Design and immunogenicity of a Pan-SARS-CoV-2 synthetic DNA vaccine

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Abstract:
First generation COVID-19 vaccines matched to the original Wuhan-Hu-1 (WT) strain are showing reduced efficacy against emerging SARS-CoV-2 variants of concern (VOC). In response, next generation vaccines either matched to a single variant or designed to provide broader coverage across the VOC group are being developed. The latter pan-SARS-CoV-2 approach may offer substantial advantages in terms of cross-strain protection, immune coverage, reduced susceptibility to escape mutants, and non-restricted geographical use. Here we have employed our SynCon® design technology to construct a DNA vaccine expressing a pan-Spike immunogen (INO-4802) to induce broad immunity across SARS-CoV-2 variants. Compared to WT and VOC-matched vaccines which showed limited cross-neutralizing activity, INO-4802 induced potent neutralizing antibodies and T cell responses against WT as well as B.1.1.7, P.1, and B.1.351 VOCs in a murine model. In addition, a hamster vaccination model showed enhanced humoral responses against VOCs in a heterologous pWT prime/INO-4802 boost setting. These results demonstrate the potential of the pan-SARS-CoV-2 vaccine, INO-4802 to induce cross-reactive immune responses against emerging VOCs as either a standalone vaccine, or as a potential boost for individuals previously immunized with WT-matched vaccines.

Introduction:
COVID-19 remains a global pandemic. To date SARS-CoV-2 has infected over 148 million people, and over 3.1 million people have succumbed to disease [1]. Concerningly, SARS-CoV-2 variants containing novel mutations impacting virological and epidemiological characteristics are driving an increased level COVID-19 morbidity and mortality in many parts of the world [2-4]. Several variants have become a focus of attention. The B.1.1.7 (United Kingdom), B.1.351 (South African), and P.1 (Brazilian) variants have rapidly become dominant in some regions (reviewed in [5]). Importantly, some of the mutations associated with these VOCs enhance resistance to neutralizing antibodies induced after infection or vaccination [6-10]. Recent clinical data has revealed a significant decrease in efficacy of vaccines against novel mutations in the SARS-CoV-2 spike protein [6, 7, 11]. A recent Phase 3 clinical trial investigating the Vaxzevria vaccine demonstrated low efficacy (21.9%) against the circulating B.1.351 VOC in South Africa [12]. Additionally, reports of fully vaccinated individuals suffering breakthrough infections with SARS-CoV-2 variants further highlight the risk variants pose [13, 14]. Pseudotyped virus and live virus neutralization results have showed reduced or in some cases loss of neutralizing antibody capacity against B.1.351. Multiple growing lines of evidence are converging on the need to effectively address new variants and for strategies to refine vaccine designs for further emerging VOC challenges [15, 16].

Vaccine strategies to address the critical global challenge of VOCs are being developed and tested. These include immunogens based on an individual variant (matched strain) or designs that aim to provide pan-SARS-CoV-2 variant coverage. We utilized our SynCon® DNA-based vaccine technology for rapid design, generation, and synthesis of new vaccine candidates to meet the challenge of emerging SARS-CoV-2 variants. The vaccine candidates were generated based on surveys of emerging variants with the goal of generating a pan-SARS-CoV-2 antigen which can demonstrate effective cross protection to multiple variants of concern. From the candidate pool we selected a vaccine which encodes a novel set of SARS-CoV-2 spike changes reflective of globally observed mutations from multiple lineages and containing critical receptor binding domain (RBD) changes present in variants of concern circulating worldwide. In previous studies, immunization of small and large animal models with DNA vaccines encoding SARS-CoV-2 S

protein (INO-4800, currently in Phase 2/3 clinical trials) provided protection against disease challenge with the matched virus [17].

Here, we report on the design and broad immunogenicity of a next-generation pan-SARS-CoV-2 vaccine, INO-4802 and its use in prime and heterologous boost regimens targeting emerging SARS-CoV-2 variants of concern in preclinical animal models. Studies highlight the use of rational design processes to identify a single immunogen encoded in plasmid DNA and delivered by CELLECTRA® electroporation which can induce broadly cross-reactive neutralizing antibodies against SARS-CoV-2 variants.

Results:

Design strategy of a pan-SARS-CoV-2 vaccine

The SynCon design strategy employed to create a pan-SARS-CoV-2 vaccine candidate is described in a step by step manner (**Fig. 1a**). SARS-CoV-2 genome sequence entries (derived from GISAID) covering a four-month period (October 2020-January 2021) were sampled from multiple geographic regions (Brazil, Canada, India, Italy, Japan, Nigeria, South Africa, United Kingdom, United States) to provide a broadly representative pool of current and emerging variants. Mutations in the SARS-CoV-2 Spike sequences were aggregated for each region. The results from these regions were then aggregated to determine a common set of overlapping mutations from emerging variations in SARS-CoV-2 Spike protein sequences to generate a single SARS-CoV-2 SynCon Spike immunogen. Additional amino acid changes were added in the receptor binding domain (RBD) of the SynCon SARS-CoV-2 Spike to reflect those observed in multiple variants of concern. A tandem proline mutation (K986P/V987P) named “2P” was then added to the SynCon SARS-CoV-2 Spike to augment protein stability [18, 19].The final single construct containing all changes was termed INO-4802. An unrooted phylogenetic comparison of the translated INO-4802 amino acid sequence shows INO-4802 occupying a position in the tree that skews it towards multiple VOCs, but is not identical with any, reflective of its consensus-based derivation (**Fig. 1b**). Plasmids matched to WT (pWT) and B.1.351 (pB.1.351) variants show identity to the matched Spike glycoprotein sequences as expected (**Table 1**). GISAID accession numbers of entries used in the analysis are available in the Supplemental Materials.

To confirm expression of the immunogens we measured *in vitro* Spike protein production in HEK-293T cells after transfection with the corresponding plasmid constructs by Western blot analysis using a cross-reactive antibody against the RBD region of the SARS-CoV-2 S protein on cell lysates. Western blots of the lysates of HEK-293T cells transfected with pWT, pB.1.351, or INO-4802 constructs revealed bands approximate to the predicted Spike protein molecular weight, 140–142 kDa, (**Fig. 1c**). Similar results were observed when using antibodies specific to the S1 or S2 regions of the spike as well (**Fig. S1a**). Spike transgene RNA expression was also confirmed by RT-PCR analysis of COS-7 cells transfected with pWT, pB.1.351, and INO-4802 plasmids (**Fig. S1b**). In summary, *in vitro* studies revealed the expression of the Spike transgene at both the RNA and protein level after transfection of cell lines with all three candidate vaccine constructs.

Vaccination with INO-4802 induces binding and neutralizing antibodies against SARS-CoV-2 variants

The immunogenicity of the pWT, pB.1.351, and INO-4802 DNA vaccines was evaluated in the BALB/c mouse model. Mice were dosed with 10 µg plasmid DNA (pDNA) on day 0 and 14, and sera samples were collected on day 21 for evaluation.

IgG binding titers against the full Spike protein of the WT and variants including B.1.1.7, P.1, and B.1.351 were evaluated by ELISA. Immunization with pWT, pB.1.351, and INO-4802 induced similar antibody binding titers against the WT and B.1.1.7 variants (**Fig. 2a and c**). Compared to pWT vaccinated animals, there was a small increase in binding titers against P.1 and B.1.351 antigens in mice receiving the pB.1.351 and INO-4802 vaccines.

