# A Newcastle disease virus (NDV) expressing membrane-anchored spike as a cost-effective inactivated SARS-CoV-2 vaccine

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## Abstract

A successful SARS-CoV-2 vaccine must be not only safe and protective but must also meet the demand on a global scale at low cost. Using the current influenza virus vaccine production capacity to manufacture an egg-based inactivated Newcastle disease virus (NDV)/SARS-CoV-2 vaccine would meet that challenge. Here, we report pre-clinical evaluations of an inactivated NDV chimera stably expressing the membrane-anchored form of the spike (NDV-S) as a potent COVID-19 vaccine in mice and hamsters. The inactivated NDV-S vaccine was immunogenic, inducing strong binding and/or neutralizing antibodies in both animal models. More importantly, the inactivated NDV-S vaccine protected animals from SARS-CoV-2 infections or significantly attenuated SARS-CoV-2 induced disease. In the presence of an adjuvant, antigen-sparing could be achieved, which would further reduce the cost while maintaining the protective efficacy of the vaccine.

**Keywords**

COVID-19 vaccine, egg-based SARS-CoV-2 vaccine, antigen-sparing, adjuvant, pre-clinical study, mouse-adapted SARS-CoV-2, hamster model

## Introduction

A severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) vaccine is urgently needed to mitigate the current coronavirus disease 2019 (COVID-19) pandemic worldwide. Numerous vaccine approaches are being developed (1-4), however, many of them are not likely to be cost-effective and affordable by low-income countries and under-insured populations. This could be of concern in the long run, as it is crucial to vaccinate a larger population than the high-income minority to establish herd immunity and effectively contain the spread of the virus. Among all the SARS-CoV-2 vaccine candidates, an inactivated vaccine might have the advantage over live vaccines of having a better safety profile in vulnerable individuals. In addition, inactivated vaccines could be combined with an adjuvant for better protective efficacy and dose-sparing to meet the large global demand. However, the current platform to produce the inactivated whole virion SARS-CoV-2 vaccine requires the propagation of the virus in cell culture under BSL-3 conditions (3) and only very few BSL-3 vaccine production facilities exist, limiting the scaling. Excessive inactivation procedures might have to be implemented to ensure the complete inactivation of the virus, at the risk of losing antigenicity of the vaccine. Many viral vector vaccines against coronaviruses have been developed, but they can only be tested as live vaccines (4-9). In addition, the efficacy of certain viral vectors could be dampened by pre-existing immunity to the viral backbone in the human population. Most recombinant protein vaccines require cumbersome manufacturing procedures that would be difficult for their inexpensive mass manufacturing. Genetic vaccines (mRNA and DNA vaccines) have a great promise, but as they have been developed only recently, their performance in humans is uncertain.

We have previously reported the construction of Newcastle disease virus (NDV)-based viral vectors expressing a pre-fusion spike protein, whose transmembrane domain and cytoplasmic tail were replaced with those from the NDV fusion (F) protein (S-F chimera) (10). We have shown that these NDV vector vaccines grow well in embryonated chicken eggs, and that the SARS-CoV-2 spike (S) proteins are abundantly incorporated into the NDV virions. The NDV vector, based on a vaccine virus strain against an avian pathogen, overcomes the abovementioned limitation for viral vector vaccines and allows the manufacturing of the vaccine prior to its inactivation under BSL-2 conditions. In this study, we investigated an attenuated recombinant NDV expressing the membrane-anchored S-F chimera (NDV-S)

as an inactivated SARS-CoV-2 vaccine candidate with and without an adjuvant in mice and hamsters. We found that the S-F chimera expressed by the NDV vector is very stable with no antigenicity loss after 3 weeks of 4°C storage in allantoic fluid. The beta-propiolactone (BPL) inactivated NDV-S vaccine is immunogenic, inducing high titers of S-specific antibodies in both animal models. Furthermore, the effects of a clinical-stage investigational liposomal suspension adjuvant (R-enantiomer of the cationic lipid DOTAP, R-DOTAP) (11-14), as well as an MF-59 like oil-in-water emulsion adjuvant (AddaVax) were also evaluated in mice. Both adjuvants were shown to achieve dose sparing (>10 fold) in mice. The vaccinated animals were protected from SARS-CoV-2 infection or SARS-CoV-2 induced disease. This is encouraging as the existing global egg-based production capacity for inactivated influenza virus vaccines could be utilized immediately to rapidly produce egg-based NDV-S vaccine with minimal modifications to their production pipelines. Most importantly, this class of products is amenable to large-scale production at low cost and has an excellent safety profile in infants, pregnant women and the elderly (15-17). Alternatively, the NDV-S and other chimeric NDV vaccines can also be manufactured in cultured cells such as Vero cells (18).

## Materials and Methods

### Plasmids

The construction of NDV_LS/L289A_S-F rescue plasmid has been described in a previous study (10). Briefly, the sequence of the ectodomain of the S without the polybasic cleavage site ($^{682}$RRAR$^{685}$ to A) was amplified from pCAGGS plasmid (19) encoding the codon-optimized nucleotide sequence of the S gene (GenBank: MN908947.3) of a SARS-CoV-2 isolate (Wuhan-Hu-1/2020) by polymerase chain reaction (PCR), using primers containing the gene end (GE), gene start (GS) and a Kozak sequences at the 5' end (20). The nucleotide sequence of the transmembrane domain (TM) and the cytoplasmic tail (CT) of the NDV_LaSota fusion (F) protein was codon-optimized for mammalian cells and synthesized by IDT (gBlock). The amplified S ectodomain was fused to the TM/CT of F through a GS linker (GGGGS). Additional nucleotides were added at the 3' end to follow the "rule of six" of paramyxovirus genome. The S-F gene was inserted between the P and M gene of pNDV_LaSota (LS) L289A mutant (NDV_LS/L289A) antigenomic cDNA by in-Fusion cloning (Clontech). The recombination product was transformed into NEB® Stable Competent E. coli (New England Biolabs, Inc.) to generate the NDV_LS/L289A_S-F rescue plasmid. The plasmid was purified using PureLink™ HiPure Plasmid Maxiprep Kit (Thermo Fisher Scientific).

### Cells and viruses

BSRT7 cells stably expressing the T7 polymerase were kindly provided by Dr. Benhur Lee at ISMMS. The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; Gibco) containing 10% (vol/vol) fetal bovine serum (FBS) and 100 unit/ml of penicillin and 100 µg/ml of streptomycin (P/S; Gibco) at 37°C with 5% $CO_2$. SARS-CoV-2 isolate USA-WA1/2020 (WA-1, BEI Resources NR-52281) used for hamster challenge were propagated in Vero E6 cells (ATCC CRL-1586) in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 2% fetal bovine serum (FBS), 4.5 g/L D-glucose, 4 mM L-glutamine, 10 mM Non-Essential Amino Acids, 1 mM Sodium Pyruvate, and 10 mM HEPES at 37 °C. All experiments with live SARS-CoV-2 were performed in the Centers for Disease Control and Prevention (CDC)/US Department of Agriculture (USDA)-approved biosafety level 3 (BSL-3) biocontainment facility of the Global Health and Emerging Pathogens Institute at the Icahn School of Medicine at Mount Sinai in accordance with institutional biosafety requirements.

**Rescue of NDV LaSota expressing the spike of SARS-CoV-2**

To rescue NDV_LS/L289A_S-F, six-well plates of BSRT7 cells were seeded 3 x $10^5$ cells per well the day before transfection. The next day, 4 µg of pNDV_LS/L289A_S-F, 2 µg of pTM1-NP, 1 µg of pTM1-P, 1 µg of pTM1-L and 2 µg of pCI-T7opt were re-suspended in 250 µl of Opti-MEM (Gibco). The plasmid cocktail was then gently mixed with 30 µL of TransIT LT1 transfection reagent (Mirus). The mixture was incubated at room temperature (RT) for 30 min. Toward the end of the incubation, the growth medium of each well was replaced with 1 ml of Opti-MEM. The transfection complex was added dropwise to each well and the plates were incubated at 37°C with 5% $CO_2$. The supernatant and the cells from transfected wells were harvested at 48 h post-transfection, and briefly homogenized by several strokes using an insulin syringe. Two hundred microliters of the homogenized mixture were injected into the allantoic cavity of 8- to 10-day old specific-pathogen-free (SPF) embryonated chicken eggs. The eggs were incubated at 37°C for 3 days before being cooled at 4°C overnight. The allantoic fluid was collected and clarified by centrifugation. The rescue of NDV was determined by hemagglutination (HA) assay using 0.5% chicken or turkey red blood cells. The RNA of the positive samples was extracted and treated with DNase I (Thermo Fisher Scientific). Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed to amplify the transgene. The sequences of the transgenes were confirmed by Sanger Sequencing (Genewiz). Recombinant DNA experiments were performed in accordance with protocols approved by the Icahn School of Medicine at Mount Sinai Institutional Biosafety Committee (IBC).

