Rapid development of a synthetic DNA vaccine for COVID-19

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Abstract
The coronavirus family member, SARS-CoV-2 has been identified as the causal agent for the outbreak of viral pneumonia disease, COVID-19 which first emerged in mid-December 2019 in the city of Wuhan in central China. As of February 25, 2020 there are 80,994 people infected and 2,760 deaths, and documented human-to-human transmission across multiple continents. At this time, no vaccine is available to control further dissemination of the disease. We have previously developed a synthetic DNA vaccine targeting the MERS coronavirus Spike (S) protein that was deployed in response to the MERS outbreak in South Korea. This vaccine induced potent antibody and CTL responses, and provided protection in a NHP challenge model. In the clinic, the vaccine generated humoral immunity including neutralizing antibody responses, as well as T cell immunity. Here we build on this prior work and report on the rapid development of a synthetic DNA-based vaccine targeting the major surface antigen Spike protein of SARS-CoV-2. The engineered construct, INO-4800 induced robust expression of the Spike protein in vitro, and generated antibody and T cell responses following a single immunization in mice and guinea pigs. This preliminary dataset identifies INO-4800 as a potential COVID-19 vaccine candidate, supporting further study for mobilization against this emerging disease threat.

Introduction
COVID-19, known previously as 2019-nCoV pneumonia or disease, has rapidly emerged as a global threat to public health, joining severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome (MERS) in a growing number of coronavirus-associated illnesses which have jumped from animals to people. There are at least seven identified coronaviruses that infect humans. In December 2019 the city of Wuhan in China became the epicenter for an outbreak of the novel coronavirus, SARS-CoV-2. SARS-CoV-2 was isolated and sequenced from human airway epithelial cells from infected patients. Disease symptoms can range from mild flu-like to severe cases with life-threatening pneumonia. The global situation is dynamically evolving, and on January 30, 2020 the World Health Organization declared COVID-19 as a public health emergency of international concern (PHEIC). As of February 25, 2020 there are 80,994 people infected and 2,760 deaths. Infections have
spread to multiple continents. Human-to-human transmission has been observed in multiple
countries, and a shortage of disposal personal protective equipment\textsuperscript{5}, and prolonged survival times of
coronaviruses on inanimate surfaces\textsuperscript{6}, have compounded this already delicate situation and
heightened the risk of nosocomial infections. Advanced research activities must be pursued in parallel
to push forward protective modalities in an effort to protect billions of vulnerable individuals
worldwide. Currently, no licensed preventative vaccine or specific anti-viral therapy is available for
COVID–19.
To address the urgent need for a medical countermeasure to prevent the further dissemination of
SARS-CoV–2 we have employed a synthetic DNA-based vaccine approach. Synthetic DNA vaccines are
highly amenable to accelerated developmental timelines due to the ability to quickly design multiple
candidates for preclinical testing, scalable manufacturing of large quantities of the drug product, and
the possibility to leverage established regulatory pathways to the clinic. Synthetic DNA is
temperature-stable and cold-chain free, important features for delivery to resource-limited settings\textsuperscript{7}.
Specifically for the development of a COVID–19 vaccine candidate, we leveraged prior experiences in
developing vaccine approaches to SARS-CoV\textsuperscript{8}, and our own experience in developing a MERS-CoV
vaccine (INO–4700)\textsuperscript{9,10}, as well as taking advantage of our rapid vaccine design and manufacturing
pathway previously utilized for the Zika vaccine candidate, GLS–5700\textsuperscript{11}, which was advanced to the
clinic in under 7 months. INO–4700 and GLS–5700 vaccines are currently in clinical testing.
Here, we build on knowledge gained from previous immunization strategies targeting SARS and MERS
coronavirus family members to design COVID–19 synthetic DNA vaccine candidates\textsuperscript{8,10}. Previous
studies indicated immunization of small and large animal models with DNA vaccines encoding MERS-
CoV or SARS-CoV spike (S) protein provided protection against disease challenge with the matched
virus\textsuperscript{8,10}. In subjects immunized with INO–4700 (MERS-CoV S protein vaccine) durable neutralizing
antibodies and T cell immune responses were measured, and a seroconversion rate of 96% was
recorded\textsuperscript{9}. INO–4700 Phase 1/2a testing is continuing in South Korea, and a larger Phase 2 study is
being planned to begin in the Middle East, both areas which have been most affected by MERS infections. The SARS-CoV-2 spike is most similar in sequence and structure to SARS-CoV spike protein\textsuperscript{12}, and shares a global protein fold architecture with the MERS-CoV spike protein \textit{(Figure 1)}.. Unlike glycoproteins of HIV and influenza, the prefusion form of the coronavirus trimeric spike is conformationally dynamic, fully exposing the receptor-binding site infrequently\textsuperscript{13}. The receptor-binding site is a vulnerable target for neutralizing antibodies. In fact, MERS nAbs targeted at the receptor-binding domain (RBD) tend to have greater neutralizing potency than other epitopes\textsuperscript{14}. A recent report demonstrated that one neutralizing anti-SARS antibody could cross-react to the RBD of SARS-CoV-2\textsuperscript{15}. These data suggest that the SARS-CoV-2 RBD is an important target for vaccine development. Recent data has revealed SARS-CoV-2 S protein binds the same host receptor, ACE-2 as SARS-CoV S protein\textsuperscript{12}.