We proceeded to measure the functional ability of the antibodies raised in the vaccinated animals to neutralize SARS-CoV-2. Neutralizing antibody levels against SARS-CoV-2 were measured by a pseudotyped virus neutralization assay in the sera of immunized mice (**Fig. 2b**). In the pWT vaccinated animals, while neutralizing activity was similar for B.1.1.7, and P.1 variants as compared to WT, there was a 3-fold reduction in average ID_{50} for the B.1.351 variant. These results are in line with other vaccines matched to the WT spike antigen [7, 20]. In the animals receiving the matched p.B.1.351 vaccine, neutralizing activity was similar for P.1 and B.1.351 variants as compared to WT, and a greater than 7-fold reduction in average ID_{50} for the B.1.1.7 variant. In contrast to the matched vaccines, INO-4802 vaccinated mice demonstrated strong neutralizing activity against all variants assessed. In addition, there was significantly higher neutralizing activity against P.1 and B.1.351 variants in the sera of INO-4802 vaccinated mice, compared to the matched pWT vaccine. Compared to the animals receiving the matched pB.1.351 vaccine, sera from INO-4802 vaccinated animals displayed significantly higher neutralization titers against all the variants tested.

Comparison of the sera from INO-4802 vaccinated mice sera to human convalescent sera (HCS) collected in 2020 from donors in the United States demonstrates similar neutralizing activity against the WT and B.1.1.7 variants. While neutralizing activity against the P.1 and B.1.351 variants demonstrates enhanced activity in the INO-4802 vaccinated mice sera compared with HCS (P.1 962 ± 703-1317 vs 279 ± 157-495 and B.1.351 765 ± 552 1058 vs 24 ± 12-46 ID_{50} mean ± range for INO-4802 and HCS, respectively).

In summary, INO-4802 demonstrates significantly enhanced neutralizing activity against P.1 and B.1.351 variants while maintaining a strong response against the WT and B.1.1.7 variants, indicating an advantage over variant-matched vaccines (**Fig. 2d**).

INO-4802 stimulates T cell activity against Global SARS-CoV-2 Variants

SARS-CoV-2 challenge data in T cell-depleted animals, along with studies indicating reduced disease incidence in individuals harboring pre-existing cross-reactive T cells with SARS-CoV-2 support the importance of COVID-19 vaccines induced cellular immunity [21-23]. We therefore sought to examine T cell responsiveness after INO-4802 vaccination. Splenocytes from mice vaccinated with pWT, pB.1.351, or INO-4802 were stimulated with peptides spanning the WT, B.1.1.7, P.1, and B.1.351 variant Spike proteins. pWT, pB.1.351 and INO-4802 demonstrated induction of comparable T cell responses as measured by IFNγ ELISpot against all variants (**Fig. 3a**). A similar broad T cell reactivity across SARS-CoV-2 VOC was recently reported in human INO-4800 vaccinees [24]. The phenotype of CD4 and CD8 T cell responses was characterized by intracellular cytokine staining on splenocytes isolated from INO-4802-vaccinated mice. Antigen-specific T cells producing IFNγ were observed in both CD4 and CD8 compartments (**Fig.**

3b & 3d). Additionally, CD8 T cells showed expression of CD107a, a marker of cytolytic potential (**Fig. 3c**). The balance of T_H1 and T_H2 expressing cells was evaluated based on cytokine expression profile for T_H1 driving IFNγ and T_H2 driving IL4 production. CD4 T cells showed greater expression of the canonical T_H1 cytokine IFNγ relative to IL-4 (**Fig. 3d-f**), consistent with T_H1-skewed T cell responses following INO-4802 vaccination. Further Th1 vs Th2 evaluation was performed by measuring the induction of IgG2A and IgG1 isotype antibodies. ELISA assay results revealed a higher percentage of IgG2A antibodies compared to IgG1 antibodies in animals vaccinated with pWT and INO-4802, indicative of a T_H1-biased response (**Fig. S2**).

Circulating T follicular helper (Tfh) cells are largely representative of a memory CD4 T cell population in the blood that correlates with neutralizing antibody responses, and Tfh cells have been found to be increased in the blood of mice receiving SARS-CoV-2 mRNA vaccines [25-27]. Accordingly, we sought to determine whether Tfh cells were induced following administration of the vaccines. Mice receiving two doses of INO-4802 showed a significantly higher frequency of Tfh cells in circulation relative to mice receiving control pVax (**Fig. 3g**), suggesting enrichment of circulating Tfh cells at an early timepoint post vaccination.

Heterologous boost with INO-4802 in Syrian hamsters against Global SARS-CoV-2 variants

The Syrian Golden hamster is permissible to SARS-CoV-2 infection and is the gold standard small animal model for assessing COVID-19 prophylactics [28-32]. We employed this model to test whether INO-4802 vaccine could boost immunity generated by first generation immunogens based on the WT Spike sequence. We tested the immunogenicity of the pWT in hamsters 236 days after receiving 2 doses of the pWT construct (**Fig. 4a**). Prior to boosting hamsters were split randomly into two groups of four. We compared the scenario of heterologous (INO-4802 construct) to homologous (pWT construct) boost in terms of magnitude and breadth of humoral responses targeting the VOCs. VOC antigen binding titers were increased across all variants tested (**Fig. 4b**). We observed enhanced levels in the INO-4802 group compared to the homologous boost group (pWT GMTs 21,044 and INO-4802 109,350). As the pre-boost levels in the INO-4802 group were slightly higher than the homologous group we assessed the fold changes in binding titers between groups. We observed a trend towards higher log2-fold increase of endpoint binding titers after boost with INO-4802 (3.6 – 4.4 log2-fold change) compared to boost with pWT (2.0 – 2.4 log2 fold change). These results suggested a broad humoral response of greater magnitude after boosting with the pan-SARS-CoV-2 construct compared to homologous construct.

Discussion:

Here we present the preclinical immunogenicity results for a pan-SARS-CoV-2 DNA vaccine, INO-4802 designed with SynCon technology to provide broad immune responses against SARS-CoV-2 Spike antigen on emerging VOCs. In both a primary and heterologous boost vaccine regimen, INO-4802 induced broadly neutralizing antibodies and T cell responses against WT, B.1.1.7, P.1, and B.1.351 SARS-CoV-2 Spike variants in BALB/c mice. In contrast, the cross-neutralizing activity for strain matched vaccines (pWT and pB.1.351) were limited. Initial data demonstrates the potential of INO-4802 as a pan-SARS-CoV-2 vaccine countermeasure to emerging VOCs.

Current COVID-19 vaccines authorized for emergency use in the US were designed early in the pandemic to match the initial outbreak SARS-CoV-2 strain. The natural mutation rate in RNA viruses inevitably spurred selection for new variants and accumulating evidence points to some critical variants showing enhanced spread, ability to escape known neutralizing antibodies, or

leading to higher hospitalization rates [10, 33, 34]. Vaccine trials have already reported reduced efficacy in geographical regions in which the B.1.351 VOC is present [12, 35, 36]. In response, vaccines matched to B.1.351 have been designed [37]. In addition to the pan-SARS-CoV-2 vaccine INO-4802, we also tested a DNA vaccine matched to the B.1.351 VOC as a comparator matched VOC strain design. However, while we observed immune responses to the matched antigen, functional antibody responses to other tested variants was reduced (**Fig. 2b**).These data indicate that focusing on single variant, matched strain approach is to some extent effective but may limit cross-protective potential against diverse novel VOCs, at worst becoming obsolete if facing rapid variant shifts during the development process. This concern is not theoretical. Nearly simultaneous occurrences of B.1.1.7 and B.1.351, followed by P.1 a short time later, show how rapidly the variant landscape can shift. Recent SARS-CoV-2 infection rate surges in India demonstrate the speed at which changes can occur [38]. This is particularly concerning when considering these variants emerged in the time required for design, preclinical evaluation, and Phase 3 efficacy evaluation before currently authorized vaccines could be distributed at a large scale globally. Coupled with previously observed global spread of these variants despite active monitoring, efforts to simultaneously address multiple variants require wide-ranging strategies that generate needed boosters with extended windows of efficacy in the face of a potentially changing challenge.