**Preparation of concentrated virus**

Before concentrating the virus, allantoic fluids were clarified by centrifugation at 4,000 rpm using a Sorvall Legend RT Plus Refrigerated Benchtop Centrifuge (Thermo Fisher Scientific) at 4°C for 30 min to remove debris. Live virus in the allantoic fluid was pelleted through a 20% sucrose cushion in NTE buffer (100 mM NaCl, 10 mM Tris-HCl, 1 mM EDTA, pH 7.4) by ultra-centrifugation in a Beckman L7-65 ultracentrifuge at 25,000 rpm for two hours at 4°C using a Beckman SW28 rotor (Beckman Coulter, Brea, CA, USA). Supernatants were aspirated off and the pellets were re-suspended in PBS (pH 7.4). The protein content was determined using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific). To prepare inactivated concentrated viruses, 1 part of 0.5 M disodium phosphate (DSP) was mixed with 38 parts of the allantoic fluid to stabilize the pH. One part of 2% beta-propiolactone (BPL) was added dropwise to the mixture during shaking, which gave a final concentration of 0.05% BPL. The treated allantoic fluid was mixed thoroughly and incubated on ice for 30 min. The mixture was then placed in a 37 °C water bath for two hours shaken every 15 min. The inactivated allantoic fluid was clarified by centrifugation at 4,000 rpm for 30 minutes. The loss of infectivity was confirmed by the lack of growth (determined by HA assay) of the virus from 10-day old embryonated chicken eggs that were inoculated with inactivated virus preparations. The inactivated viruses were concentrated as described above.

**Evaluation of stability of the S-F in the allantoic fluid**

The allantoic fluid containing the NDV_LS/L289A_S-F virus was harvested and clarified by centrifugation. The clarified allantoic fluid was aliquoted into 15 ml volumes. Week (wk) 0 allantoic fluid was concentrated immediately after centrifugation as described above through a 20% sucrose cushion. The pelleted virus was re-suspended in 300 µL phosphate buffered saline (PBS) and stored at -80°C. The other three aliquots of the allantoic fluid were maintained at 4°C to test the stability of the S-F construct. Wk 1, 2 and 3 samples were collected consecutively on a weekly basis, and concentrated virus was prepared in 300 µL PBS using the same method. The protein content of the concentrated virus from wk 0, 1, 2, and 3 was determined using BCA assay after one free-thaw from -80°C. One microgram of each concentrated virus preparation was resolved on a 4-20% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad) and the S-F protein and the NDV hemagglutinin-neuraminidase (HN) protein were detected by Western blot.

**Western Blot**

Concentrated live or inactivated virus samples were mixed with Novex™ Tris-Glycine SDS Sample Buffer (2X) (Thermofisher Scientific) with NuPAGE™ Sample Reducing Agent (10X) (Thermofisher Scientific). One or two micrograms of the concentrated viruses were heated at 95 °C for 5 min before being resolved on 4-20% SDS-PAGE (Bio-Rad) using the Novex™ Sharp Pre-stained Protein Standard

(ThermoFisher Scientific) as the protein marker. To perform Western blots, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (GE healthcare). The membrane was blocked with 5% non-fat dry milk in PBS containing 0.1% v/v Tween 20 (PBST) for 1 h at room temperature (RT). The membrane was washed with PBST on a shaker three times (10 min at RT each time) and incubated with an S-specific mouse monoclonal antibody 2B3E5 (provided by Dr. Thomas Moran at ISMMS) or an HN-specific mouse monoclonal antibody 8H2 (MCA2822, Biorad) diluted in PBST containing 1% bovine serum albumin (BSA), overnight at 4°C. The membranes were then washed with PBST on a shaker 3 times (10 min at RT each time) and incubated with secondary sheep anti-mouse IgG linked with horseradish peroxidase (HRP) diluted (1:2,000) in PBST containing 5% non-fat dry milk. The secondary antibody was discarded and the membranes were washed with PBST on a shaker three times (10 min at RT each time). Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) was added to the membrane, the blots were imaged using the Bio-Rad Universal Hood Ii Molecular imager (Bio-Rad) and processed by Image Lab Software (Bio-Rad).

**Immunization and challenge study in BALB/c mice**

Seven-week old female BALB/cJ mice (Jackson Laboratories) were used in this study. Experiments were performed in accordance with protocols approved by the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee (IACUC). Mice were divided into 10 groups (n=5) receiving the inactivated virus without or with an adjuvant at three different doses intramuscularly. The vaccination followed a prime-boost regimen in a 2-week interval. Specifically, group 1, group 2 and group 3 received 5 µg, 10 µg and 20 µg inactivated NDV-S vaccine (total protein) without the adjuvant, respectively; Group 4, group 5 and group 6 received low doses of 0.2 µg, 1 µg and 5 µg of inactivated NDV-S vaccine, respectively, combined with 300 µg/mouse of R-DOTAP (PDS Biotechnology); Group 7, group 8 and group 9 mice received 0.2 µg, 1 µg and 5 µg of inactivated NDV-S vaccine, respectively, with 50 µl/mouse of AddaVax (Invivogen) as the adjuvant. Group 10 received 20 µg inactivated WT NDV as the vector-only control. The SARS-CoV-2 challenge was performed at the University of North Carolina by Dr. Ralph Baric's group in a Biosafety Level 3 (BSL-3) facility. Mice were intranasally (i.n challenged 19 days after the boost using a mouse-adapted SARS-CoV-2 strain (1, 21) at 7.5 x $10^4$ plaque forming units (PFU). Weight loss was monitored for 4 days.

**Immunization and challenge study in golden Syrian hamsters**

Eight-week old female golden Syrian hamsters were used in this study. Experiments were performed in accordance with protocols approved by the Icahn School of Medicine at Mount Sinai Institutional Animal Care and Use Committee (IACUC). Five groups (n=8) of hamsters were included. The inactivated

vaccines were given intramuscularly following a prime-boost regimen in a 2-week interval. Group 1 received 10 μg of inactivated NDV-S vaccine; group 2 received 5 μg of inactivated NDV-S vaccine combined with 50 μl of AddaVax per hamster; group 3 hamsters received 10 μg of inactivated WT NDV as vector-only control. A healthy control group receiving no vaccine was also included. Twenty-four days after the boost, hamsters were challenged intranasally with $10^4$ PFU of the USA-WA1/2020 SARS-CoV-2 strain in a Biosafety Level 3 (BSL-3) facility. Weight loss was monitored for 5 days.

**Lung titers**

Lung lobes of mice were collected and homogenized in PBS. A plaque assay was performed to measure viral titer in the lung homogenates as described previously (1, 21). Geometric mean titers of plaque forming units (PFU) per lobe were calculated using GraphPad Prism 7.0.

**Enzyme Linked Immunosorbent Assays (ELISAs)**

Mice were bled pre-boost and 11 days after the boost. Hamsters were bled pre-boost and 26 days after the boost. Sera were isolated by low-speed centrifugation. ELISAs were performed as described previously (19). Briefly, Immulon 4 HBX 96-well ELISA plates (Thermo Fisher Scientific) were coated with 2 μg/ml of recombinant trimeric S protein (50ul per well) in coating buffer (SeraCare Life Sciences Inc.) overnight at 4°C. The next day, all plates were washed 3 times with 220 μL PBS containing 0.1% (v/v) Tween-20 (PBST) and blocked in 220 μL blocking solution (3% goat serum, 0.5% non-fat dried milk powder, 96.5% PBST) for 1 h at RT. Both mouse sera and hamster sera were 3-fold serially diluted in blocking solution starting at 1:30 followed by a 2 h incubation at RT. ELISA plates were washed 3 times with PBST and incubated in 50 μL per well of sheep anti-mouse IgG-horseradish peroxidase (HRP) conjugated antibody (GE Healthcare) or goat anti-hamster IgG-HRP conjugated antibody (Invitrogen) diluted (1:3,000) in blocking solution. Plates were washed 3 times with PBST and 100 μL of *o*-phenylenediamine dihydrochloride (SigmaFast OPD, Sigma) substrate was added per well. After developing the plates for 10 min, 50 μL of 3 M hydrochloric acid (HCl) was added to each well to stop the reactions. The optical density (OD) was measured at 492 nm on a Synergy 4 plate reader (BioTek) or equivalents. An average of OD values for blank wells plus three standard deviations was used to set a cutoff for plate blank outliers. A cutoff value was established for each plate that was used for calculating the endpoint titers. The endpoint titers of serum IgG responses was graphed using GraphPad Prism 7.0.