Here we describe the design and initial preclinical testing of COVID-19 synthetic DNA vaccine candidates. We show the expression of the SARS-CoV-2 S antigen RNA and protein after \textit{in vitro} transfection of COS and 293T cells, respectively with the vaccine candidates. We followed the induction of immunity by the selected immunogens in mice and guinea pigs. The data demonstrate that the synthetic DNA SARS-CoV-2 S antigen vaccine induces robust cellular and humoral host immune responses that can be observed within days following a single immunization. T cell and B cell responses are highly potent for the SARS-CoV-2 S protein and responses cross react at a lower level against SARS-CoV S protein.

\textbf{Results}

\textbf{Design and synthesis COVID-19 synthetic DNA vaccine constructs}

Four spike protein sequences were retrieved from the first four available SARS-CoV-2 full genome sequences published on GISAID (Global Initiative on Sharing All Influenza Data). Three Spike sequences were 100% matched and one was considered an outlier (98.6% sequence identity with the other sequences). After performing a sequence alignment, the SARS-CoV-2 spike glycoprotein sequence was generated and an N-terminal IgE leader sequence was added. The highly optimized DNA sequence encoding SARS-CoV-2 IgE-spike was created using Inovio’s proprietary \textit{in silico} Gene
Optimization Algorithm to enhance expression and immunogenicity. The optimized DNA sequence was synthesized, digested with BamHI and XhoI, and cloned into the expression vector pGX0001 under the control of the human cytomegalovirus immediate-early promoter and a bovine growth hormone polyadenylation signal. The resulting plasmids were designated as pGX9501 and pGX9503, designed to encode the SARS-CoV-2 S protein from the 3 matched sequences and the outlier sequence, respectively (Figure 2a).

**In vitro characterization of COVID-19 synthetic DNA vaccine constructs**

We measured the expression of the encoded SARS-CoV-2 spike transgene at the RNA level in COS-7 cells transfected with pGX9501 and pGX9503. Using the total RNA extracted from the transfected COS-7 cells we confirmed expression of the spike transgene by RT-PCR (Figure 2b). **In vitro** spike protein expression in 293T cells was measured by Western blot analysis using a cross-reactive antibody against SARS-CoV S protein on cell lysates. HEK-293T cells transfected with pGX9501 or pGX9503 constructs expressed the S protein at the predicted molecular weight, 140-142 kDa (Figure 2c). In immunofluorescent studies the S protein was detected in 293T cells transfected with pGX9501 or pGX9503 (Figure 2d). In summary, **in vitro** studies revealed the expression of the Spike protein at both the RNA and protein level after transfection of cell lines with the candidate vaccine constructs.