In the design process of INO-4802, we utilized a broad antigen design strategy using SynCon technology as a potential mitigation solution to the limited coverage provided by a matched strain approach. A single construct design consisting of an antigen representative of multiple viral strains while retaining sufficient identity to generate effective and broad immunity is a favorable solution that presents fewer production barriers as defined by cost of goods and formulation complexity. Application of the SynCon antigen design platform in conjunction with rational design choices allowed us to generate a pan-SARS-CoV-2 vaccine design, INO-4802 (**Fig. 1a, b**). We evaluated this approach in terms of the broadness of the immune response induced by INO-4802 against the VOC.

As detailed in this study, humoral immunogenicity results in BALB/c mice demonstrated the consensus-based approach provided a broad cross-reactive neutralizing antibody responses against all the VOC tested (**Fig. 2b**). While the matched pWT construct and INO-4802 performed similarly in neutralization assays against WT and B.1.1.7-matched pseudotyped virus, strain-matched vaccination with pB.1.351 construct showed poor overall performance against B.1.1.7. This may be due to the unique cluster of mutations in the B.1.351 Spike compared to either WT or to INO-4802, making it a less ideal antigen for promoting a B.1.1.7-directed humoral response. B.1.351 has multiple unique changes to the N-terminal domain (NTD) which along with the RBD contains potent neutralization sites [39]. Generation of neutralizing response to B.1.351 alone may come at the cost of a lack of neutralizing antibody response to more diverse lineages. INO-4802 demonstrated superiority to both pWT and pB.1.351 in inducing functional antibodies against P.1 and B.1.351-matched pseudoviruses (**Fig. 2d**). The greater magnitude of neutralizing activity induced by INO-4802 compared to pB.1.351 against P.1 VOC may be less surprising than those against B.1.351 VOC, as the pB.1.351 was strain-matched in this case. Added design features in INO-4802 to promote antigen stability, notably the 2P mutation, may be an important structural advantage to account for this observed difference. Comparison of INO-4802 vaccinated mouse sera with COVID-19 HCS demonstrated a stark increase in neutralizing activity against the P.1 and B.1.351 variant highlighting INO-4802's ability to provide broad neutralizing activity against multiple variants.

Vaccination with pWT, pB1.351 and INO-4802 resulted in comparably strong IFNγ ELISpot responses against WT and VOC-matched peptide pools in a murine model (Fig. 3). Lack of differentiation between the vaccine constructs in respect to level of T cell immunity against VOC Spike antigens was expected. The highly diverse and linear epitope dependent T cell compartment is less impacted than the structurally dependent functional antibody response. The results are supported by the maintenance of INO-4800 T cell immunity against the same panel of VOC [24], as well as data in COVID-19 exposed donors and vaccinees showing a negligible impact of SARS-CoV-2 variants on CD4 and CD8 T cell responses [40]. In addition to ELISpot analysis, we performed additional functional T cell assays. INO-4802 vaccination induced CD107a/IFN-γ+ cross-reactive CD8 T cells and Tfh cells, cell populations involved in cytotoxic and augmenting B cell responses, respectively (**Figure 3b & c**). Importantly, Inovio's plasmid-encoded antigens and *in vivo* electroporation technology have repeatedly been shown to drive cellular immune responses in humans [41-43]. The cross-reactive T cell results may also play an important role in effective cross-protection, in a similar manner to that observed in individuals during the H1N1 influenza pandemic. In a study of healthy individuals who contracted pH1N1 influenza during the 2009 pandemic, the presence of pre-existing T cells responsive to conserved epitopes in pH1N1 correlated with less severe clinical outcomes even when effective cross-neutralizing antibodies were not present [44].

In addition to the use of INO-4802 as a prime vaccine, we assessed employing it in a heterologous boost regimen (**Fig 4a**). INO-4802 boosting was assessed approximately 8 months after priming with pWT vaccine in the Syrian Golden hamster model. Delaying the INO-4802 boost by approximately 8 months provide time for the maturation of the immune response the first round of vaccination and potentially antigenic imprinting to WT spike antigen. Encouragingly, initial humoral immunogenicity readout suggests strong boost of binding antibody titers across the panel of WT and VOC antigens. The increase in binding titers against all the VOCs tested was greater than same dose boost of pWT. Further studies are ongoing and planned to fully investigate INO-4802 as a boost to first generation vaccine responses generated by INO-4800 and other platform technologies.

Some limitations of the current study include the lack of efficacy testing in a challenge model. Challenge studies to assess efficacy in appropriate animal models are currently in planning for INO-4802. Importantly, it is encouraging that pseudotyped virus nAb titers measured here exceed levels reported to correlate with protection in relevant animal models (**Fig. 2b** & [45]). The pseudotyped virus testing panel used in this study consisted of currently known VOCs. Over time, additional novel VOCs are expected to emerge or to rise in importance through changing epidemiology. As they emerge, these new variants will be interrogated similarly in our assay platform as a continuous screening read-out.

We have reported on the design and testing of a pan-SARS-CoV-2 vaccine, INO-4802. High levels of cross-reactive neutralizing activity against multiple VOCs was demonstrated by pseudotyped virus neutralization, superior to that shown by strain-matched vaccines. Additionally, INO-4802 has shown utility in both prime and heterologous boost immunization scenarios. Vaccination with INO-4802 led to cross-reactive T cell immune responses as well as circulating populations of Tfh cells correlative of both memory and neutralizing antibody responses. In summary, INO-4802 represents a potentially useful pan-SARS-CoV-2 vaccine approach against multiple variants of concern and the data presented in this report supports its further development.

Methods:

INO-4802 and other plasmid constructs

The synthetic consensus (SynCon®) sequence for the SARS-CoV-2 spike glycoprotein with additional focused RBD changes and 2P mutation was codon-optimized using Inovio's proprietary optimization algorithm. A Kozak sequence (GCCACC) immediately 5' of the start codon in addition to restriction sites for subcloning of the construct into pGX0001 vector (5' BamHI and 3' XhoI) were added. Sequence analysis was performed using custom Python scripts (Python Software Foundation, https://www.python.org/) and assembly was performed using Geneious Prime® 2020.2.3 (Build 2020-08-25, Biomatters Ltd., Auckland NZ). Dual proline mutations (K986P/V987P, 2P) were added to the INO-4802 sequence. An IgE leader sequence replaced the endogenous SARS-CoV-2 Spike signal peptide sequence. The coding sequence was codon-optimized using Inovio's proprietary optimization algorithm. Included in the construct synthesis was the addition of a Kozak sequence (GCCACC) immediately 5' of the start codon in addition to restriction sites for subcloning of the construct into pGX0001 vector (5' BamHI and 3' XhoI). The optimized DNA sequence was synthesized (Genscript, Piscataway NJ), digested with BamHI and XhoI, and cloned into the expression vector under the control of the cytomegalovirus immediate-early promoter, generating INO-4802. Strain-matched spike sequences (pWT, pB.1.351) were similarly optimized and cloned into identical restriction site locations into the pGX0001 backbone. Pseudovirus plasmids were designed and constructed as previously described [24].

Cell lines and *In-vitro* plasmid expression

HEK-293T (ATCC® CRL-3216™) and African Green monkey kidney COS-7 (ATCC® CRL-1651™) 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.

