Newcastle disease virus (NDV) expressing the spike protein of SARS-CoV-2 as vaccine candidate

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Abstract

Due to the lack of protective immunity of humans towards the newly emerged SARS-CoV-2, this virus has caused a massive pandemic across the world resulting in hundreds of thousands of deaths. Thus, a vaccine is urgently needed to contain the spread of the virus. Here, we describe Newcastle disease virus (NDV) vector vaccines expressing the spike protein of SARS-CoV-2 in its wild type or a pre-fusion membrane anchored format. All described NDV vector vaccines grow to high titers in embryonated chicken eggs. In a proof of principle mouse study, we report that the NDV vector vaccines elicit high levels of antibodies that are neutralizing when the vaccine is given intramuscularly. Importantly, these COVID-19 vaccine candidates protect mice from a mouse-adapted SARS-CoV-2 challenge with no detectable viral titer and viral antigen in the lungs.

Keywords

Viral vector vaccine, intramuscular administration, neutralizing antibodies, mouse-adapted SARS-CoV-2, live COVID-19 vaccine, coronavirus vaccine

Research in context

36 **Evidence before this study**

37 The spike (S) protein of the SARS-CoV-2 is the major antigen that notably induces neutralizing
38 antibodies to block viral entry. Many COVID-19 vaccines are under development, among them viral
39 vectors expressing the S protein of SARS-CoV-2 exhibit many benefits. Viral vector vaccines have the
40 potential of being used as both live or inactivated vaccines and they can induce Th1 and Th2-based
41 immune responses following different immunization regimens. Additionally, viral vector vaccines can be
42 handled under BSL-2 conditions and they grow to high titers in cell cultures or other species restricted-
43 hosts. For a SARS-CoV-2 vaccine, several viral vectors are being tested, such as adenovirus, measles
44 virus and Modified vaccinia Ankara.

45

46 **Added value of this study**

47 The NDV vector vaccine against SARS-CoV-2 described in this study has advantages similar to those of
48 other viral vector vaccines. But the NDV vector can be amplified in embryonated chicken eggs, which
49 allows for high yields and low costs per dose. Also, the NDV vector is not a human pathogen, therefore
50 the delivery of the foreign antigen would not be compromised by any pre-existing immunity in humans.
51 Finally, NDV has a very good safety record in humans, as it has been used in many oncolytic virus trials.
52 This study provides an important option for a cost-effective SARS-CoV-2 vaccine.

53

54 **Implications of all the available evidence**

55 This study informs of the value of a viral vector vaccine against SARS-CoV-2. Specifically, for this NDV
56 based SARS-CoV-2 vaccine, the existing egg-based influenza virus vaccine manufactures in the U.S. and
57 worldwide would have the capacity to rapidly produce hundreds of millions of doses to mitigate the
58 consequences of the ongoing COVID-19 pandemic.

59

60 **Introduction**

61 The unprecedented coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory
62 syndrome coronavirus 2 (SARS-CoV-2) has resulted in ~16.3 million infections with more than half a
63 million deaths since the end of 2019 as of July 26th 2020, and continues to pose a threat to public health.
64 To mitigate the spread of the virus, social distancing, mask-wearing, and the lockdown of cities, states or
65 even countries were practiced, with a heavy price paid both medically and economically. Unfortunately,
66 due to the relative lack of pre-existing immunity of humans to this virus, no countermeasures will be
67 completely effective without a vaccine. Because of the urgent need for an effective SARS-CoV-2 vaccine,
68 many candidates are being developed using various vaccine platforms, including mRNA vaccines (1, 2),
69 inactivated whole virus vaccines (3), subunit vaccines, DNA vaccines and viral vector vaccines (4). These