**Robust humoral immune responses to SARS-CoV-2 S protein antigens measured in mice immunized with INO-4800**

Since candidate design, it has been observed that newly published SARS-CoV-2 Spike protein sequences match pGX9501 with >99.7% amino acid sequence identity. pGX9501 was therefore selected as the vaccine construct to advance to immunogenicity studies, due to the broader coverage it would likely provide compared to the outlier, pGX9503. pGX9501 was subsequently termed INO-4800. The immunogenicity of INO-4800 was evaluated in BALB/c mice, post-administration to the TA muscle followed with CELLECTRA® delivery device16. The reactivity of the sera from a group of mice immunized with INO-4800 was measured against a panel of SARS-CoV-2 and SARS-CoV antigens (Figure 3). Analysis revealed robust IgG binding against SARS-CoV-2 S protein antigens, with limited cross-reactivity to SARS-CoV S protein antigens, in the serum of INO-4800 immunized mice. We proceeded to measure the serum IgG binding endpoint titers in mice immunized with pDNA against
recombinant SARS-CoV–2 spike protein S1+S2 regions (Figure 4a&b) and recombinant SARS-CoV–2 spike protein receptor binding domain (RBD) (Figure 4c&d). Robust endpoint titers were observed in the serum of mice at day 14 after immunization with a single dose of INO–4800 (Figure 4b-d). Detection of robust humoral immune response to SARS-CoV–2 S protein in guinea pigs after intradermal delivery of INO–4800

We assessed the immunogenicity of INO–4800 in the Hartley guinea pig model, an established model for intradermal vaccine delivery. 100 µg of pDNA was administered by Mantoux injection to the skin and followed by CELLECTRA® delivery device on day 0 as described in the methods section. On day 14 anti-spike protein binding of serum antibodies was measured by ELISA. Immunization with INO–4800 revealed a robust immune response in respect to SARS-CoV–2 S1+2 protein binding IgG levels in the serum (Figure 5a&b). The endpoint SARS-CoV–2 S protein binding titer at day 14 was 10,530 and 21 in guinea pigs treated with 100 µg INO–4800 or pVAX (control), respectively (Figure 5b).

In summary, immunogenicity testing in both mice and guinea pigs revealed the COVID–19 vaccine candidate, INO–4800, was capable of eliciting antibody responses to SARS-CoV–2 spike protein. Early detection of cross-reactive cellular immune responses against SARS-CoV–2 and SARS-CoV in mice immunized with INO–4800

We assayed T cell responses against SARS-CoV–2, SARS-CoV, and MERS-CoV S antigens by IFN-γ ELISpot. Groups of BALB/c mice were sacrificed at days 4, 7, or 10 post-INO–4800 administration (2.5 or 10 µg of pDNA), splenocytes were harvested, and a single-cell suspension was stimulated for 20 hours with pools of 15-mer overlapping peptides spanning the SARS-CoV–2, SARS-CoV, and MERS-CoV spike protein. Day 7 post-INO–4800 administration, we measured T cell responses of 205 and 552 SFU/10^6 splenocytes against SARS-CoV–2 for the 2.5 and 10 µg doses, respectively (Figure 6a). Higher magnitude responses of 852 and 2193 SFU/10^6 splenocytes against SARS-CoV–2 were observed on Day 10 post-INO–4800 administration. Additionally, we assayed the cross-reactivity of the cellular response elicited by INO–4800 against SARS-CoV, observing detectable, albeit lower, T cell responses on both Day 7 (74 [2.5 µg dose] and 140 [10 µg dose] SFU/10^6 splenocytes) and Day 10 post-
administration (242 [2.5 µg dose] and 588 [10 µg dose] SFC/10^6 splenocytes) (Figure 6b). Interestingly, no cross-reactive T cell responses were observed against MERS-CoV peptides (Figure 6c).

**BALB/c SARS-CoV–2 epitope mapping**

We performed epitope mapping on the splenocytes from BALB/c mice receiving the 10 µg INO-4800 dose. Thirty matrix mapping pools were used to stimulate splenocytes for 20 hours and immunodominant responses were detected in multiple peptide pools (Figure 7a). The responses were deconvoluted to identify several epitopes (H2-K^d^) clustering in the receptor binding domain and in the S2 domain (Figure 7b). Interestingly, one SARS-CoV–2 H2-K^d^ epitope, PHGVVFLHV, was observed to be overlapping and adjacent to the SARS-CoV human HLA-A2 restricted epitope VVFLHVVTYV (Ahmed SF et al. Viruses 2020).