**Micro-neutralization assay**

All neutralization assays were performed in the biosafety level 3 (BSL-3) facility following institutional guidelines as described previously (19, 22). Briefly, serum samples were heat-inactivated at 56°C for 60

minutes prior to use. Vero E6 cells were maintained in culture using DMEM supplemented with 10% fetal bovine serum (FBS). Twenty-thousands cells per well were seeded in a 96-well cell culture plate the night before the assay. Pooled sera in technical duplicates were serially diluted by 3-fold starting at 1:20 in a 96-well cell culture plate and each dilution was mixed with 600 times the 50% tissue culture infectious dose ($TCID_{50}$) of SARS-CoV-2 (USA-WA1/2020, BEI Resources NR-52281). Serum-virus mixture was incubated for 1 h at RT before being added to the cells for another hour of incubation in a 37°C incubator. The virus-serum mixture was removed and the corresponding serum dilution was added to the cells. The cells were incubated for 2 days and fixed with 100 µL 10% formaldehyde per well for 24 h before taken out of the BSL-3 facility. The staining of the cells was performed in a BSL-2 biosafety cabinet. The formaldehyde was carefully removed from the cells. Cells were washed with 200 µL PBS once before being permeabilized with PBS containing 0.1% Triton X-100 for 15 min at RT. Cells were washed with PBS and blocked in PBS containing 3% dry milk for 1h at RT. Cells were then stained with 100 µL per well of a mouse monoclonal anti-NP antibody (1C7), kindly provided by Dr. Thomas Moran at ISMMS, at 1µg/ml for 1h at RT. Cells were washed with PBS and incubated with 100 µL per well anti-mouse IgG HRP (Rockland) secondary antibody at 1:3,000 dilution in PBS containing 1% dry milk for 1h at RT. Finally, cells were washed twice with PBS and the plates were developed using 100 µL of SigmaFast OPD substrate. Ten minutes later, the reactions were stopped using 50 µL per well of 3M HCI. The OD 492 nm was measured on a Biotek SynergyH1 Microplate Reader. Non-linear regression curve fit analysis (The top and bottom constraints were set at 100% and 0%) over the dilution curve was performed to calculate 50% of inhibitory dilution ($ID_{50}$) of the serum using GraphPad Prism 7.0.

## Results

### The design and concept of NDV-based inactivated SARS-CoV-2 vaccines

We have previously reported the construction of NDV-based SARS-CoV-2 vaccine candidates, among which NDV vectors expressing the S-F chimera (10) showed higher abundance of the spike protein in the NDV particles than the NDV vector expressing just the wild type (WT) S protein. The final construct also had a mutation (L289A) in the F protein of NDV which was shown to facilitate HN-independent fusion of the virus (Fig. 1A). To develop an NDV-based inactivated SARS-CoV-2 vaccine, the existing global influenza virus vaccine production capacity could be employed as both influenza virus and NDV grow to high titers in embryonated chicken eggs. With minor modifications to the manufacturing process of inactivated influenza virus vaccines, NDV-S vaccine can be purified by zonal sucrose density centrifugation. Egg-grown influenza virus vaccines are inactivated by formalin or beta-propiolactone (BPL) treatment. For the inactivated NDV-S vaccine we chose BPL inactivation because it is believed to

be a less disrupting inactivation process. Such inactivated NDV-S vaccines will display SARS-CoV-2 spike proteins together with HN and F NDV proteins on the surface of the whole inactivated virion. Due to the large size, the spike proteins are likely immunodominant relative to the HN and F proteins. The inactivated NDV-S vaccine could be administered intramuscularly, with an adjuvant for dose sparing. This approach should be suited to safely induce spike-specific protective antibodies (Fig. 1B).

**The spike protein expressed on NDV virions is stable in allantoic fluid**

The stability of the antigen could be of concern as the vaccine needs to be purified and inactivated through a temperature-controlled (~4°C) process. The final product is often formulated and stored in liquid buffer at 4°C. To examine the stability of the S-F protein, allantoic fluid containing the NDV-S live virus was aliquoted into equal volumes (15 ml), and stored at 4°C. Samples were collected weekly (wk 0, 1, 2, 3) and concentrated through a 20% sucrose cushion. The concentrated virus was re-suspended in equal amounts of PBS. The total protein content of the 4 aliquots was comparable among the preparations (wk 0: 0.94 mg/ml; wk 1: 1.04 mg/ml; wk 2: 0.9 mg/ml; wk 3: 1.08 mg/ml). The cold stability of the S-F construct was evaluated by Western blot with the anti-S monoclonal antibody, 2B3E5. As compared to the stability of the NDV HN protein, the Spike protein remained stable when kept in allanotic fluid at 4 °C (Fig. 2A). Moreover, the inactivation procedure using 0.05% BPL did not cause any loss of antigenicity of the S-F, as evaluated by Western blot (Fig. 2B). These observations demonstrate that the membrane anchored S-F chimera expressed by the NDV vector is very stable without degradation at 4°C for 3 weeks or when treated with BPL for inactivation.

**Inactivated NDV-S vaccine induced high titers of binding and neutralizing antibodies in mice**

For a pre-clinical evaluation of the inactivated NDV-S vaccine, immunogenicity as well as the dose sparing ability of the adjuvants were investigated in mice. The vaccines were administered intramuscularly, following a prime-boost regimen in a 2-week interval. Specifically, for the three unadjuvanted groups, mice were intramuscularly immunized with increasing doses of inactivated NDV-S vaccine at 5 μg, 10 μg or 20 μg per mouse. Two adjuvants were tested here, a clinical-stage adjuvant, liposomal suspension of the pure R-enantiomer of the cationic lipid DOTAP (R-DOTAP) and the MF59-like oil-in-water emulsion adjuvant AddaVax. Each adjuvant was combined with low doses of NDV-S vaccines at 0.2 μg, 1 μg and 5 μg. Mice receiving 20 μg of inactivated WT NDV were used as vector-only (negative) controls (Fig. 3A). Mice were bled pre-boost (2 weeks after prime) and 11 days post-boost to examine antibody responses by ELISA using a trimeric full-length S protein as the substrate (19), and micro-neutralization assay using the USA-WA1/2020 strain of SARS-CoV-2 (Fig.3A). After one immunization, all vaccination groups developed S-specific antibodies. The boost greatly increased the

antibody titers of all NDV-S immunization groups. Immunization with R-DOTAP combined with 5 μg of vaccine induced the highest antibody titer. Immunization with one microgram of vaccine formulated with R-DOTAP or AddaVax and 5 μg of vaccine with AddaVax induced comparable levels of binding antibody, which is also similar to the titers induced by 20 μg of vaccine without an adjuvant. As expected, immunization with the inactivated wild type NDV virus did not induce S-specific antibody responses (Fig. 3B). We performed microneutralization assays to determine the neutralizing activity of serum antibodies collected from vaccinated mice. Except for mice immunized with the WT NDV, sera from all mice immunized with NDV-S vaccine showed neutralizing activity against the SARS-CoV-2 USA-WA1/2020 strain. The neutralization titers induced by immunization with of 1 μg of vaccine with R-DOTAP ($ID_{50}$ of ~476) and 5 μg of vaccine with AddaVax groups ($ID_{50}$ of ~515) appeared to be the highest and were comparable to each other. These titer levels are also in the higher range of human convalescent serum neutralization titers as measured in our previous studies (19, 23). Interestingly, although the group receiving 5 μg of vaccine with R-DOTAP developed the most abundant binding antibodies detected by ELISA, these sera were not the most neutralizing ones suggesting R-DOTAP might have a different impact on immunogenicity compared to AddaVax. R-DOTAP is an immune modulator, that induces the production of important cytokines and chemokines and enhances cytolytic T cells when combined with proteins. It is possible that with more antigen, the immune responses were skewed towards the induction of non-neutralizing antibodies (Fig. 3C). In any case, these results demonstrated that inactivated NDV-S vaccine expressing the membrane anchored S-F was immunogenic inducing potent binding and neutralizing antibodies. Importantly, at least 10-fold dose sparing was achieved with an adjuvant in mice.

**The inactivated NDV-S vaccine protects mice from infection by a mouse-adapted SARS-CoV-2**

To evaluate vaccine-induced protection, mice were challenged 19 days post-boost using a mouse-adapted SARS-CoV-2 virus (1, 21) (Fig. 3A). Weight loss was monitored for 4 days post infection, at which point the mice were euthanized to assess pulmonary virus titers. Only the negative control group receiving the WT NDV was observed to lose notable weight (~10%) by day 4 post-infection, while all the vaccinated groups showed no weight loss (Fig.4A). Viral titers in the lung at 4 days post challenge were also measured. As expected, the negative control group given the WT NDV exhibited the highest viral titer of $>10^4$ PFU/lobe. Groups receiving 5 μg of unadjuvanted vaccine or 0.2 μg of vaccine with R-DOTAP showed detectable but low viral titers in the lung, while all the other groups were fully protected showing no viral loads (Fig. 4B). These results are encouraging as immunization with 0.2 μg of vaccine adjuvanted with AddaVax conferred a level of protection that was equal to that induced by immunization with 10 μg of vaccine without an adjuvant. Although 0.2 μg of vaccine with R-DOTAP did not induce sterilizing

immunity, approximately a 1000-fold reduction of viral titer in the lungs was achieved. To conclude, the inactivated NDV-S exhibits great potential as a cost-effective vaccine as it induces protective immunity against the SARS-CoV-2 at very low doses with an adjuvant.