For in vitro protein expression by Western blot, human embryonic kidney cells, $5.5x10^5$ 293T were transfected with 2.5µg pDNA in 6-well plates using Lipofectamine 3000 (Invitrogen #L3000015) transfection reagent following the manufacturer's protocol. Transfections were performed in duplicate wells for each plasmid DNA. Forty-eight hours later cell lysates were harvested using Cell Signaling Cell Lysis Buffer (#9803) and duplicate transfection lysates pooled for expression analysis. 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 antibodies (S1, Sino Biological #40591-T62; S2, Invitrogen #PA1-41165; RBD, Sino Biological #40592-MP01) then visualized with horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit IgG (Bethyl) (GE Amersham). Beta actin was detected using Santa Cruz # SC-47778.

For *in vitro* RNA expression by qRT-PCR, transfections, RNA purification, cDNA synthesis, and qPCR assay were performed as previously described [17, 24]. PCR was performed using a single set of primers and probes recognizing the RNA products of all three plasmids (pS-spike forward ATGATCGCCCAGTACACATC, pS-spike reverse CACGCCGATGCCATTAAATC, pS-spike probe AT CACCAGTGGCTGGACATTTGGA). In a separate reaction, the same quantity of sample cDNA was subjected to PCR using primers and a probe designed (β-actin Forward – GTGACGTGGACATCCGTA AA; β-actin Reverse – CAGGGCAGTAATCTCCTTCTG; β-actin Probe – TACCCTGGCATTGCTGACAGGATG) for COS-7 cell line β-actin sequences. The primers and probes were synthesized by Integrated DNA Technologies, Inc. and the probes were labeled with 56-FAM and Black Hole Quencher 1.

In-vivo immunogenicity

BALB/c mice (6 weeks old, Jackson Laboratory, Bar Harbor, ME) and Syrian Golden Hamsters (8 weeks old, Envigo, Indianapolis, IN) were housed at Acculab (San Diego, CA). Mice (n=8/group) received 30 µL intramuscular (IM) injection of 10 µg pDNA immediately followed by electroporation (EP) in the tibialis anterior (TA) muscle on days 0 and 14 of the experiment. Hamsters (n=4/group) received 60 µL IM injection of 90 µg pDNA immediately followed by EP into the TA on days 0, 14 and 236 of the experiment. The CELLECTRA® EP treatment consists of two sets of pulses with 0.2 Amp constant current. Second pulse set is delayed 4 s. Within each set there are two 52 ms pulses with a 198 ms delay between the pulses. Mice were euthanized on day 21 for terminal blood collection and spleens were harvested for cellular assays. Serum was collected from hamsters on days 236 (pre-boost) and 244 (post-boost) by jugular blood collection for pseudovirus-neutralization assay. All animal treatments and procedures were performed at Acculab, and animal testing and research complied with all relevant ethical regulations and studies received ethical approval by the Acculab Institutional Animal Care and Use Committees (IACUC).

Antigen binding Assays/ELISA's

Binding ELISAs were performed as described previously [24] except different variants of SARS-CoV-2 S1+S2 spike proteins were used for plate coating. Binding titers were determined after background subtraction of animals vaccinated with mock vector. The S1+S2 wild-type spike protein (Acro Biosystems #SPN-C52H8) contained amino acids 16-1213 of the full spike protein (Accession #QHD43416.1) with F817P, A892P, A899P, A942P, K986P, V987P, R683A and R685A mutations to stabilize the trimeric prefusion state. The B.1.1.7 and B.1.351, and P.1 S1+S2 variant proteins (Acro Biosystems #SPN-C52H6, #SPN-C52Hc, and #SPN-C52Hg, respectively) additionally contained the following proline substitutions for trimeric protein stabilization: F817P, A892P, A899P, A942P, K986P, and V987P. The B.1.1.7 protein contained the following variant-specific amino acid substitutions: HV69-70del, Y144del, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H; and the B.1.351 protein contained the following substitutions: L18F, D80A, D215G, R246I, K417N, E484K, N501Y, D614G, A701V; and the P.1 protein contains: L18F,T20N,P26S,D138Y,R190S,K417T,E484K,N501Y,D614G,H655Y,T1027I,V1176F. Half-area assay plates were coated using 25 µL of 1 µg/mL of protein. Secondary antibodies included IgG (Sigma #A4416), IgG2A (Abcam #ab98698), and IgG1 (Abcam #ab98693) at 1:10,000 dilution.

Pseudovirus production

SARS-CoV-2 pseudotyped stocks encoding for the WT, B.1.1.7, P.1, or B.1.351 Spike protein (**Table 2**) were produced using HEK 293T cells transfected with Lipofectamine 3000 (ThermoFisher) using IgE-SARS-CoV-2 S plasmid variants (Genscript) co-transfected with pNL4-3.Luc.R-E- plasmid (NIH AIDS reagent) at a 1:8 ratio. Cell supernatants containing pseudotyped viruses were harvested after 72h, steri-filtered (Millipore Sigma), and aliquoted for storage at -80°C.

SARS-CoV-2 Pseudotyped neutralization

CHO cells stably expressing ACE2 (ACE2-CHOs) to allow permissiveness to SARS-CoV-2, were seeded at 10,000 cells/well. SARS-CoV-2 pseudotyped stocks were titered to yield greater than 30 times the cell only control relative luminescence units (RLU) 72h post- infection. Sera from vaccinated mice were heat inactivated and serially diluted two-folds starting at 1:16 dilution. Sera were incubated with SARS-CoV-2 pseudotyped virus for 90 min at room temperature. After incubation, sera-pseudovirus mixture was added to ACE2-CHOs and allowed to incubate in a

standard incubator (37 degree Celsius, 5% CO_2) for 72h. After 72h, cells were lysed using Bright-Glo™ Luciferase Assay (Promega) and RLU was measured using an automated luminometer. Neutralization titers (ID_{50}) were calculated using GraphPad Prism 8 and defined as the reciprocal serum dilution at which RLU were reduced by 50% compared to RLU in virus control wells after subtraction of background RLU in cell control wells.

COVID-19 Convalescent Serum samples

Two sets of convalescent donor sera were used in this study, each consisting of 10 donors from the USA. One set included donors that tested positive for SARS-CoV-2 infection in March-April 2020, while the other set consisted donors that tested positive in October 2020. Samples were collected between 14 and 71 days from the onset of symptoms. 15 donors (75%) had disease symptoms classified as mild, 1 donor (5%) was asymptomatic, and 4 donors (20%) had moderate disease symptoms. Seven donors were male, and thirteen were female. Ages ranged from 19 to 60 years. Serum samples were sourced from BioIVT.

SARS-CoV-2 spike ELISpot assay

Mouse Peripheral mononuclear cells (PBMCs) post-vaccination with DNA vaccinations were stimulated *in vitro* with 15-mer peptides (overlapping by 9 amino acids) spanning the full-length Spike protein sequence of the indicated variants. Variant peptide pools (GenScript, custom) included the following changes to match published deletions/mutation in each variant: B.1.1.7 variant (delta69-70, delta144, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H), P.1 variant L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, V1176F), B.1.351 variant (L18F, D80A, D215G, delta242-244, R246I, K417N, E484K, N501Y, D614G, A701V); Cells were incubated overnight in an incubator with peptide pools at a concentration of 1 µg per ml per peptide in a precoated ELISpot plate, (MabTech, Mouse IFNγ ELISpot Plus). Cells were washed off, and the plates were developed via a biotinylated anti-IFN-γ detection antibody followed by a streptavidin-enzyme conjugate resulting in visible spots. Each spot corresponds to an individual cytokine-secreting cell. After plates were developed, spots were scanned and quantified using the CTL S6 Micro Analyzer (CTL) with *ImmunoCapture* and *ImmunoSpot* software. Values are shown as the background-subtracted average of measured triplicates. The ELISpot assay qualification determined that 12 spot forming units was the lower limit of detection. Thus, anything above this cutoff is considered to be a signal of an antigen specific cellular response.