In summary, rapid and robust T cell responses against SARS-CoV–2 S protein epitopes were detected in mice immunized with INO-4800.

**Discussion**

The novel coronavirus, SARS-CoV–2, has spread across China, and as of this writing, a rapidly increasing number of infections and associated COVID–19 disease are being reported across the globe. Currently, there are no COVID–19 vaccines available, and global dissemination of SARS-CoV–2 may continue until there is a high level of herd immunity within the human population. Here, we have described accelerated preclinical development of a synthetic DNA-based COVID–19 vaccine, INO-4800 to combat this emerging infectious disease. Synthetic DNA vaccine design and synthesis was immediately initiated upon public release of the SARS-CoV–2 genome sequences on January 11, 2020. All *in vitro* and *in vivo* studies described in the current manuscript were executed within 6 weeks of the SARS-CoV–2 genome sequence becoming available. Our data support the expression and immunogenicity of the INO-4800 synthetic DNA vaccine candidate in multiple animal models. Robust humoral and T cells responses were observed in mice after a single dose. In guinea pigs we employed clinical delivery parameters, and observed robust antibody titers after a single dose.

Halting a rapidly emerging infectious disease requires an orchestrated response from the global
health community and requires improved strategies to accelerate vaccine development. In response to the 2019/2020 coronavirus outbreak we immediately employed our highly adaptable synthetic DNA medicine platform. The design and manufacture of synthetic DNA vaccines for novel antigens is a plug and play process in which we insert the target antigen sequence into a highly characterized and clinically-tested plasmid vector backbone (pGX0001). The construct design and engineering parameters have been optimized for in vivo gene expression, and previously applied to MERS, EBOV, Zika and Lassa DNA vaccine constructs which are all undergoing clinical testing\textsuperscript{7,9,11,19,20}.

Based upon our previous experience developing a vaccine against MERS coronavirus, and previous published studies of SARS vaccines, SARS-CoV-2 S protein was chosen as the antigen target. The SARS-CoV-2 S protein is a class I membrane fusion protein, which the major envelope protein on the surface of coronaviruses. Initial studies have already been performed which indicate SARS-CoV-2 interaction with its host receptor (ACE-2) can be blocked by antibodies\textsuperscript{21}. In vivo immunogenicity studies in both mouse and guinea pig models revealed robust levels of S protein-reactive IgG in the serum of INO-4800 immunized animals. In addition to full-length S1+S2 and S1, INO-4800 immunization induced potent RBD binding antibodies (Figures 3&4), a domain known to be a target for neutralizing antibodies from SARS-CoV convalescent patients\textsuperscript{22,23}. Evaluation of S2 binding and neutralizing antibodies are planned in future studies as these reagents become available.

In addition to humoral responses, cellular immune responses have been shown to be associated with more favorable recovery in MERS-CoV infection\textsuperscript{24}, and are likely to be important against SARS-CoV infection\textsuperscript{25}. Here, we showed the induction of T cell responses against SARS-CoV-2 as early as day 7 post-vaccine delivery. Rapid cellular responses have the potential to lower viral load and could potentially reduce the spread of SARS-CoV-2 and the associated COVID-19 illness.

In addition to the ability of INO-4800 to rapidly elicit humoral and cellular responses following a single immunization, our synthetic DNA medicine platform has several synergistic characteristics which position it well to respond to disease outbreaks, such as COVID-19. As mentioned previously, the ability to design and synthesize candidate vaccine constructs means that in vitro and in vivo testing
can potentially begin within days of receiving the viral sequence, allowing for an accelerated response to vaccine development. The well-defined and established production processes for DNA plasmid manufacture result in a rapid and scalable manufacture process which has the potential to circumvent the complexities of conventional vaccine production in eggs or cell culture. The cost of goods related to DNA manufacture is also significantly lower than currently seen for mRNA-based technologies. We have recently published on the stability profile afforded to our products through the use of our optimized DNA formulation\(^7\). The stability characteristics mean that our DNA drug product is non-frozen and can be stored for 4.5+ years at 2–8°C, room temperature for 1 year and 1 week at 37°C, while maintaining potency at temperatures upwards of 60°C. In the context of a pandemic outbreak, the stability profile of a vaccine plays directly to its ability to be deployed and stockpiled in an efficient and executable manner.