70 vaccine candidates are designed to essentially target the spike (S) protein of the SARS-CoV-2 (5), which
71 is the major structural protein displayed on the surface of the SARS-CoV-2. The S protein mediates the
72 entry of the virus via binding to the angiotensin converting enzyme 2 (ACE2) receptor in humans. The S
73 protein is also the most important antigen of the virus that harbors many B cell and T cell epitopes (6-9).
74 Neutralizing antibodies, most of which target the receptor-binding domain (RBD), can be induced by the
75 S protein (9, 10). However, to eventually contain the virus spread worldwide, not only the efficacy, but
76 also the cost and scalability of the vaccine are crucial, especially in low and middle income countries with
77 limited resources.
78
79 Here, we report the construction and characterization of Newcastle disease virus (NDV) vectors
80 expressing the SARS-CoV-2 S protein. NDV belongs to the genus of *Avulavirus* in the family of
81 *Paramyxoviridae*, it is an avian pathogen, typically causing no symptoms in humans although mild
82 influenza-like symptoms or conjunctivitis have been described in rare cases. The lentogenic NDV vaccine
83 strain such as the LaSota strain, in addition to be avirulent in birds, has been used as an oncolytic agent
84 and a vaccine vector (11-15). As a large negative strand RNA virus, NDV is stable and well tolerates
85 transgenes into its genome. NDV vectors have been successfully used to express the spike protein of other
86 coronaviruses (16, 17). The NDV platform is also appealing, because the virus grows to high titers in
87 embryonated chicken eggs, which are also used to produce influenza virus vaccines. Humans typically
88 lack pre-existing immunity toward the NDV, which makes the virus preferable over other viral vectors
89 that are human pathogens, such as human adenovirus, measles virus or Modified Vaccinia Ankara (MVA).
90 The lentogenic NDV vector has proven to be safe in humans as it has been tested extensively in human
91 trials (18-20). Most importantly, at low cost, NDV vector vaccines could be generated in embryonated
92 chicken eggs quickly under biosafety level 2 (BSL-2) conditions to meet the vast demand on a global
93 scale. In this study, we have successfully rescued NDV vectors expressing two forms of the spike protein
94 of SARS-CoV-2, the wild type (WT) S and a chimeric version containing the ectodomain (with the
95 polybasic cleavage site deleted) of the spike and the transmembrane domain and cytoplasmic domain of
96 the NDV F (pre-fusion S-F chimera). We have shown that WT S and S-F were well expressed from the
97 NDV as transgenes in infected cells. While both WT S and S-F were displayed on the surface of the NDV
98 particles, the incorporation of the S-F into NDV particles was substantially improved compared to that of
99 the WT S, as expected. A proof of concept study in mice using three live NDV vectors expressing the
100 spike protein (NDV_LS_S, NDV_LS_S-F and NDV_LS/L289A_S-F) showed that high titers of binding
101 and neutralizing antibodies were induced. All three NDV vector vaccines fully protected mice from
102 challenge with a SARS-CoV-2 mouse-adapted strain, showing no detectable viral titers and viral antigens
103 in the lungs at day four post-challenge. To conclude, we have developed promising cost-effective SARS-

104 CoV-2 vaccine candidates using the NDV LaSota strain as the viral vector, which could be generated to
105 high yield under BSL-2 conditions.
106
107 **Materials and Methods**
108 **Plasmids**
109 The sequence of the wild type S was amplified from pCAGGS plasmid (21) encoding the codon-
110 optimized nucleotide sequence of the spike gene (GenBank: MN908947.3) of a SARS-CoV-2 isolate by
111 PCR, using primers containing the gene end (GE), gene start (GS) and a Kozak sequences at the 5' end
112 (22). To construct the S-F chimera, the ectodomain of the S without the polybasic cleavage site (CS,
113 ^{682}RRAR^{685} to A) (22) was generated by PCR. A mammalian cell codon-optimized nucleotide sequence of
114 the transmembrane domain (TM) and the cytoplasmic tail (CT) of the NDV LaSota fusion (F) protein was
115 synthesized commercially (gBlock, Integrated DNA technologies). The S ectodomain (no CS) was fused
116 to the TM/CT of F through a GS linker (GGGGS). The sequence was again modified by adding GE, GS
117 and a Kozak sequence at the 5'. Additional nucleotides were added at the 3' of both inserts to follow the
118 "rule of six". The transgenes were inserted between the P and M gene of pNDV LaSota (LS) wild type or
119 the L289A (15, 22, 23) mutant (NDV_LS/L289A) antigenomic cDNA by in-Fusion cloning (Clontech).
120 The recombination products were transformed into NEB® Stable Competent E. coli (NEB) to generate
121 NDV_LS_S, NDV_LS_S-F and NDV_LS/L289A_S-F rescue plasmids. The plasmids were purified using
122 QIAprep Spin Miniprep kit (Qiagen) for Sanger sequencing (Macrogen). Maxipreps of rescue plasmids
123 were purified using PureLink™ HiPure Plasmid Maxiprep Kit (Thermo Fisher Scientific).
124
125 **Cells**
126 BSRT7 cells stably expressing the T7 polymerase were kindly provided by Dr. Benhur Lee at ISMMS.
127 The cells were maintained in Dulbecco's Modified Eagle's medium (DMEM; Gibco) containing 10%
128 (vol/vol) fetal bovine serum (FBS) and 100 unit/ml of penicillin/streptomycin (P/S; Gibco) at 37°C with 5%
129 CO_2. Vero E6 cells were obtained from American Type Culture Collection (ATCC, CRL-1586). Vero E6
130 cells were also maintained in DMEM containing 10% FBS with 100 unit/ml P/S at 37 °C with 5% CO_2.
131
132 **Rescue of NDV LaSota expressing the spike protein of SARS-CoV-2**
133 Six-well plates of BSRT7 cells were seeded 3 x 10^5 cells per well the day before transfection. The next
134 day, a transfection cocktail was prepared consisting of 250 µl of Opti-MEM (Gibco) including 4 µg of
135 pNDV_LS_S or pNDV_LS_S-F or pNDV_LS/L289A_S-F, 2 µg of pTM1-NP, 1 µg of pTM1-P, 1 µg of
136 pTM1-L and 2 µg of pCI-T7opt. Thirty µl of TransIT LT1 (Mirus) were added to the plasmid cocktail
137 and gently mixed by pipetting three times and incubated at room temperature (RT) for 30 min. Toward