**The inactivated NDV-S vaccine confers protection against SARS-CoV-2 infection in a hamster model**

Golden Syrian hamsters have been characterized as a useful small animal model for COVID-19 as they are susceptible to SARS-CoV-2 infections and manifest SARS-CoV-2 induced diseases (24, 25). Here, we conducted a pilot study that assessed the immunogenicity and protective efficacy of the inactivated NDV-S vaccine in hamsters. Female golden Syrian hamsters were immunized by a prime-boost regimen in a 2-week interval via the intramuscular administration route. Twenty-four days after the booster immunization, hamsters were intranasally infected with $10^4$ PFU of SARS-CoV-2 (USA-WA1/2020) virus. Four groups of hamsters were included in this pilot study. Group 1 was immunized with 10 μg of inactivated NDV-S vaccine per animal without adjuvants. Group 2 received 5 μg of inactivated NDV-S vaccine with AddaVax as an adjuvant. Group 3 was immunized with 10 μg of inactivated WT NDV as the vector-only negative control group. Group 4, which was not vaccinated and was mock-challenged with PBS, was included as the healthy control group (Fig. 5A). Serum IgG titers sampled prior to the booster immunization and at 2 days post-infection (dpi) were measured by ELISA. One immunization with NDV-S vaccine with or without the adjuvant successfully induced spike-specific antibodies. Since there was no seroconversion from infection at 2 dpi indicated by baseline level of the WT NDV sera, the increase in titers at 2 dpi as compared to titers after vaccine priming most likely represent vaccine-induced antibody levels after the boost. As expected, the boost substantially increased the antibody titers in the NDV-S vaccination groups, whereas the WT NDV sera showed negligible binding signals (Fig. 5B). Nevertheless, we cannot exclude a contribution from a rapid production of S antibodies by vaccine-induced memory B cells after exposure to SARS-CoV-2. Hamsters were challenged and weight loss was monitored for 5 days. The WT NDV group lost up to 15% of weight by 5 dpi. Animals receiving 10 μg of inactivated NDV-S vaccine lost ~10% of weight by 3 dpi, at which point body weights started to recover. Animals receiving 5 μg of inactivated NDV-S vaccine with AddaVax only lost weight by 2 dpi, at which point body weights started to recover (Fig. 5C). These data suggested inactivated NDV-S vaccine could effectively attenuate the symptoms of SARS-CoV-2 induced diseases in hamsters.

**Discussion**

We have previously reported NDV-based SARS-CoV-2 live vaccines expressing two forms of spike protein (S and S-F)(10). Since the S-F showed superior incorporation into NDV particles, we investigated

its potential of being used as an inactivated vaccine in this study. The NDV-S was found to be very stable when stored at 4°C for 3 weeks with no loss of antigenicity of the S-F protein. In mice, we have shown a total amount of inactivated NDV-S vaccine as low as 0.2 μg could significantly reduce viral titers in the lung when combined with R-DOTAP, by approximately a factor of 1000, while the adjuvant AddaVax conferred even better protection. NDV-S vaccine at 1 μg with either adjuvant elicited potent neutralizing antibodies and resulted in undetectable viral titers in the lung after SARS-CoV-2 challenge. These pre-clinical results demonstrate that antigen-sparing greater than 10-fold can be achieved in a mouse model, providing valuable input for clinical trials in humans. In a pilot hamster experiment, the inactivated NDV-S vaccine is also immunogenic inducing high titers of spike-specific antibodies. Since hamsters are much more susceptible to SARS-CoV-2 infection, the group receiving the WT NDV lost up to 15% of weight by day 5, while both NDV-S vaccinated groups with or without the adjuvant greatly attenuated SARS-CoV-2 induced disease determined by the weight loss. The AddaVax adjuvant again enhanced vaccine-induced protection, resulting in weight loss only on 2 dpi of the group. We did not evaluate the adjuvant R-DOTAP, as the dosing was not well determined for this model by the time of this study. However, R-DOTAP as well as additional adjuvants will be evaluated in combination with the inactivated NDV-S vaccine in future studies. In addition, we will examine other outcomes of SARS-CoV-2 induced disease in hamsters, such as viral titers in nasal washes or lungs.

We have shown promising protection by immunization with inactivated NDV-S in both the mouse and the hamster models. Even though sterilizing immunity might not always be induced, the trade-off for having an affordable and widely available effective vaccine that reduces the symptoms of COVID-19 should be much preferred over a high-cost vaccine that is limited to high income populations. Most importantly, the egg-based production of NDV-S vaccine only requires only minor modifications to the current inactivated influenza virus vaccine manufacturing process. The cost of goods should be similar to that of a monovalent inactivated influenza virus vaccine (a fraction of the cost of a quadrivalent seasonal influenza virus vaccine), or even lower due to dose sparing with an adjuvant that is inexpensive to manufacture.

**Conflict of interest statement**

The Icahn School of Medicine at Mount Sinai has filed patent applications entitled “RECOMBINANT NEWCASTLE DISEASE VIRUS EXPRESSING SARS-COV-2 SPIKE PROTEIN AND USES THEREOF”.

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**Reference**

1. Corbett KS, Edwards D, Leist SR, Abiona OM, Boyoglu-Barnum S, Gillespie RA, Himansu S, Schafer A, Ziwawo CT, DiPiazza AT, Dinnon KH, Elbashir SM, Shaw CA, Woods A, Fritch EJ, Martinez DR, Bock KW, Minai M, Nagata BM, Hutchinson GB, Bahl K, Garcia-Dominguez D, Ma L, Renzi I, Kong WP, Schmidt SD, Wang L, Zhang Y, Stevens LJ, Phung E, Chang LA, Loomis RJ, Altaras NE, Narayanan E, Metkar M, Presnyak V, Liu C, Louder MK, Shi W, Leung K, Yang ES, West A, Gully KL, Wang N, Wrapp D, Doria-Rose NA, Stewart-Jones G, Bennett H, Nason MC, Ruckwardt TJ, et al. 2020. SARS-CoV-2 mRNA Vaccine Development Enabled by Prototype Pathogen Preparedness. bioRxiv doi:10.1101/2020.06.11.145920.
2. Jackson LA, Anderson EJ, Rouphael NG, Roberts PC, Makhene M, Coler RN, McCullough MP, Chappell JD, Denison MR, Stevens LJ, Pruijssers AJ, McDermott A, Flach B, Doria-Rose NA, Corbett KS, Morabito KM, O'Dell S, Schmidt SD, Swanson PA, 2nd, Padilla M, Mascola JR, Neuzil KM, Bennett H, Sun W, Peters E, Makowski M, Albert J, Cross K, Buchanan W, Pikaart-Tautges R, Ledgerwood JE, Graham BS, Beigel JH, m RNASG. 2020. An mRNA Vaccine against SARS-CoV-2 - Preliminary Report. N Engl J Med doi:10.1056/NEJMoa2022483.
3. Gao Q, Bao L, Mao H, Wang L, Xu K, Yang M, Li Y, Zhu L, Wang N, Lv Z, Gao H, Ge X, Kan B, Hu Y, Liu J, Cai F, Jiang D, Yin Y, Qin C, Li J, Gong X, Lou X, Shi W, Wu D, Zhang H, Zhu L, Deng W, Li Y, Lu J, Li C, Wang X, Yin W, Zhang Y, Qin C. 2020. Rapid development of an inactivated vaccine candidate for SARS-CoV-2. Science doi:10.1126/science.abc1932.
4. Zhu FC, Li YH, Guan XH, Hou LH, Wang WJ, Li JX, Wu SP, Wang BS, Wang Z, Wang L, Jia SY, Jiang HD, Wang L, Jiang T, Hu Y, Gou JB, Xu SB, Xu JJ, Wang XW, Wang W, Chen W.

2020. Safety, tolerability, and immunogenicity of a recombinant adenovirus type-5 vectored COVID-19 vaccine: a dose-escalation, open-label, non-randomised, first-in-human trial. Lancet 395:1845-1854.

5. DiNapoli JM, Kotelkin A, Yang L, Elankumaran S, Murphy BR, Samal SK, Collins PL, Bukreyev A. 2007. Newcastle disease virus, a host range-restricted virus, as a vaccine vector for intranasal immunization against emerging pathogens. Proc Natl Acad Sci U S A 104:9788-93.

6. Liu RQ, Ge JY, Wang JL, Shao Y, Zhang HL, Wang JL, Wen ZY, Bu ZG. 2017. Newcastle disease virus-based MERS-CoV candidate vaccine elicits high-level and lasting neutralizing antibodies in Bactrian camels. J Integr Agric 16:2264-2273.

7. Li K, Li Z, Wohlford-Lenane C, Meyerholz DK, Channappanavar R, An D, Perlman S, McCray PB, Jr., He B. 2020. Single-Dose, Intranasal Immunization with Recombinant Parainfluenza Virus 5 Expressing Middle East Respiratory Syndrome Coronavirus (MERS-CoV) Spike Protein Protects Mice from Fatal MERS-CoV Infection. mBio 11.