Additional preclinical studies are ongoing to further characterize INO-4800 in small and larger animals. Availability of reagents is a major challenge for development of vaccines against newly emerging infectious diseases. For initial vaccine expression studies, we successfully used a cross-reactive SARS-CoV polyclonal antibody for detection. Once the tools become available, studies will begin to determine the functionality of the antibodies raised in animals immunized with INO-4800 in terms of virus neutralization. The ability of INO-4800 immunization to mediate protection against viral challenge will be assessed in multiple animal models. Even though we are still in the early days of this outbreak the scientific community has been rapidly mobilized, and we believe the tools to do these studies will be available soon.

In summary, the initial result describing the immunogenicity of COVID-19 vaccine candidate, INO-4800 are promising, and it is particularly encouraging to measure robust antibody and T cell levels at an early time point after a single dose of the vaccine.

Methods

Cell lines Human embryonic kidney (HEK)-293T and COS-7 cell lines were obtained from ATCC (Old Town Manassas, VA). All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin.
\textit{In vitro RNA expression (qRT-PCR)} In vitro mRNA expression of the plasmid was demonstrated by transfection of COS–7 with serially diluted plasmids followed by analysis of the total RNA extracted from the cells using reverse transcription and PCR. Transfections of four concentrations of the plasmid were performed using FuGENE® 6 transfection reagent (Promega) which resulted in final masses ranging between 80 and 10 ng/well. The transfections were performed in duplicate. Following 18 to 26 hours of incubation the cells were lysed with RLT Buffer (Qiagen). Total RNA was isolated from each well using the Qiagen RNeasy kit following the kit instructions. The resulting RNA concentration was determined by OD_{260/280} and samples of the RNA were diluted to 10 ng/µL. One hundred nanograms of RNA was then converted to cDNA using the High Capacity cDNA Reverse Transcription (RT) kit (Applied Biosystems) following the kit instructions. RT reactions containing RNA but no reverse transcriptase (minus RT) were included as controls for plasmid DNA or cellular genomic DNA sample contamination. Eight µL of sample cDNA were then subjected to PCR using primers and probes that are specific to the target sequence. In a separate reaction, the same quantity of sample cDNA was subjected to PCR using primers and probe designed for COS–7 cell line β-actin sequences. Using a QuantStudio 7 Flex Real Time PCR Studio System (Applied Biosystems), samples were first subjected to a hold of 1 minute at 95°C and then 40 cycles of PCR with each cycle consisting of 1 second at 95°C and 20 seconds at 60°C. Following PCR, the amplifications results were analyzed as follows. The negative transfection controls, the minus RT controls, and the NTC were scrutinized for each of their respective indications. The threshold cycle (C_T) of each transfection concentration for the INO-4800 COVID-19 target mRNA and for the β-actin mRNA was generated from the QuantStudio software using an automatic threshold setting. The plasmid was considered to be active for mRNA expression if the expression in any of the plasmid transfected wells compared to the negative transfection controls were greater than 5 C_T.

\textit{In vitro protein expression (Western blot)} Human embryonic kidney cells, 293T were cultured and transfected as described previously\textsuperscript{26}. 293T cells were transfected with pDNA using TurboFectin8.0 (OriGene) transfection reagent following the manufacturer's protocol. Forty-eight hours later cell
lysates were harvested using modified RIPA cell lysis buffer. Proteins were separated on a 4-12% BIS-TRIS gel (ThermoFisher Scientific), then following transfer, blots were incubated with an anti-SARS-CoV spike protein polyclonal antibody (Novus Biologicals) then visualized with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (GE Amersham). (Immunofluorescence of transfected 293T cells) For *in vitro* staining of Spike protein expression 293T cells were cultured on 4-well glass slides (Lab-Tek) and transfected with 3 µg/well of pDNA using TurboFectin8.0 (OriGene) transfection reagent following the manufacturer's protocol. Cells were fixed 48hrs after transfection with 10% Neutral-buffered Formalin (BBC Biochemical, Washington State) for 10 min at room temperature (RT) and then washed with PBS. Before staining, chamber slides were blocked with 0.3% (v/v) Triton-X (Sigma), 2% (v/v) donkey serum in PBS for 1hr at RT. Cells were stained with a rabbit anti-SARS-CoV spike protein polyclonal antibody (Novus Biologicals) diluted in 1% (w/v) BSA (Sigma), 2% (v/v) donkey serum, 0.3% (v/v) Triton-X (Sigma) and 0.025% (v/v) 1g/ml Sodium Azide (Sigma) in PBS for 2hrs at RT. Slides were washed three times for 5 min in PBS and then stained with donkey anti-rabbit IgG AF488 (lifetechnologies) for 1hr at RT. Slides were washed again and mounted and covered with DAPI-Fluoromount (SouthernBiotech).