INO-4802 SARS-CoV-2 spike flow cytometry assays

Mouse splenocytes were also used for intracellular cytokine staining (ICS) analysis and visualized using flow cytometry. One million splenocytes in 200 µL complete RPMI media were stimulated for six hours (37 °C, 5% CO_2) with DMSO (negative control), PMA and ionomycin (positive control, 100 ng/mL and 2 µg/mL, respectively), or with the indicated peptide pools (225 ug/mL). Variant peptide pools included the following changes to match published deletions/mutation in each variant: B.1.1.7 variant (delta69-70, delta144, N501Y, A570D, D614G, P681H, T716I, S982A, D1118H), P.1 variant L18F, T20N, P26S, D138Y, R190S, K417T, E484K, N501Y, D614G, H655Y, V1176F), B.1.351 variant (L18F, D80A, D215G, K417N, E484K, N501Y, D614G, A701V). After stimulation, cells were washed in PBS for live/dead staining (Life Technologies Live/Dead aqua fixable viability dye), and then stained with extracellular markers. The cells were then fixed and permeabilized (eBioscience™ Intracellular Fixation and Permeabilization Buffer Set) and then stained for the indicated intracellular cytokines using fluorescently-conjugated antibodies (**Table S1**). In a separate flow cytometry assay, circulating T follicular helper (Tfh) cells were assessed in vaccinated mice using a whole blood staining strategy. 100 uL of whole blood was

obtained 2 weeks after the second dose of either INO-4802 or pVax. Whole blood was directly stained using the same viability dye and fluorescently-conjugated antibodies described above for CD3, CD4, and CD8. The antibody cocktail also included the canonical Tfh markers CXCR5-biotin (BD Biosciences), PD-1 PE-CF594 (BD Biosciences), and ICOS BV650 (BD Biosciences) in the presence of Fc block (BD Biosciences). Following a 45-minute incubation at 4 C, whole blood was lysed using FACS Lysing Solution (BD Biosciences) according to the manufacturer's instructions. Cells were then stained with streptavidin-BV421 (BD Biosciences) for 40 minutes at 4 C, fixed, and acquired on a FACSCelesta flow cytometer (BD Biosciences). Data were analyzed using FlowJo software. Tfh cells were identified as $CD4^+CXCR5^+PD\text{-}1^+$ T cells.

Data analysis

GraphPad Prism 8.1.2 (GraphPad Software, San Diego, USA) was used for graphical and statistical analysis of data sets. P values of < 0.05 were considered statistically significant. A nonparametric Mann-Whitney test was used to assess statistical significance when comparing two groups and a by Kruskal-Wallis test (ANOVA) with Dunn multiple comparisons test when comparing three or more groups.

Reporting summary

Further information on experimental design is available.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Figure Legends:

Figure 1. Design strategy of a pan-SARS-CoV-2 vaccine a) SARS-CoV-2 spike glycoprotein sequences were sampled from multiple countries (Brazil, Canada, India, Italy, Japan, Nigeria, South Africa, the United Kingdom, and the United States) and the most prevalent mutations were aggregated for each location. The regional mutations were analyzed and aggregated to generate the SynCon spike. Lastly, in addition to the global SynCon changes to the spike (indicated in orange), supplemental variant of concern (VOC) changes were placed in the receptor binding domain (RBD, indicated in pale green) and the 2P mutation was added (indicated in gray) producing INO-4802 (pan-SARS-CoV-2). **b)** An unrooted phylogenetic tree comparing protein sequences derived from INO-4802, pB.1.351, and pWT spikes (black, gray, and red respectively) as well as spike sequences from a sampling of circulating variants (black) including current VOCs (blue). **c)** Analysis of in vitro expression of Spike protein after transfection of 293T cells with empty vector (pVax), pWT, INO-4802, or pB.1.351 plasmid by Western blot. Control proteins and 293T cell lysates were resolved on a gel and probed with a polyclonal anti-SARS-CoV-2 Spike RBD Protein. Blots were stripped then probed with an anti-β-actin loading control. Bands were detected at the expected SARS-COV-2 Spike antigen molecular weight of about 180 kDa inclusive of glycosylation.

Figure 2. INO-4802 pan-SARS-CoV-2 vaccine-induced humoral immune responses against SARS-CoV-2 VOC. BALB/c mice were immunized on days 0 and 14 with 10μg of pWT, pB.1.351, or INO-4802 and sera samples were collected at day 21 for evaluation of antibody responses as described in the methods. **a)** Sera IgG binding titers against the indicated Spike proteins for pWT, pB.1.351, or INO-4802 vaccinated mice (n of 8 each). Data shown represent geometric mean titer values (GMT+/- 95% CI) for each group of 8 mice. **b)** Sera pseudovirus neutralization ID_{50} titers against the indicated SARS-CoV-2 variant for pWT, pB.1.351, or INO-4802 vaccinated mice (n of 8 per group) or human convalescent sera samples (n of 20). Each data point represents the mean

of technical duplicates for individual samples. Dashed lines represent the limit of detection (LOD) of the assay. Samples below LOD were plotted at the number equivalent to half of the lowest serum dilution. *P < 0.05, **P < 0.005, ***P < 0.001 determined by Kruskal-Wallis test (ANOVA) with Dunn multiple comparisons test. **c)** IgG binding data represented as group means for each variant tested. **d)** Pseudovirus neutralization ID_{50} titer data represented as group means for each variant tested.

Figure 3. INO-4802 pan-SARS-CoV-2 vaccine-induced cellular immune response against SARS-CoV-2 variants. Splenocytes isolated from mice were collected 1 week after receiving the second dose of either pWT, pB.1.351, or INO-4802. **a)** Splenocytes were stimulated with peptide pools spanning the entire Spike proteins of the WT, B.1.1.7, P.1, or B.1.351 variants and cellular responses were measured by IFNγ ELISpot assay. Mean +/- SD IFNγ SFUs/million splenocytes of experimental triplicates are shown. **b-e)** Intracellular cytokine staining was employed for CD4+ and CD8+ T cell activation. Expression levels of IFNγ, CD107a and IL-4 were analyzed. **f)** A representative graph showing correlation of T_H1 (IFNγ) versus T_H2 (IL-4) cytokine expression in the CD4 compartment of INO-4802 treated animals restimulated with either the WT, B.1.1.7, P.1, or B.1.351 peptide pools. **g)** Frequencies of circulating Tfh cells ($CXCR5^+PD\text{-}1^+$) in CD4 T cells 2 weeks after the second dose of either pWT or INO-4802. *P < 0.05, (Mann-Whitney test).

Figure 4. Heterologous boost with INO-4802 pan-SARS-CoV-2 vaccine induces humoral immune responses against SARS-CoV-2 variants in Syrian Hamsters: a) Experimental design. Syrian hamsters (n=8) were immunized with 90 µg of pWT on days 0 and 14. On day 236 animals were boosted with 90 µg of pWT or INO-4802 b) Pre- and post-boost sera IgG binding titers against the indicated SARS-CoV-2 Spike antigens. Symbols represent endpoint binding titers for individual animals and lines and bars represent group GMT +/- 95%CI. Values indicate log2 fold changes of GMTs from pre- to post-boost.