138 the end of the incubation, the medium was replaced with 1 ml of Opti-MEM. The transfection complex
139 was added dropwise to each well and the plates were incubated at 37°C with 5% CO_2. Forty-eight hours
140 post transfection, the supernatant and the cells were harvested and briefly homogenized by several strokes
141 with an insulin syringe. Two hundred microliters of the cell/supernatant mixture were injected into the
142 allantoic cavity of 8- to 10-day old specific pathogen free (SPF) embryonated chicken eggs. The eggs
143 were incubated at 37°C for 3 days before being cooled at 4°C overnight. The allantoic fluid was collected
144 and clarified by low-spin centrifugation to remove debris. The presence of the rescued NDV was
145 determined by hemagglutination (HA) assay using 0.5% chicken or turkey red blood cells. The RNA of
146 the positive samples was extracted and treated with DNase I (Thermo Fisher Scientific). Reverse
147 transcriptase-polymerase chain reaction (RT-PCR) was performed to amplify the transgenes. The
148 sequences of the transgenes were confirmed by Sanger Sequencing (Genewiz).
149
150 **Immunofluorescence assay (IFA)**
151 Vero E6 cells were seeded onto 96-well tissue culture plates at 2.5 x 10^4 cells per well. The next day, cells
152 were washed with 100 µl warm phosphate buffered saline (PBS) and infected with 50 µl of allantoic fluid
153 at 37°C for 1h. The inocula were removed and replaced with 100 µl of growth medium. The plates were
154 then incubated at 37°C. Sixteen to eighteen hours after infection, the cells were washed with 100 µl of
155 warm PBS and fixed with 4% methanol-free paraformaldehyde (PFA) (Electron Microscopy Sciences) for
156 15 min at 4°C. The PFA was discarded, cells were washed with PBS and blocked in PBS containing 0.5%
157 bovine serum albumin (BSA) for 1 hour at 4°C. The blocking buffer was discarded and surface proteins
158 were stained with anti-NDV rabbit serum or SARS-CoV-2 spike receptor-binding domain (RBD) specific
159 human monoclonal antibody CR3022 (24, 25) for 2h at RT. The primary antibodies were discarded, cells
160 were then washed 3 times with PBS and incubated with goat anti-rabbit Alexa Fluor 488 or goat anti-
161 human Alexa Fluor 488 secondary antibodies (Thermo Fisher Scientific) for 1h at RT. The secondary
162 antibodies were discarded, cells were washed again 3 times with PBS and images were captured using an
163 EVOS fl inverted fluorescence microscope (AMG).
164
165 **Virus titration**
166 Stocks of NDV expressing the S or S-F proteins were titered using an immunofluorescence assay (IFA).
167 Briefly, Vero cells were seeded onto 96-well (Denville) tissue culture plates at 2.5 x 10^4 cells/well the day
168 before infection. The next day, five-fold serial dilutions of each virus stocks were prepared in a separate
169 96-well plate in Opti-MEM (Gibco). Medium in the 96-well plate was removed and the cells were washed
170 with 100 µL of warm PBS. Fifty µL of the virus dilutions were added to each well. The plates were
171 incubated at 37°C for one hour and shaken every 15 minutes to ensure the cells were infected evenly. The