**Animals** Female, 6 week old C57/BL6 and BALB/c mice were purchased from Charles River Laboratories (Malvern, PA) and The Jackson Laboratory (Bar Harbor, ME). Female, 8 week old Hartley guinea pigs were purchased from Elm Hill Labs (Chelmsford, MA). All animals were housed in the animal facility at The Wistar Institute Animal Facility or Acculab Life Sciences (San Diego, CA). All animal protocols were approved by the Wistar Institute or Acculab Institutional Animal Care and Use Committees (IACUC). For mouse studies, on day 0 doses of 2.5, 10 or 25 µg pDNA were administered to the tibialis anterior (TA) muscle by needle injection followed by CELLECTRA® *in vivo* electroporation (EP). The CELLECTRA® EP delivery consists of two sets of pulses with 0.2 Amp constant current. Second pulse sets is delayed 3 seconds. Within each set there are two 52 ms pulses with a 198 ms delay between the pulses. On days 0 and 14 blood was collected. Parallel groups of mice were serially sacrificed on days 4, 7, and 10 post-immunization for analysis of cellular immune responses. For guinea pig studies, on day 0, 100 µg pDNA was administered to the skin by Mantoux
injection followed by CELLECTRA® in vivo EP. Blood was collected on day 0 and 14.

Antigen binding ELISA ELISAs were performed to determine sera antibody binding titers. Nunc ELISA plates were coated with 1 µg/ml recombinant protein antigens in Dulbecco’s phosphate-buffered saline (DPBS) overnight at 4°C. Plates were washed three times then blocked with 3% bovine serum albumin (BSA) in DPBS with 0.05% Tween 20 for 2 hours at 37°C. Plates were then washed and incubated with serial dilutions of mouse or guinea pig sera and incubated for 2 hours at 37°C. Plates were again washed and then incubated with 1:10,000 dilution of horse radish peroxidase (HRP) conjugated anti-guinea pig IgG secondary antibody (Sigma-Aldrich, cat. A7289) or (HRP) conjugated anti-mouse IgG secondary antibody (Sigma-Aldrich) and incubated for 1 hour at RT. After final wash plates were developed using SureBlue™ TMB 1-Component Peroxidase Substrate (KPL, cat. 52–00–03) and the reaction stopped with TMB Stop Solution (KPL, cat. 50–85–06). Plates were read at 450 nm wavelength within 30 minutes using a Synergy HTX (BioTek Instruments, Highland Park, VT). Binding antibody endpoint titers (EPTs) were calculated as previously described in Bagarazzi M et al. 201227.

Binding antigens tested included, SARS-CoV-2 antigens: S1 spike protein (Sino Biological 40591-V08H), S1+S2 ECD spike protein (Sino Biological 40589-V08B1), RBD (University of Texas, at Austin (McLellan Lab.)); SARS-COV antigens: Spike S1 protein (Sino Biological 40150-V08B1), S (1–1190) (Immune Tech IT–002–001P) and Spike C-terminal (Meridian Life Science R18572).