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AUTHOR CONTRIBUTIONS

Conceptualization K.E.B. J.J.K., D.B.W., L.M.H., V.M.A., D.E., R.K., S.J.R., C.C.R., K.S., T.R.F.S.; Methodology V.M.A., R.K., S.J.R., C.C.R., K.S., T.R.F.S., J.W., M.Y., J.F, R.F. ; Investigation V.M.A., K.S., D.E., J.N.W., B.S., I.M., M.V., A.D., M.V., Z.E., P.P., D.A.; Resources K.E.B. J.J.K., L.M.H.; Writing-original draft preparation V.M.A., D.E., S.J.R., C.C.R., B.S., K.S., T.R.F.S., J.T., J.W.; Writing-review and editing K.E.B., J.J.K., D.B.W., L.M.H., S.J.R., C.C.R., T.R.F.S., J.T.; Project administration D.B.W., S.J.R., J.J.K., L.M.H., T.R.F.S., K.E.B.

COMPETING INTERESTS

C.C.R., V.M.A., R.K., K.S., J.T., B.S., D.E., J.W., I.M., A.D., Z.E., P.P., D.A. M.Y., J.F., R.F., M.V., J.J.K., L.M.H., S.J.R., T.R.F.S., K.E.B. are employees of Inovio Pharmaceuticals and as such receive salary and benefits, including ownership of stock and stock options, from the company. D.B.W. 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 D.B.W. 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.

ADDITIONAL INFORMATION
Supplementary information is available upon request to Corresponding author.

Supplemental Figure Legends:

Supplemental Figure 1. *In vitro* expression of pDNA. a) Analysis of *in vitro* expression of Spike protein after transfection of 293T cells with empty vector (pVax), pWT, INO-4802, or pB.1.351 plasmid by Western blot. Control proteins and 293T cell lysates were resolved on a gel and probed with a polyclonal anti-SARS-CoV-2 Spike RBD Protein. Blots were stripped then probed with an anti-β-actin loading control. **b)** *In vitro* expression of RNA by RT-PCR assay. RNA extracts from COS-7 cells transfected in duplicate with pWT, INO-4802, or pB.1.351. Extracted RNA was analyzed by RT-PCR using a PCR assays designed for SARS-CoV-2 spike 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).

Supplemental Figure 2. IgG isotype profile of INO-4802 humoral immunogenicity against SARS-CoV-2 VOC. BALB/c mice were immunized on days 0 and 14 with 10μg of pWT or INO-4802 as described in the methods. Protein antigen binding of IgG2A and IgG1 from mice at day 21 (7 days post second immunization). Data shown represent OD450 nm values for sera at a 1:1350 dilution (linear range of binding for all groups/protein antigens) for each group of mice. Protein antigens are SARS-CoV-2 full length spike proteins (Black circle-WT, Red Square-B.1.1.7, Green Triangle-P.1, Blue Diamond-B.1.351) representing the full mutational profile of each VOC as described in the methods.

Table 1. Vaccine candidate plasmid descriptions

Vaccine Construct	Design
pWT	Matched to Wuhan sequence
pB.1.351	Matched to B.1.351
INO-4802	Pan-SARS-CoV-2
pVax	Empty insert plasmid control

Table 2. Spike glycoprotein mutations in antigens

Pseudoviral construct	Mutations
WT	Original Wuhan sequence (NC_045512)
B.1.1.7	Δ69-70/Δ144/N501Y/A570D/D614G/P681H/ T716I/S982A/D1118H
P.1	L18F/T20N/P26S/D138Y/R190S/K417/E484K/ N501Y/D614G/H655Y/T1027I/V1176F
B.1.351	L18F/D80A/D215G/Δ242-244/R246I/K417N/ E484K/N501Y/D614G/A701V