172 inoculum was removed and 100 µL of DMEM containing 10% FBS with 100 unit/ml P/S was added. The
173 plates were incubated at 37°C overnight for 16 to 18 hours. The next day, the media were aspirated off
174 and cells were washed once with 100 µL of warm PBS. IFA was performed as described above to staining
175 NDV surface glycoproteins. Infected fluorescent cells were counted starting from the undiluted wells until
176 a well down the dilution with a countable number of cells was found. The fluorescent cells in the entire
177 well were counted. Titer of the virus (focus forming unit, FFU per ml) was determined by the following
178 formula:

179 Titer (FFU/ml) = No. of fluorescent cells x Dilution factor x (1000uL/volume of infection)

180

181 **Virus concentration**

182 Allantoic fluids were clarified by centrifugation at 4,000 rpm using a Sorvall Legend RT Plus
183 Refrigerated Benchtop Centrifuge (Thermo Fisher Scientific) at 4 °C for 30 min. Viruses in the allantoic
184 fluid were pelleted through a 20% sucrose cushion in NTE buffer (100 mM NaCl, 10 mM Tris-HCl, 1
185 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4) by centrifugation in a Beckman L7-65
186 ultracentrifuge at 25,000 rpm for 2h at 4°C using a Beckman SW28 rotor (Beckman Coulter, Brea, CA,
187 USA). Supernatants were aspirated and the pellets were re-suspended in PBS (pH 7.4). The protein
188 content was determined using the bicinchoninic acid (BCA) assay (Thermo Fisher Scientific).

189

190 **Western Blot**

191 Concentrated virus samples were mixed with Novex™ Tris-Glycine SDS Sample Buffer (2X) (Thermo
192 Fisher Scientific) with NuPAGE™ Sample Reducing Agent (10X) (Thermo Fisher Scientific). The
193 samples were heated at 95 °C for 5 min. Two microgram of concentrated viruses were resolved on 4-20%
194 sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels (Biorad) using the
195 Novex™ Sharp Pre-stained Protein Standard (Thermo Fisher Scientific) as the marker. Proteins were
196 transferred onto polyvinylidene difluoride (PVDF) membrane (GE healthcare). The membrane was
197 blocked with 5% dry milk in PBS containing 0.1% v/v Tween 20 (PBST) for 1h at RT. The membrane
198 was washed with PBST on a shaker 3 times (10 min at RT each time) and incubated with primary
199 antibodies diluted in PBST containing 1% BSA overnight at 4°C. To detect the spike protein of SARS-
200 CoV-2, a mouse monoclonal antibody 2B3E5 kindly provided by Dr. Thomas Moran at ISMMS was used,
201 while the HN protein was detected by a mouse monoclonal antibody 8H2 (MCA2822, Bio-Rad). The
202 membranes were then washed with PBST on a shaker 3 times (10 min at RT each time) and incubated
203 with sheep anti-mouse IgG linked with horseradish peroxidase (HRP) diluted (1:2,000) in PBST
204 containing 5% dry milk for 1h at RT. The secondary antibody was discarded and the membranes were

205 washed with PBST on a shaker 3 times (10 min at RT each time). Pierce™ ECL Western Blotting
206 Substrate (Thermo Fisher Scientific) was added to the membrane, the blots were imaged using the Bio-
207 Rad Universal Hood Ii Molecular imager (Bio-Rad) and processed by Image Lab Software (Bio-Rad).

208

209 **Mice immunizations**

210 Ten-week old female BALB/cJ mice (Jackson Laboratories) were used. Experiments were performed in
211 accordance with protocols approved by the Icahn School of Medicine at Mount Sinai Institutional Animal
212 Care and Use Committee (IACUC). Mice were divided into 9 groups (n=5) receiving four different
213 concentrated live viruses at two doses (10 µg and 50 µg) intramuscularly (i.m). Specifically, group 1 (10
214 µg per mouse) and 2 (50 µg per mouse) were given wild type NDV_LS; group 3 (10 µg per mouse) and 4
215 (50 µg per mouse) received NDV_LS_S; group 5 (10 µg) and 6 (50 µg) received NDV_LS_S-F and
216 group 7 (10 µg per mouse) and 8 (50 µg per mouse) received NDV_LS/L289A_S-F. Group 9 given PBS
217 was used as the negative controls. A prime-boost immunization regimen was used for all the groups in a
218 3-week interval.