IFN-γ ELISpot Spleens from mice were collected individually in RPMI1640 media supplemented with 10% FBS (R10) and penicillin/streptomycin and processed into single cell suspensions. Cell pellets were re-suspended in 5 mL of ACK lysis buffer (Life Technologies, Carlsbad, CA) for 5 min at room temperature, and PBS was then added to stop the reaction. The samples were again centrifuged at 1,500 g for 10 min, cell pellets re-suspended in R10, and then passed through a 45 µm nylon filter before use in ELISpot assay. ELISpot assays were performed using the Mouse IFN-γ ELISpotPLUS plates (MABTECH). 96-well ELISpot plates pre-coated with capture antibody were blocked with R10 medium overnight at 4°C. 200,000 mouse splenocytes were plated into each well and stimulated for 20 hours with pools of 15-mer peptides overlapping by 9 amino acid from the SARS-CoV-2, SARS-CoV, or MERS-
CoV Spike proteins (5 peptide pools per protein). Additionally, matrix mapping was performed using peptide pools in a matrix designed to identify immunodominant responses. Cells were stimulated with a final concentration of 5 μL of each peptide/well in RPMI + 10% FBS (R10). The spots were developed based on manufacturer’s instructions. R10 and cell stimulation cocktails (Invitrogen) were used for negative and positive controls, respectively. Spots were scanned and quantified by ImmunoSpot CTL reader. Spot-forming unit (SFU) per million cells was calculated by subtracting the negative control wells.

**Structural modeling** The structural models for SARS-CoV and MERS-CoV were constructed from PDB IDs 6acc and 5x59 in order to assemble a prefusion model with all three RBDs in the down conformation. The SARS-CoV-2 structural model was built by using SARS-CoV structure (PDB id:6acc) as a template. Rosetta remodel simulations were employed to make the appropriate amino acid mutations and to build *de novo* models for SARS-CoV-2 loops not structurally defined in the SARS-CoV structure. Amino acid positions neighboring the loops were allowed to change backbone conformation to accommodate the new loops. The structural figures were made using PyMOL.

**Statistics** All statistical analyses were performed using GraphPad Prism 7 or 8 software (La Jolla, CA). These data were considered significant if p <0.05. The lines in all graphs represent the mean value and error bars represent the standard deviation. No samples or animals were excluded from the analysis. Randomization was not performed for the animal studies. Samples and animals were not blinded before performing each experiment.

**Data Availability** The authors declare that all other relevant data supporting the findings of the study are available in this article and its Supplementary Information files, or from the corresponding author.

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Declarations
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Author Contributions
TRFS, AP, SR, PP and JY designed experiments, analyzed the data, and prepared the manuscript. IM, DE, JW, MY, DA, AD, MV, KS, XZ, SW, NC, ENG, EP, ARA, ELR, NT, JC, MAB, YW, DP, KYK, SR, MK and JC designed and performed experiments. NW, DW, JSM developed reagents. ASB, TH, KM, XZ, ENG, EP, ELR, DWK aided in experimental design and manuscript review. DBW, JJK, LHH, JB and KEB provided supervision and oversaw final manuscript preparation.

*Competing Interests* TRFS, IM, JJK, JY, DE, SR, DA, JW, AD, MV, MY, KS, PP, TH, ASB, JB, JJK, LHH and KEB are employees of Inovio Pharmaceuticals and as such receive salary and benefits, including ownership of stock and stock options, from the company. DBW has received grant funding, participates in industry collaborations, has received speaking honoraria, and has received fees for consulting, including serving on scientific review committees and board services. Remuneration received by DBW includes direct payments or stock or stock options, and in the interest of disclosure he notes potential conflicts associated with this work with Inovio and possibly others. In addition, he has a patent DNA vaccine delivery pending to Inovio. All other authors report there are no competing interests.

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Figures
Comparison of SARS-CoV-2, SARS-CoV and MERS-CoV spike glycoproteins. a) Amino acid alignment of coronavirus spike proteins including 11 SARS-CoV-2 sequences with mutations (GISAID). Grey bars indicate identical amino acids and colored bars represent mutations relative to Wuhan-Hu-1. RBD, Cleavage Site, Fusion Peptide and TransMembrane domains are indicated in red. b) Structural models for SARS-CoV-2, SARS and MERS glycoproteins with one chain represented as cartoon and two chains represented as surface. RBD of SARS-CoV-2 is colored yellow.
Figure 2