8. Liniger M, Zuniga A, Tamin A, Azzouz-Morin TN, Knuchel M, Marty RR, Wiegand M, Weibel S, Kelvin D, Rota PA, Naim HY. 2008. Induction of neutralising antibodies and cellular immune responses against SARS coronavirus by recombinant measles viruses. Vaccine 26:2164-74.

9. Koch T, Dahlke C, Fathi A, Kupke A, Krahling V, Okba NMA, Halwe S, Rohde C, Eickmann M, Volz A, Hesterkamp T, Jambrecina A, Borregaard S, Ly ML, Zinser ME, Bartels E, Poetsch JSH, Neumann R, Fux R, Schmiedel S, Lohse AW, Haagmans BL, Sutter G, Becker S, Addo MM. 2020. Safety and immunogenicity of a modified vaccinia virus Ankara vector vaccine candidate for Middle East respiratory syndrome: an open-label, phase 1 trial. Lancet Infect Dis 20:827-838.

10. Weina Sun SRL, Stephen McCroskery, Yonghong Liu, Stefan Slamanig, Justine Oliva, Fatima Amanat, Alexandra Schaefer, Kenneth Dinnon III, Adolfo Garcia-Sastre, Florian Krammer, Ralph S. Baric, Peter Palese. 2020. Newcastle disease virus (NDV) expressing the spike protein of SARS-CoV-2 as vaccine candidate. BioRxiv doi: https://doi.org/10.1101/2020.07.26.221861.

11. Vasievich EA, Ramishetti S, Zhang Y, Huang L. 2012. Trp2 peptide vaccine adjuvanted with (R)-DOTAP inhibits tumor growth in an advanced melanoma model. Mol Pharm 9:261-8.

12. Smalley Rumfield C, Pellom ST, Morillon Ii YM, Schlom J, Jochems C. 2020. Immunomodulation to enhance the efficacy of an HPV therapeutic vaccine. J Immunother Cancer 8.

13. Riehl M, Harms M, Hanefeld A, Baleeiro RB, Walden P, Mader K. 2017. Combining R-DOTAP and a particulate antigen delivery platform to trigger dendritic cell activation: Formulation development and in-vitro interaction studies. Int J Pharm 532:37-46.

14. Gandhapudi SK, Ward M, Bush JPC, Bedu-Addo F, Conn G, Woodward JG. 2019. Antigen Priming with Enantiospecific Cationic Lipid Nanoparticles Induces Potent Antitumor CTL Responses through Novel Induction of a Type I IFN Response. J Immunol 202:3524-3536.

15. Langley JM, Wang L, Aggarwal N, Bueso A, Chandrasekaran V, Cousin L, Halperin SA, Li P, Liu A, McNeil S, Mendez LP, Rivera L, Innis BL, Jain VK. 2015. Immunogenicity and Reactogenicity of an Inactivated Quadrivalent Influenza Vaccine Administered Intramuscularly to Children 6 to 35 Months of Age in 2012-2013: A Randomized, Double-Blind, Controlled, Multicenter, Multicountry, Clinical Trial. J Pediatric Infect Dis Soc 4:242-51.

16. Madan A, Ferguson M, Rheault P, Seiden D, Toma A, Friel D, Soni J, Li P, Innis BL, Schuind A. 2017. Immunogenicity and safety of an AS03-adjuvanted H7N1 vaccine in adults 65years of age and older: A phase II, observer-blind, randomized, controlled trial. Vaccine 35:1865-1872.

17. Wang L, Chandrasekaran V, Domachowske JB, Li P, Innis BL, Jain VK. 2016. Immunogenicity and Safety of an Inactivated Quadrivalent Influenza Vaccine in US Children 6-35 Months of Age During 2013-2014: Results From A Phase II Randomized Trial. J Pediatric Infect Dis Soc 5:170-9.

18. Arifin MA, Mel M, Abdul Karim MI, Ideris A. 2010. Production of Newcastle disease virus by Vero cells grown on cytodex 1 microcarriers in a 2-litre stirred tank bioreactor. J Biomed Biotechnol 2010:586363.

19. Amanat F, Stadlbauer D, Strohmeier S, Nguyen THO, Chromikova V, McMahon M, Jiang K, Arunkumar GA, Jurczyszak D, Polanco J, Bermudez-Gonzalez M, Kleiner G, Aydillo T, Miorin L, Fierer DS, Lugo LA, Kojic EM, Stoever J, Liu STH, Cunningham-Rundles C, Felgner PL, Moran T, Garcia-Sastre A, Caplivski D, Cheng AC, Kedzierska K, Vapalahti O, Hepojoki JM, Simon V, Krammer F. 2020. A serological assay to detect SARS-CoV-2 seroconversion in humans. Nat Med 26:1033-1036.

20. Vijayakumar G, Zamarin D. 2020. Design and Production of Newcastle Disease Virus for Intratumoral Immunomodulation. Methods Mol Biol 2058:133-154.

21. Dinnon KH, Leist SR, Schafer A, Edwards CE, Martinez DR, Montgomery SA, West A, Yount BL, Hou YJ, Adams LE, Gully KL, Brown AJ, Huang E, Bryant MD, Choong IC, Glenn JS, Gralinski LE, Sheahan TP, Baric RS. 2020. A mouse-adapted SARS-CoV-2 model for the evaluation of COVID-19 medical countermeasures. bioRxiv doi:10.1101/2020.05.06.081497.

22. Amanat F, White KM, Miorin L, Strohmeier S, McMahon M, Meade P, Liu WC, Albrecht RA, Simon V, Martinez-Sobrido L, Moran T, Garcia-Sastre A, Krammer F. 2020. An In Vitro Microneutralization Assay for SARS-CoV-2 Serology and Drug Screening. Curr Protoc Microbiol 58:e108.

23. Ania Wajnberg FA, Adolfo Firpo, Deena Altman, Mark Bailey, Mayce Mansour, Meagan McMahon, Philip Meade, Damodara Rao Mendu, Kimberly Muellers, Daniel Stadlbauer, Kimberly Stone, Shirin Strohmeier, Judith Aberg, David Reich, Florian Krammer, Carlos Cordon-Cardo. SARS-CoV-2 infection induces robust, neutralizing antibody responses that are stable for at least three months. medRxiv doi:https://doi.org/10.1101/2020.07.14.20151126.

24. Imai M, Iwatsuki-Horimoto K, Hatta M, Loeber S, Halfmann PJ, Nakajima N, Watanabe T, Ujie M, Takahashi K, Ito M, Yamada S, Fan S, Chiba S, Kuroda M, Guan L, Takada K, Armbrust T, Balogh A, Furusawa Y, Okuda M, Ueki H, Yasuhara A, Sakai-Tagawa Y, Lopes TJS, Kiso M, Yamayoshi S, Kinoshita N, Ohmagari N, Hattori SI, Takeda M, Mitsuya H, Krammer F, Suzuki T, Kawaoka Y. 2020. Syrian hamsters as a small animal model for SARS-CoV-2 infection and countermeasure development. Proc Natl Acad Sci U S A doi:10.1073/pnas.2009799117.

25. Chan JF, Zhang AJ, Yuan S, Poon VK, Chan CC, Lee AC, Chan WM, Fan Z, Tsoi HW, Wen L, Liang R, Cao J, Chen Y, Tang K, Luo C, Cai JP, Kok KH, Chu H, Chan KH, Sridhar S, Chen Z, Chen H, To KK, Yuen KY. 2020. Simulation of the clinical and pathological manifestations of Coronavirus Disease 2019 (COVID-19) in golden Syrian hamster model: implications for disease pathogenesis and transmissibility. Clin Infect Dis doi:10.1093/cid/ciaa325.

**Figure legends**

**Figure 1. Design and concept of an inactivated NDV-based SARS-CoV-2 vaccine**

(A) Design of the NDV-S vaccine. The sequence of the S-F chimera (green: ectodomain of S; black: the transmembrane domain and cytoplasmic tail of NDV F protein) was inserted between the P and M gene of the NDV LaSota (NDV_LS) strain L289A mutant (NDV_LS/L289A). NDV-S: NDV_LS/L289A_S-F. The polybasic cleavage site of the S was removed ($^{682}$RRAR$^{685}$ to A). (B) The concept overview of an inactivated NDV-based SARS-CoV-2 vaccine. The NDV-S vaccine could be produced using current global influenza virus vaccine production capacity. Such an NDV-S vaccine displays abundant S protein on the surface of the virions. The NDV-S vaccine will be inactivated by beta-propiolactone (BPL). The NDV-S vaccine will be administered intramuscularly (i.m.) to elicit protective antibody responses in humans.