Figure 1

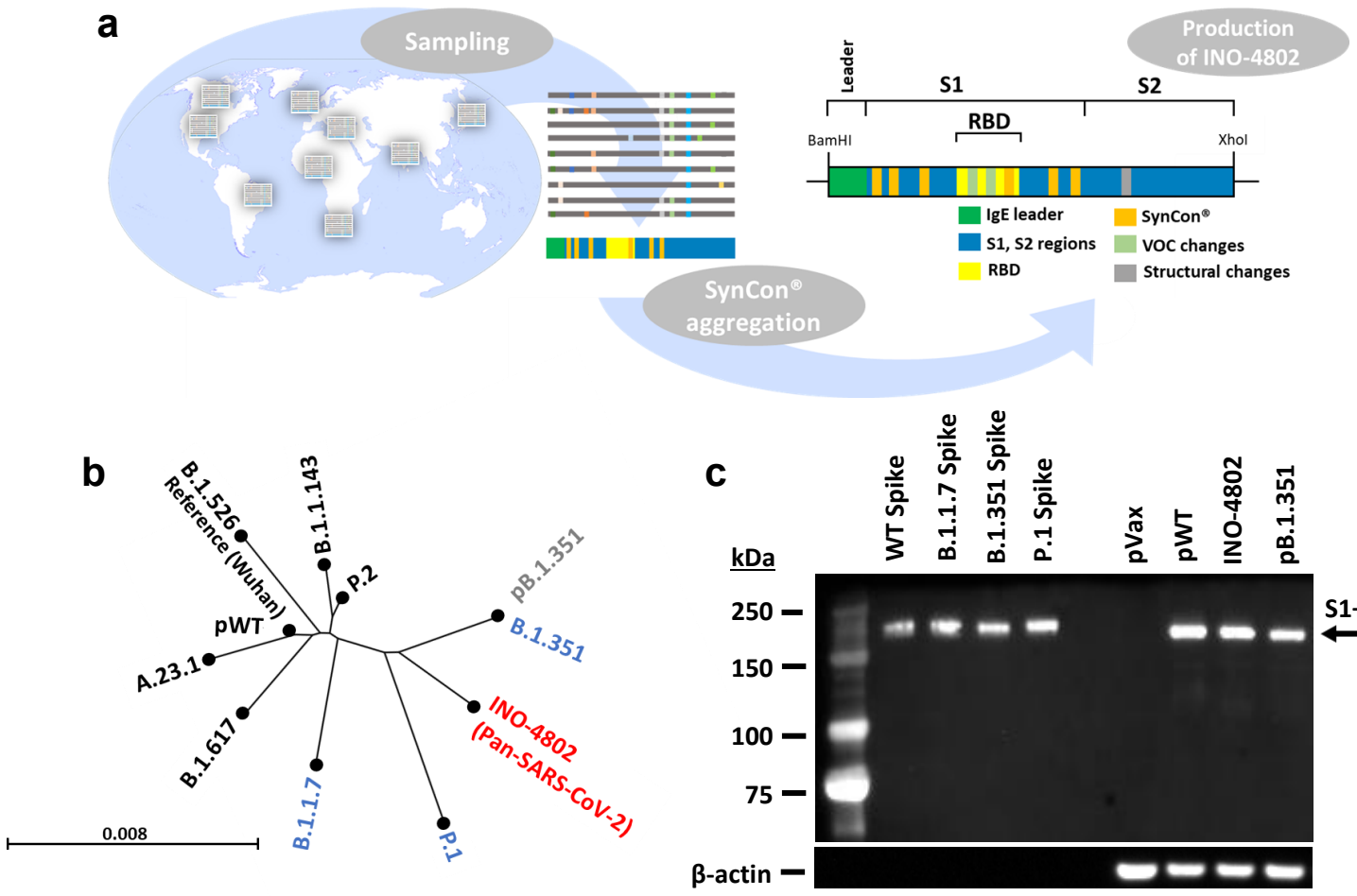

Figure 2

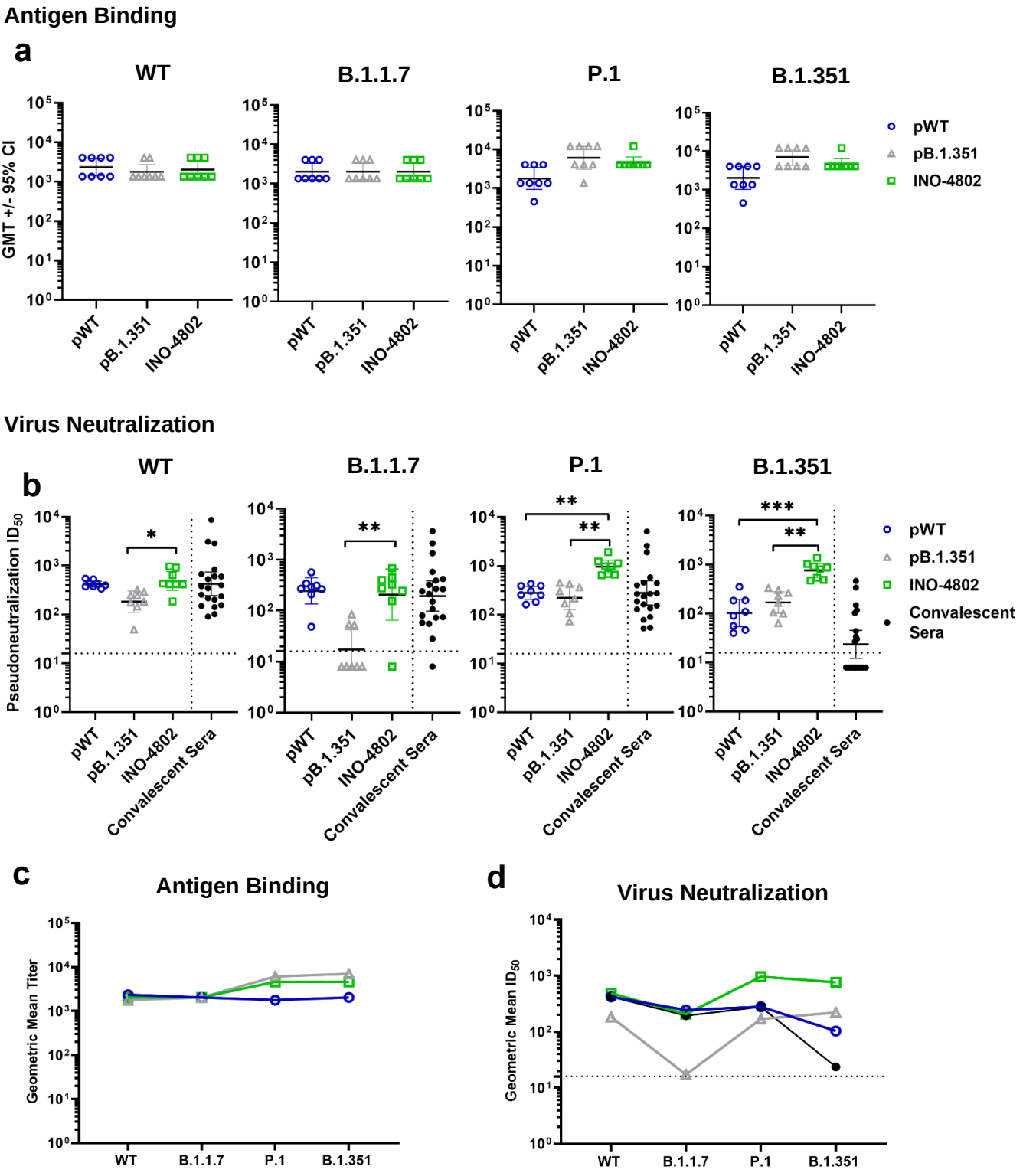

Figure 3

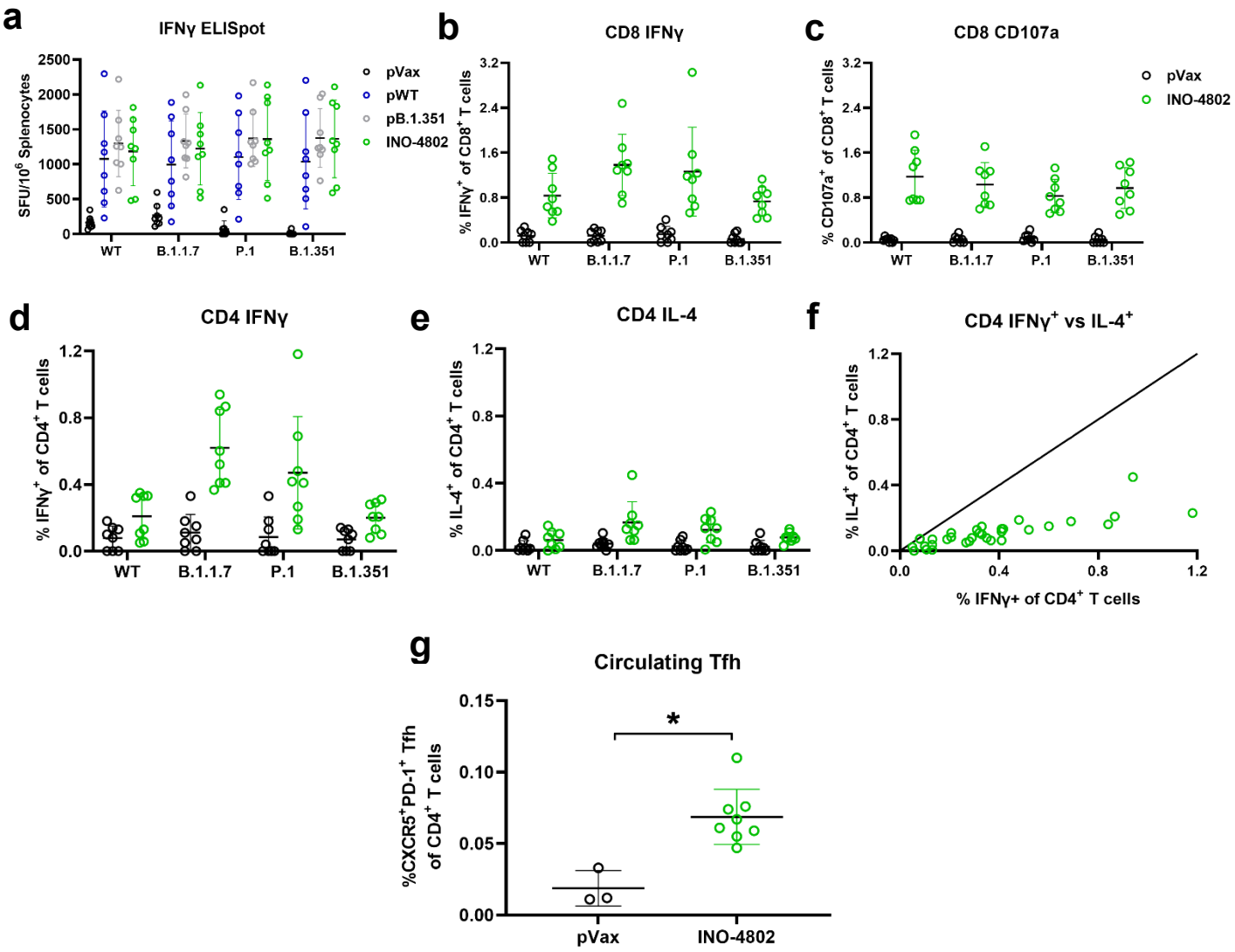

Figure 4

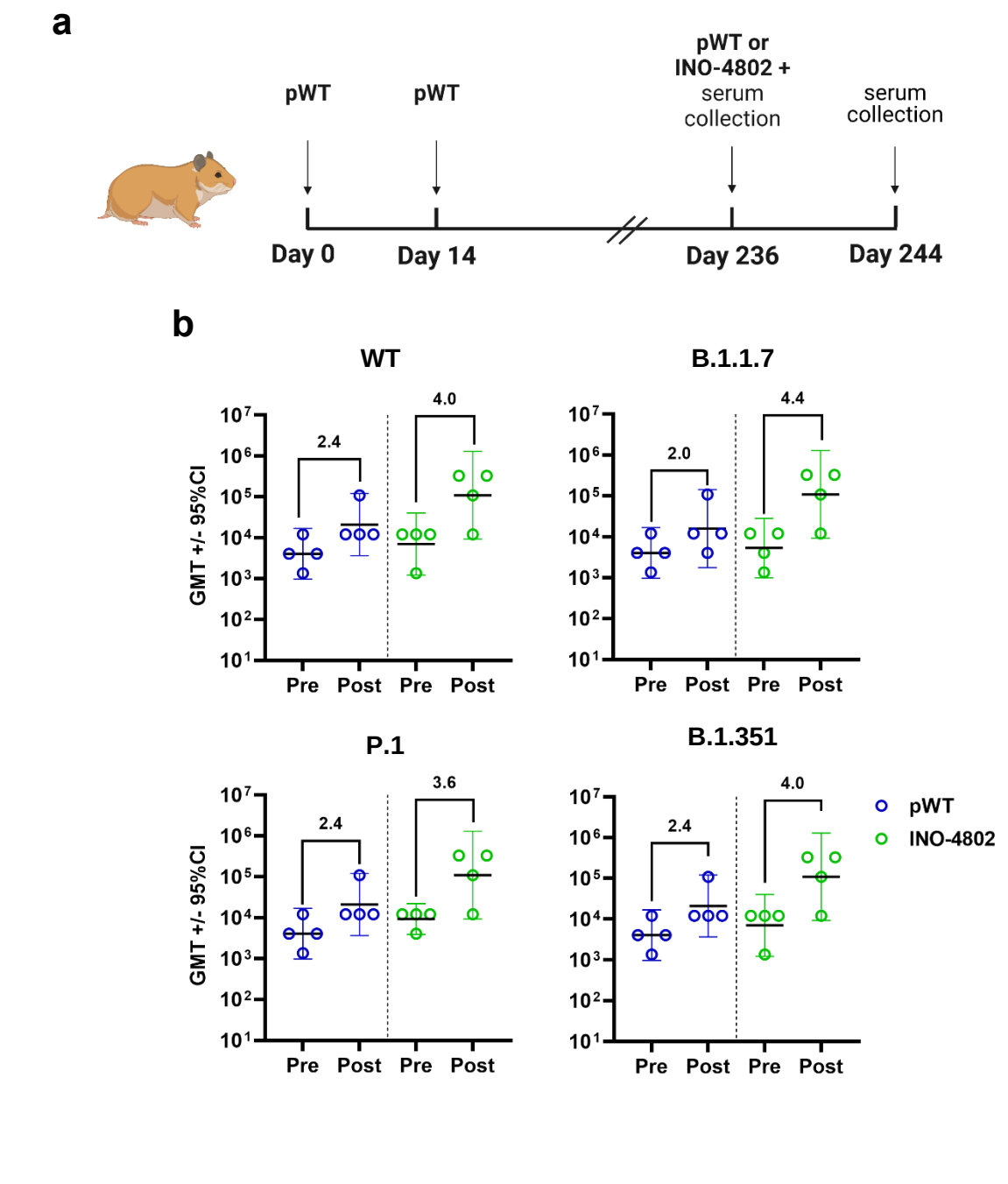

Table S1. Flow cytometry panel