219

220 **Enzyme linked immunosorbent assay (ELISA)**

221 Immunized mice were bled pre-boost and 8 days after the boost. Sera were isolated by low-speed
222 centrifugation. To perform ELISAs, Immulon 4 HBX 96-well ELISA plates (Thermo Fisher Scientific)
223 were coated with 2 µg/ml of recombinant trimeric S protein (50 µL per well) in coating buffer (SeraCare
224 Life Sciences Inc.) overnight at 4°C (21). The next day, all plates were washed 3 times with 220 µL PBS
225 containing 0.1% (v/v) Tween-20 (PBST) and 220 µL blocking solution (3% goat serum, 0.5% dry milk,
226 96.5% PBST) was added to each well and incubated for 1h at RT. Mouse sera were 3-fold serially diluted
227 in blocking solution starting at 1:30 followed by a 2 h incubation at RT. ELISA plates were washed 3
228 times with PBST and 50 µL of sheep anti-mouse IgG-horseradish peroxidase (HRP) conjugated antibody
229 (GE Healthcare) was added at a dilution of 1:3,000 in blocking solution. Then, plates were again
230 incubated for one hour at RT. Plates were washed 3 times with PBST and 100 µL of o-phenylenediamine
231 dihydrochloride (SigmaFast OPD, Sigma) substrate was added per well. After 10 min, 50 µL of 3M
232 hydrochloric acid (HCl) was added to each well to stop the reaction and the optical density (OD) was
233 measured at 492 nm on a Synergy 4 plate reader (BioTek). An average of OD values for blank wells plus
234 three standard deviations was used to set a cutoff for plate blank outliers. A cutoff value was established
235 for each plate that was used for calculating the endpoint titers. The endpoint titers of serum IgG responses
236 was graphed using GraphPad Prism 7.0.

237

238 **SARS-CoV-2 challenge in mice**

239 The SARS-CoV-2 challenge was performed at the University of North Carolina by Dr. Ralph Baric's
240 group in a Biosafety Level 3 (BSL-3) facility. Mice were challenged 11 days after the boost using a
241 mouse adapted SARS-CoV-2 strain at 10^4 plaque forming unit (PFU) intranasally (i.n) under
242 ketamine/xylazine anesthesia as described previously (1, 26).
243
244 **Lung titers**
245 Lung lobes of mice were collected and homogenized in PBS. A plaque assay was performed to measure
246 viral titer in the lung homogenates as described previously (1, 26). Geometric mean titers of plaque
247 forming units (PFU) per lobe were calculated using GraphPad Prism 7.0.
248
249 **Micro-neutralization assay**
250 All neutralization assays were performed in the biosafety level 3 (BSL-3) facility following institutional
251 guidelines as described previously (21, 27). Briefly, serum samples were heat-inactivated at 56°C for 60
252 minutes prior to use. 2X minimal essential medium (MEM) supplemented with glutamine, sodium
253 biocarbonate, 4- (2- hydroxyethyl)1- piperazineethanesulfonic acid (HEPES), and antibiotics P/S was
254 used for the assay. Vero E6 cells were maintained in culture using DMEM supplemented with 10% fetal
255 bovine serum (FBS). Twenty-thousands cells per well were seeded the night before in a 96-well cell
256 culture plate. 1X MEM was prepared from 2X MEM and supplemented with 2% FBS. Three-fold serial
257 dilutions starting at 1:20 of pooled sera were prepared in a 96-well cell culture plate and each dilution was
258 mixed with 600 times the 50% tissue culture infectious dose ($TCID_{50}$) of SARS-CoV-2 (USA-WA1/2020,
259 BEI Resources NR-52281). Serum-virus mixture was incubated for 1h at room temperature. Virus-serum
260 mixture was added to the cells for 1h and kept in a 37°C incubator. Next, the virus-serum mixture was
261 removed and the corresponding serum dilution was added to the cells with addition 1X MEM. The cells
262 were incubated for 2 days and fixed with 100 µL 10% formaldehyde per well for 24 h before taken out of
263 the BSL-3 facility. The staining of the cells was performed in a biosafety cabinet (BSL-2). The
264 formaldehyde was carefully removed from the cells. Cells were washed with 200 µL PBS once before
265 being permeabilized with PBS containing 0.1% Triton X-100 for 15 min at RT. Cells were washed with
266 PBS and blocked in PBS containing 3% dry milk for 1h at RT. Cells were then stained with 100 µL per
267 well of a mouse monoclonal anti-NP antibody (1C7), kindly provided by Dr. Thomas Moran at ISMMS,
268 at 1µg/ml for 1h at RT. Cells were washed with PBS and incubated with 100 µL per well Anti- mouse
269 IgG HRP (Rockland) secondary antibody at 1:3,000 dilution in PBS containing 1% dry milk for 1h at RT.
270 Finally, cells were washed twice with PBS and the plates were developed using 100 µL of SigmaFast
271 OPD substrate. Ten minutes later, the reactions were stopped using 50 µL per well of 3M HCI. The OD
272 492 nM was measured on a Biotek SynergyH1 Microplate Reader. Non- linear regression curve fit

