5′-ACACTAGGCTCATTACGGCGCCTTCCG-3′-BHQ) specific to the E gene. PCR amplification was performed using the SensiFAST™ Probe Lo-ROX One-Step Kit (Cat. # BIO-78005, Meridian Bioscience, Cincinnati, OH, USA) on an Applied Biosystems 7500 Real-Time PCR instrument with the following program: 48 °C for 30 min, 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, and 1 min at 55 °C.

Statistical analyses

Each measurement represents a single animal. Antibody positive and negative responses were determined using a 99.9% confidence interval cut-off value calculated for each serum dilution from the serum controls included in the assays29. Variation in paired continuous variables between multiple time points were compared using the non-parametric Friedman test with Dunn’s correction for multiple comparisons. For the latter, adjusted p-values are reported. The Wilcoxon rank sum test was performed where data for only two time points were available. All statistical tests were two-tailed. Statistical analyses and graphing were done with GraphPad PRISM version 8.3.0. (GraphPad Software Inc., San Diego, CA).

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

Data are available from the corresponding author upon request.

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AUTHOR CONTRIBUTIONS

A.Fo., G.I.G., and C.P. designed the study. R.L., C.P., A.Fr., and J.L.T. designed experiments and analyzed the data. A.Fr. and J.L.T. performed experiments. R.L. prepared the figures and manuscript. M.K. and B.G.D. developed, characterized, and validated the anti-SARS-CoV-2 monoclonal antibodies. A.C., R.B., T.O., T.P.T., T.-A.C., J.G., L.P., H.A., and M.G.L. conducted the nonhuman primate study.

COMPETING INTERESTS

A.Fo. and C.P. are co-inventors on a patent application covering a SARS-CoV-2 DNA vaccine; all rights to the vaccine have been assigned to Statens Serum Institut (SSI), a Danish national not-for-profit governmental public health institute. Other authors declare no competing interests. No particular funding was obtained for this work, which was a part of the Danish national health response to the COVID-19 pandemic.

ADDITIONAL INFORMATION

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