Tube	Channel	Marker/Cytokine
1	FITC	CD107a
2	V500	Live/Dead Fix Aqua
3	PerCP-Cy5.5	CD4
4	BV786	CD8
5	APC-Cy7	CD3
6	BV605	IFNγ
7	AlexaFluor 700	IL-2
8	AlexaFluor647	IL-4
9	V450	TNF

Figure S1

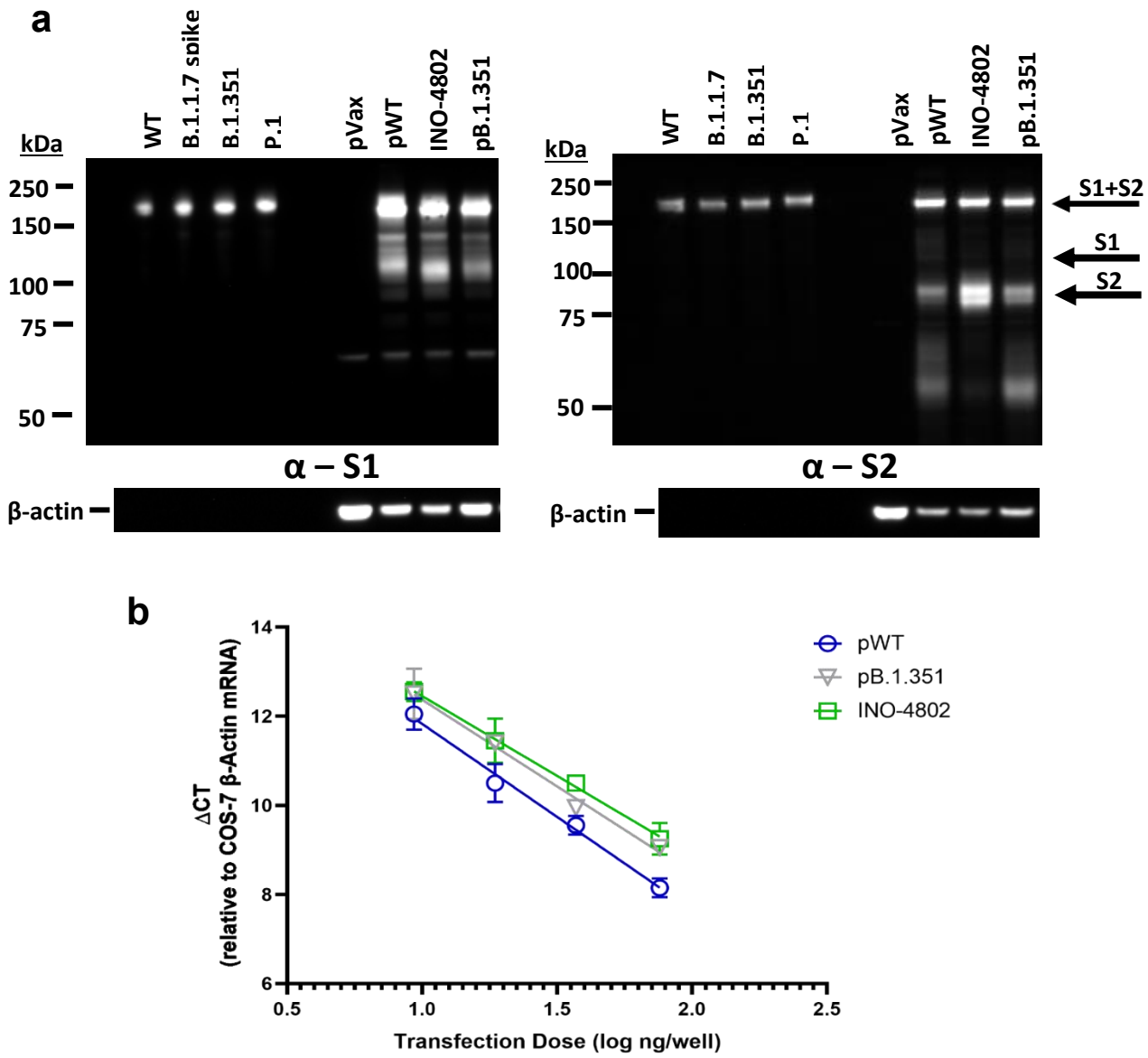

Figure S2