273 analysis (The top and bottom constraints are set at 100% and 0%) over the dilution curve was performed
274 to calculate 50% of inhibitory dilution (ID_{50}) of the serum using GraphPad Prism 7.0.
275
276 **Immunohistochemistry (IHC)**
277 The lung lobes of mice were perfused and fixed in 10% phosphate buffered formalin for 7 days before
278 transferred out of the BSL-3 facility. The fixed lungs were paraffin embedded, and sectioned at 5µm for
279 immunohistochemistry (IHC) staining (HistoWiz). IHC was performed using a rabbit SARS-CoV-2
280 nucleocapsid (N) protein (NB100-56576, Novus Biologicals). Slides were counter stained with
281 hematoxylin. All slides were examined by a board-certified veterinary pathologist (HistoWiz).
282
283 **Results**
284 **Design and rescue of NDV LaSota expressing the spike protein of SARS-CoV-2**
285 For protective immunity, the S protein is the most important antigen of SARS-CoV-2. To express S
286 antigen by the NDV LaSota vaccine strain, we designed two constructs. One is the wild type spike (S),
287 the other is the spike-F chimera (S-F). The S-F consists of the ectodomain of the S, in which the polybasic
288 cleavage site ^{682}RRAR^{685} is removed by deleting the three arginines to stabilize the protein in its pre-
289 fusion conformation (21). Importantly, to increase membrane-anchoring of the spike on the surface of the
290 NDV virions, we replaced the transmembrane domain (TM) and cytoplasmic tail (CT) of the spike with
291 those from the fusion (F) protein of NDV (Fig. 1A)(28). The nucleotide sequences of each construct were
292 inserted between the P and M genes of the antigenomic cDNA of WT NDV LaSota strain and/or NDV
293 LaSota/L289A mutant strain, in which the mutation L289A in the F protein supports HN independent
294 fusion (23). The latter NDV mutant has been safely used in humans (15) (Fig. 1B). NDV expressing the
295 spike proteins were rescued by transient transfection of BSRT7 cells followed by amplification in
296 embryonated chicken eggs. All the viruses expressing the S or S-F grew to high titers (~10^8 FFU/ml) in
297 embryonated chicken eggs (Fig. 1C), which is advantageous for the development of a low-cost vaccine.
298
299 **The spike protein is incorporated into NDV particles**
300 To validate the expression of S and S-F as transgenes, Vero E6 cells were infected with WT NDV or
301 NDV expressing the S or S-F. The surface of the cells was stained with anti-NDV rabbit serum or spike-
302 specific monoclonal antibody CR3022 that recognizes the RBD. We confirmed that only NDV expressing
303 the S or S-F showed robust expression of the spike on the cell surface, while NDV proteins were detected
304 in all virus-infected cells (Fig. 2A). This demonstrates that S and S-F are successfully expressed by the
305 NDV. To examine the incorporation of the S and S-F into the NDV virions, we concentrated the
306 NDV_LS_S, NDV_LS_S-F and NDV_LS/L289A_S-F through a 20% sucrose cushion. The pellets were