Design and expression of COVID-19 synthetic DNA vaccine constructs. a. Schematic diagram of COVID-19 synthetic DNA vaccine constructs, pGX9501 (matched) and pGX9503 (outlier (OL)) containing the IgE leader sequence and SARS-CoV-2 spike protein insert. b. RT-PCR assay of RNA extract from COS-7 cells transfected with pGX9501 & pGX9503. Extracted RNA was analyzed by RT-PCR using PCR assays designed for each target and for COS-7 β-Actin mRNA, used as an internal expression normalization gene. Delta CT (Δ CT) was calculated as the CT of the target minus the CT of β-Actin for each transfection concentration and is...
plotted against the log of the mass of pDNA transfected (Plotted as mean±SD) c. Analysis of in vitro expression of Spike protein after transfection of 293T cells with pGX9501, pGX9503 or MOCK plasmid by Western blot. 293T cell lysates were resolved on a gel and probed with a polyclonal anti-SARS Spike Protein. Blots were stripped then probed with an anti-β-actin loading control. d. In vitro immunofluorescent staining of 293T cells transfected with 3µg/well of pGX9501, pGX9503 or pVax (empty control vector). Expression of Spike protein was measured with polyclonal anti-SARS Spike Protein IgG and anti-IgG secondary (green). Cell nuclei were counterstained with DAPI (blue). Images were captured using ImageXpress Pico automated cell imaging system.

![Graph](image)

**Figure 3**

IgG binding screen of a panel of SARS-CoV-2 and SARS-CoV antigens using serum from INO-4800-treated mice. BALB/c mice were immunized on day 0 with 25 µg INO-4800 or pVAX-empty vector (Control) as described in the methods. Protein antigen binding of IgG at 1:50 and 1:250 serum dilutions from mice at day 14. Data shown represent mean OD450 nm values (mean+SD) for each group of 4 mice. Further antigen details are provided in the Methods section.
Figure 4

Humoral responses to SARS-CoV-2 S 1+2 and S RBD protein antigen in BALB/c mice after a single dose of INO-4800 BALB/c mice were immunized on day 0 with indicated doses of INO-4800 or pVAX-empty vector as described in the methods. (a) SARS-CoV-2 S1+2 or (b) SARS-CoV-2 RBD protein antigen binding of IgG in serial serum dilutions from mice at day 14. Data shown represent mean OD450 nm values (mean±SD) for each group of 8 mice (a&b) and 5 mice (c&d). Serum IgG binding endpoint titers to (b) SARS-CoV-2 S1+2 and (d) SARS-CoV-2 RBD protein. Data representative of 2 independent experiments.
Humoral responses to SARS-CoV-2 in Hartley guinea pigs after a single dose of INO-4800. Hartley guinea pigs mice were immunized on Day 0 with 100 µg INO-4800 or pVAX-empty vector as described in the methods. a. SARS-CoV-2 S protein antigen binding of IgG in serial serum dilutions at day 0 and 14. Data shown represent mean OD450 nm values (mean±SD) for the 5 guinea pigs. b. Serum IgG binding titers (mean±SD) to SARS-CoV-2 S protein at day 14. P = 0.0079, Mann-Whitney test.
Rapid induction of T cell responses in BALB/c mice post-administration of INO-4800. BALB/c mice (n=5/group) were immunized with 2.5 or 10 µg INO-4800. T cell responses were analyzed in the animals on days 4, 7, 10 for plots a&b, and day 14 for plot c. T cell responses were measured by IFN-γ ELISpot in splenocytes stimulated for 20 hours with overlapping peptide pools spanning the SARS-CoV-2 (a), SARS-CoV (b), or MERS-CoV (c) Spike proteins. Bars represent the mean +SD.
Figure 7

T cell epitope mapping after INO-4800 administration to BALB/c mice. Splenocytes were stimulated for 20 hours with SARS-CoV-2 peptide matrix mapping pools. A) T cell responses followint stimulation with matrix mapping SARS-CoV-2 peptide pools. Bars represent the mean +SD. B) Map of the SARS-Co-V-2 Spike protein and identification of immunodominant peptides in BALB/c mice. A known immunodominant SARS-CoV human epitope (HLA-A2) is included for comparison.