**Figure 2. The antigenicity of the S-F chimera is stable**

(A) Stability of the S-F chimera. Allantoic fluid containing the NDV-S virus was aliquoted into equal amounts (15 ml) and stored at 4°C. Virus from each aliquot was concentrated through a 20% sucrose cushion, re-suspended in equal amount of PBS, and then stored at - 80°C for several weeks (wk 0, wk 1,

wk 2, wk 3). One microgram of each concentrated virus was resolved onto 4-20% SDS-PAGE. Protein degradation was evaluated by Western blot using a S-specific mouse monoclonal antibody 2B3E5. HN protein of NDV was used as an NDV protein control. (B) Antigenicity of the S-F before and after BPL inactivation. Live or inactivated (using 0.05% BPL) NDV-S virus was concentrated through a 20% sucrose cushion as described previously. Two micrograms of live or BPL inactivated virus were loaded onto 4-20% SDS-PAGE. Antigenicity loss of the S-F was evaluated by western blot as described in A.

**Figure 3. Inactivated NDV-S vaccine elicits high antibody responses in mice**

(A) Immunization regimen and groups. BALB/c mice were given two immunizations via intramuscular administration route with a 2-week interval. Mice were bled pre-boost and 11 days after the boost for *in vitro* serological assays. Mice were challenged with a mouse-adapted SARS-CoV-2 strain 19 days after the boost. Ten groups described in the table were included in this study. Group 1, 2 and 3 were immunized with 5μg, 10 μg and 20 μg of vaccine, respectively; group 4, 5 and 6 were immunized with 0.2 μg, 1 μg and 5 μg of vaccine formulated with R-DOTAP, respectively; group 7, 8 and 9 were immunized with 0.2 μg, 1 μg and 5 μg of vaccine combined with AddaVax, respectively; group 10 was immunized with 20 μg of WT NDV virus as the vector-only control. (B) Spike-specific serum IgG titers. Serum IgG titers from animals after prime (pattern bars) and boost (solid bars) toward the recombinant trimeric spike protein was measured by ELISA. Endpoint titers were shown as the readout for ELISA. (C) Neutralization titers of serum antibodies. Microneutralization assays were performed to determine the neutralizing activities of serum antibodies from animals after the boost (D26) using the USA-WA1/2020 SARS-CoV-2 strain. The $ID_{50}$ of serum samples showing no neutralizing activity (WT NDV) is set as 10. (LoD: limited of detection).

**Figure 4. Inactivated NDV-S vaccine protects mice from SARS-CoV-2 infection**

(A) Weight loss of mice infected with SARS-CoV-2. Weight loss of mice challenged with a mouse-adapted SARS-CoV-2 strain were monitored for 4 days. (B) Viral titers in the lung. Lungs of mice were harvested at day 4 post-infection. Viral titers of the lung homogenates were determined by plaque assay. Geometric mean titer (PFU/lobe) was shown. (LoD: limit of detection)

**Figure 5. Inactivated NDV-S vaccine attenuates SARS-CoV-2 induced diseases in hamsters**

(A) Immunization regimen and groups. Golden Syrian hamsters were vaccinated with inactivated NDV-S following a prime-boost regimen with a 2-week interval. Hamsters were challenged 24 days after the boost with the USA-WA1/2020 SARS-CoV-2 strain. Four groups of hamsters (n=8) were included in this study. Group 1 received 10 μg of inactivated NDV-S vaccine without any adjuvants. Group 2 received 5

576 μg of inactivated NDV-S vaccine adjuvanted with AddaVax. Group 3 receiving the 10 μg of inactivated
577 WT NDV was included as vector-only (negative) control. Group 4 receiving no vaccine were mock
578 challenged with PBS as healthy controls. (B) Spike-specific serum IgG titers. Hamsters were bled pre-
579 boost and a subset of hamsters were terminally bled at 2 dpi. Vaccine-induced serum IgG titers towards
580 the trimeric spike protein were determined by ELISA. Endpoint titers were shown as the readout for
581 ELISA. (C) Weight loss of hamsters challenged with SARS-CoV-2. Weight loss of SARS-CoV-2
582 infected hamsters were monitored for 5 days.
583

Figure 1

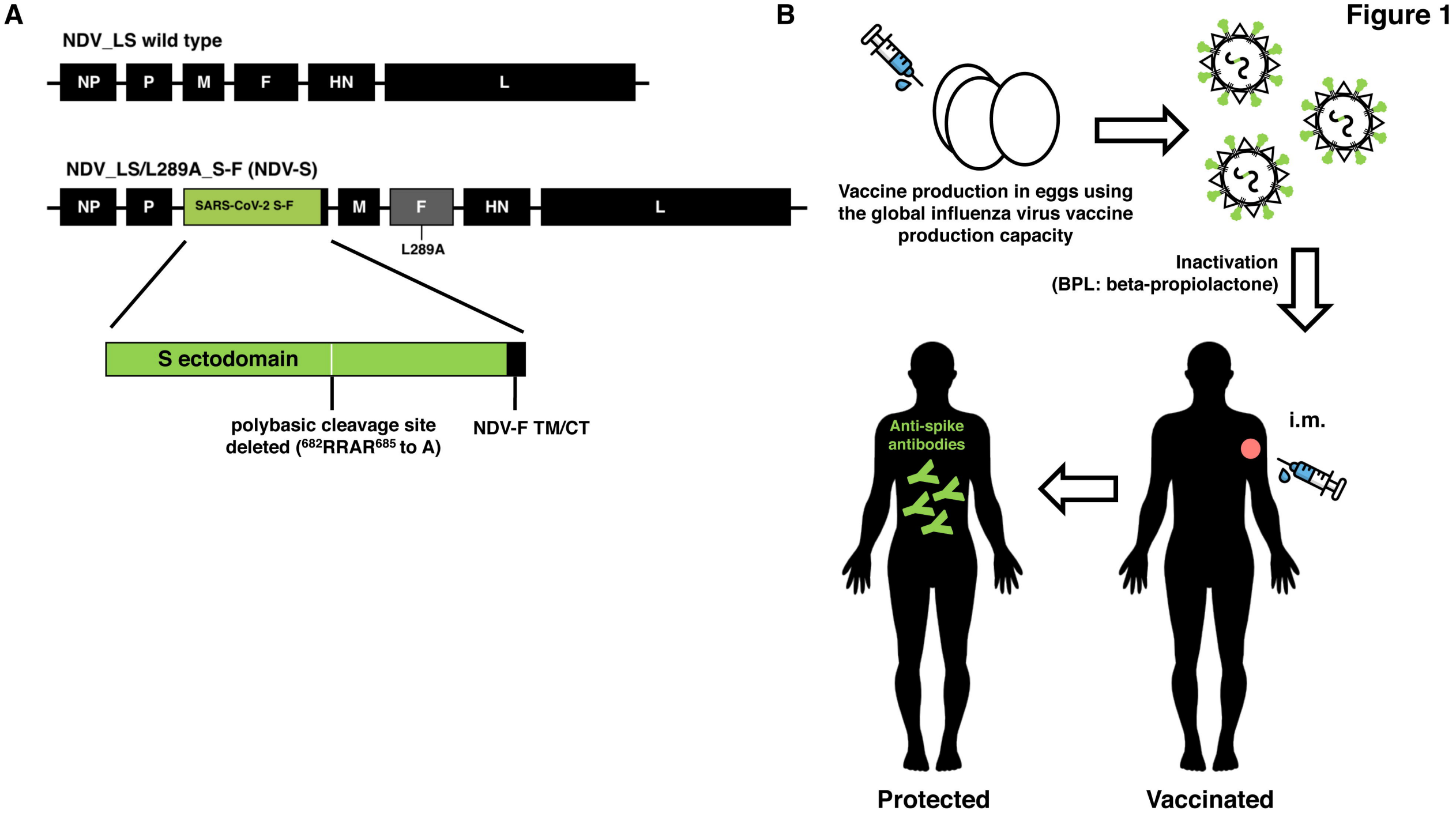
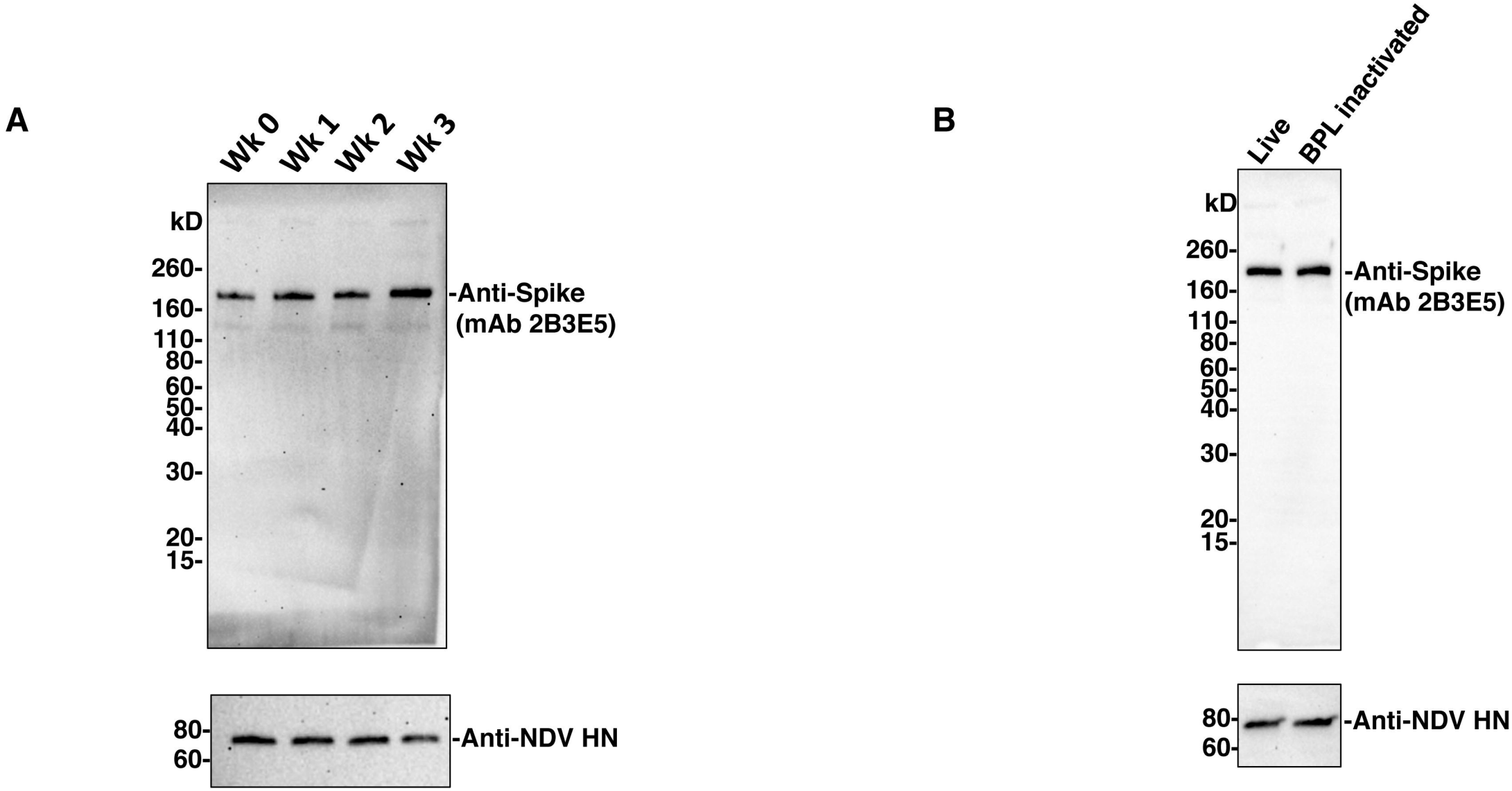
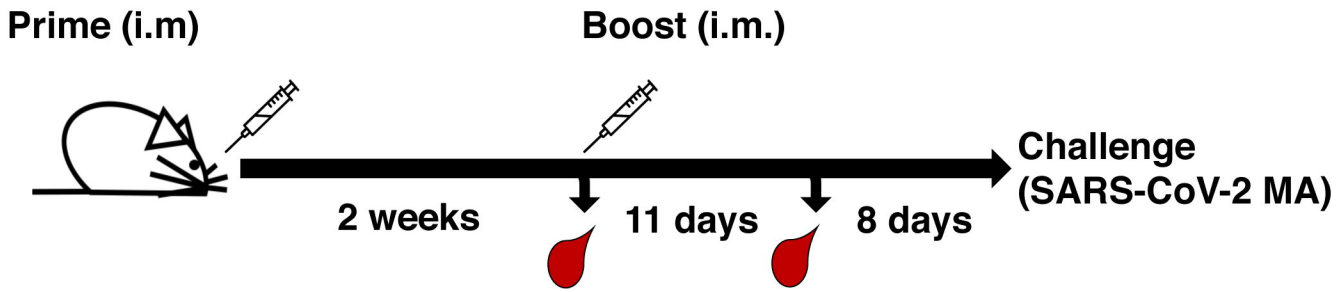

A
NDV_LS wild type
NP
P
M
F
HN
L
NDV_LS/L289A_S-F (NDV-S)
NP
P
SARS-CoV-2 S-F
M
F
HN
L
L289A
S ectodomain
polybasic cleavage site deleted (682RRAR685 to A)
NDV-F TM/CT
B
Vaccine production in eggs using the global influenza virus vaccine production capacity
Inactivation
(BPL: beta-propiolactone)
i.m.
Anti-spike antibodies
Protected
Vaccinated

Figure 2

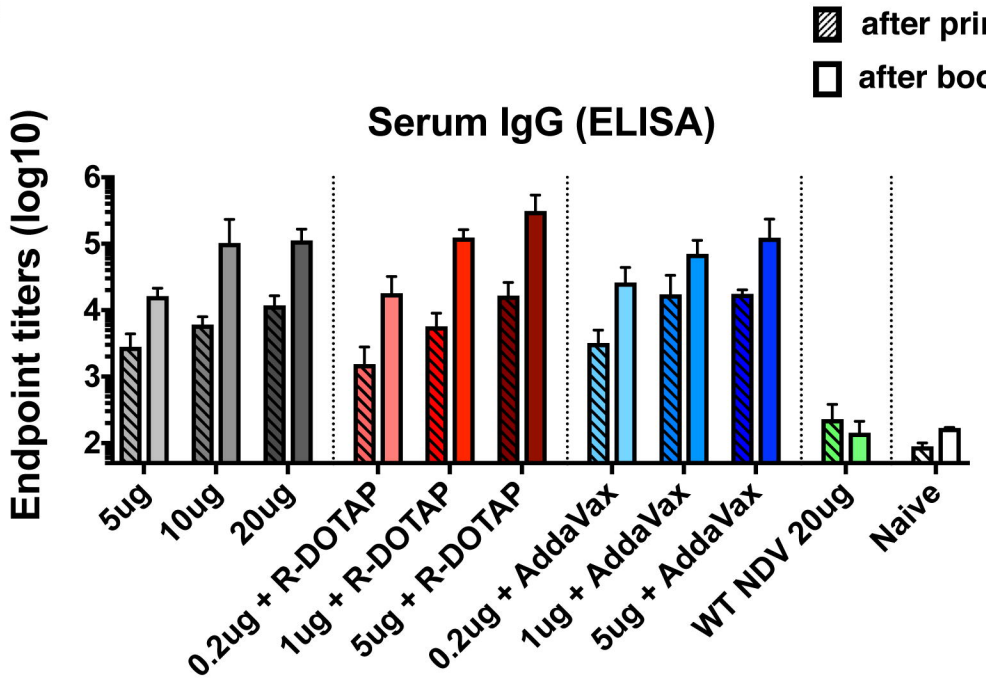

A
Wk 0
Wk 1
Wk 2
Wk 3
kD
260-
160-
110-
80-
60-
50-
40-
30-
20-
15-
-Anti-Spike
(mAb 2B3E5)
80-
60-
-Anti-NDV HN
B
Live
BPL inactivated
kD
260-
160-
110-
80-
60-
50-
40-
30-
20-
15-
-Anti-Spike
(mAb 2B3E5)
80-
60-
-Anti-NDV HN

A




| Groups (n=5) | Prime | Boost |
|---|---|---|
| 1 | 5 ug | 5 ug |
| 2 | 10 ug | 10 ug |
| 3 | 20 ug | 20 ug |
| 4 | 0.2 ug + R-DOTAP | 0.2 ug + R-DOTAP |
| 5 | 1 ug + R-DOTAP | 1 ug + R-DOTAP |
| 6 | 5 ug + R-DOTAP | 5 ug + R-DOTAP |
| 7 | 0.2 ug + AddaVax | 0.2 ug + AddaVax |
| 8 | 1 ug + AddaVax | 1 ug + AddaVax |
| 9 | 5 ug + AddaVax | 5 ug + AddaVax |
| 10 | WT NDV 20 ug | WT NDV 20 ug |

B




C

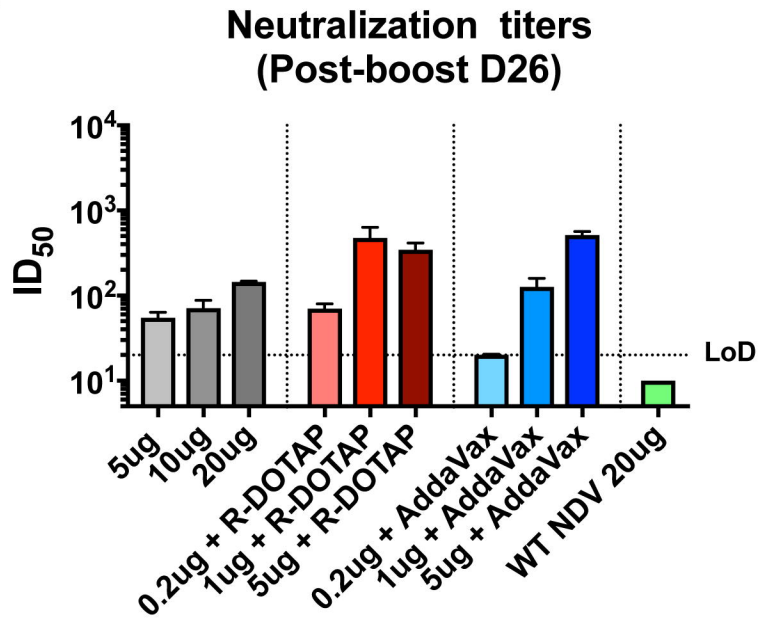
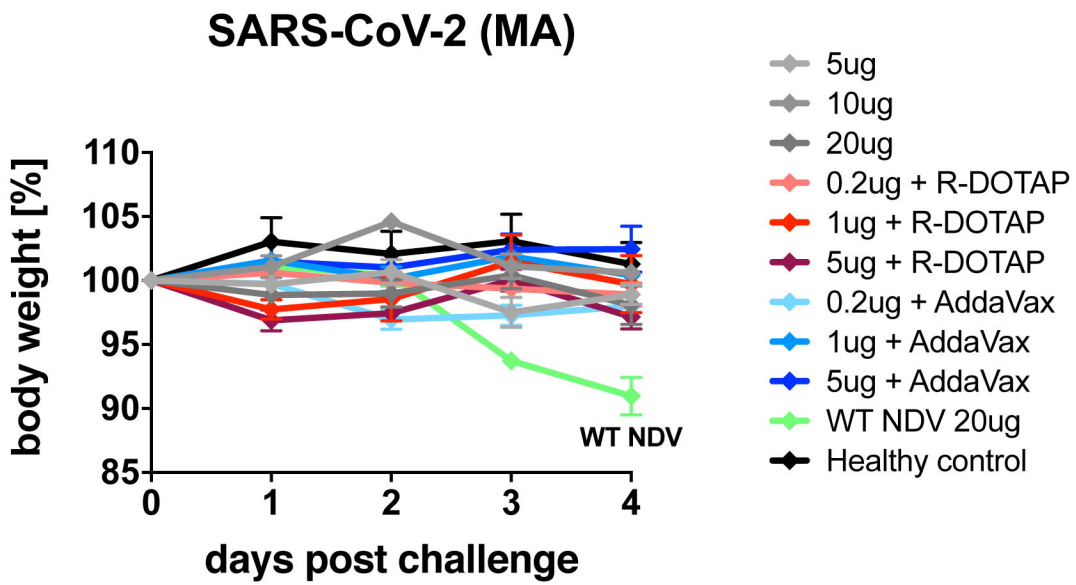



Figure 3

Figure 4

A


SARS-CoV-2 (MA)
body weight [%]
110
105
100
95
90
85
0
1
2
3
4
days post challenge
WT NDV
5ug
10ug
20ug
0.2ug + R-DOTAP
1ug + R-DOTAP
5ug + R-DOTAP
0.2ug + AddaVax
1ug + AddaVax
5ug + AddaVax
WT NDV 20ug
Healthy control

B

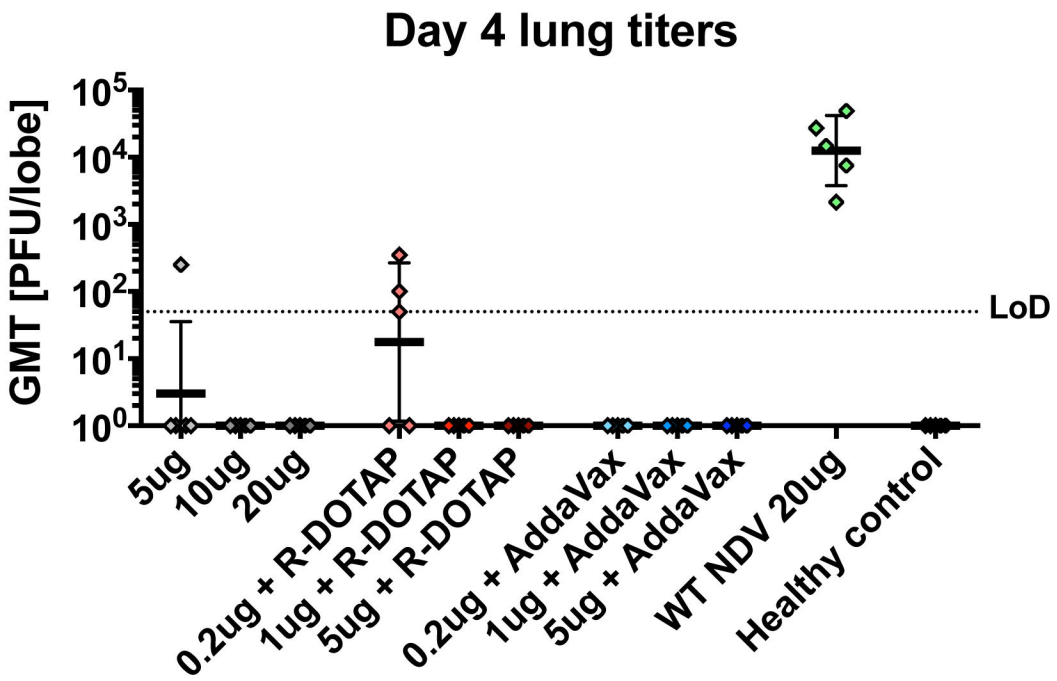
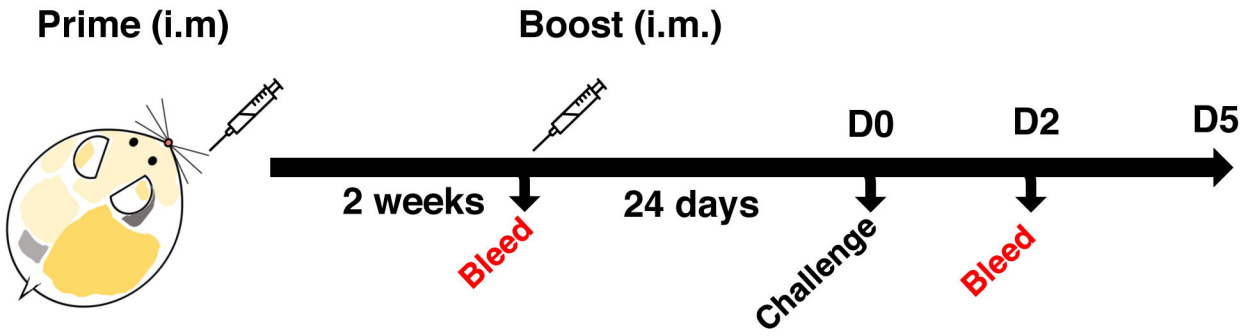

Day 4 lung titers
GMT [PFU/lobe]
$10^5$
$10^4$
$10^3$
$10^2$
$10^1$
$10^0$
LoD
5ug
10ug
20ug
0.2ug + R-DOTAP
1ug + R-DOTAP
5ug + R-DOTAP
0.2ug + AddaVax
1ug + AddaVax
5ug + AddaVax
WT NDV 20ug
Healthy control

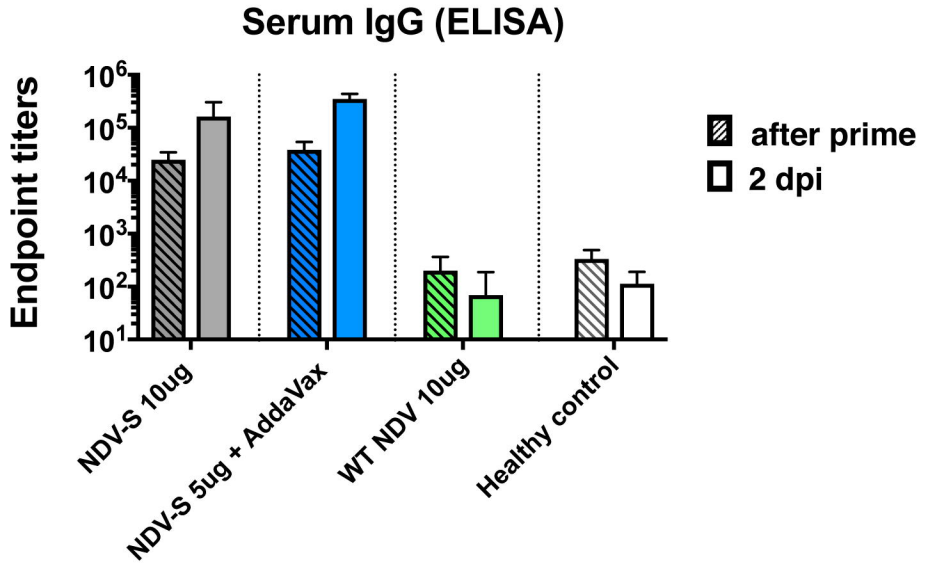
Figure 5

A




| Groups | Prime | Boost |
|---|---|---|
| 1 | NDV-S 10 ug | NDV-S 10 ug |
| 2 | NDV-S 5 ug + AddaVax | NDV-S 5 ug + AddaVax |
| 3 | WT NDV 10 ug | WT NDV 10 ug |
| 4 | Healthy control | |

B




C