307 re-suspended in PBS. The WT NDV_LS was prepared the same way and was used as the negative control.
308 The protein content of each concentrated virus was determined by BCA assay. Two micrograms of each
309 virus was resolved on an SDS-PAGE. A Western blot was performed to examine the abundance of the
310 spike using mouse monoclonal antibody 2B3E5 that binds to a linear epitope of the S1 protein. The
311 expression of the NDV viral hemagglutinin-neuraminidase (HN) protein was also shown as an internal
312 control of the concentrated viruses (Fig. 2B). As expected, both S and S-F incorporated into the NDV
313 particles. Of note, the WT S harboring the polybasic cleavage site (CS) was completely cleaved showing
314 only the S1, while the S-F was maintained at its pre-fusion S0 stage. Importantly, the S-F expressed either
315 by the WT or L289A NDV_LS backbone exhibited superior incorporation into the virions over the WT S
316 shown by much higher abundance of S-F than S1 cleaved from the WT S (Fig. 2B). This confirms that the
317 TM/CT of F in the S-F chimera indeed facilitates the membrane-anchoring of the spike as expected. Since
318 the anti-NDV rabbit sera completely neutralize focus formation of these three NDV vectors vaccines, and
319 for the fact, that the S-F constructs don't have a polybasic cleavage site, it is unlikely the expression of
320 the transgenes alters the tropism of these viruses.
321
322 **Immunization of mice with NDV LaSota expressing the spike protein elicited potently binding and**
323 **neutralizing antibodies**
324 To evaluate the immunogenicity of our NDV vectors expressing the S or S-F as vaccine candidates
325 against SARS-CoV-2, a proof of principle study was performed in mice. Specifically, BALB/c mice were
326 immunized with live NDV_LS_S, NDV_LS_S-F and NDV_LS/L289A_S-F intramuscularly, as live
327 NDV barely replicates in the muscle and causes no symptoms in mammals. Here, we used a prime-boost
328 immunization regimen in a three-week interval. Mice were bled pre-boost (after prime) and 8 days after
329 the boost for *in vitro* serological assays (Fig. 3A). Two doses (10 µg and 50 µg) of each NDV construct
330 including NDV_LS_S (group 3 and 4), NDV_LS_S-F (group 5 and 6) and NDV_LS_S-F (group 7 and 8)
331 were tested as shown in Fig. 3A. Animals vaccinated with WT NDV expressing no transgenes (group 1
332 and 2) were used as vector-only controls. Mice receiving only the PBS (group 9) were used as negative
333 controls. Mouse sera from the two bleedings were harvested. Serum IgG titers and neutralizing antibody
334 titers were measured by ELISAs and microneutralization assays, respectively. To perform ELISA, full-
335 length trimeric spike protein was coated onto ELISA plates. The endpoint titers of serum IgG were used
336 as the readout (Fig. 3B). After one immunization, all the NDV constructs expressing the spike protein
337 elicited S-binding antibodies, whereas WT NDV constructs and PBS controls show negligible antibody
338 binding signals. The second immunization significantly increased the antibody titers around 1 week after
339 the boost without showing significant difference among the three NDV constructs (Fig. 3B). The
340 neutralizing activity of the antibodies was measured in a microneutralization assay using the USA-

341 WA1/2020 SARS-CoV-2 strain. Pooled sera from each group were tested in a technical duplicate. The
342 ID_{50} value was calculated as the readout of neutralizing activity of post-boost sera (Day 29). Sera from all
343 vaccinated groups showed neutralizing activity. The neutralization titer of sera from the NDV_LS_S
344 high-dose (50 µg) vaccination group ($ID_{50} \approx 444$) appeared to be slightly higher than that from the low-
345 dose (10 µg) vaccination group ($ID_{50} \approx 178$). No substantial difference was observed between the low-
346 dose and high-dose groups using the NDV_LS_S-F and NDV_LS/L289A_S-F constructs (Fig. 3C), the
347 neutralization titers of which are comparable to that of the NDV_LS_S high-dose (50 µg) group. To
348 summarize, all the NDV vectors that were engineered to express the S or S-F elicited high titers of
349 binding and neutralizing antibodies in mice. The WT S and S-F constructs appeared to exhibit similar
350 immunogenicity when expressed by live NDV vectors that were given intramuscularly to mice.
351
352 **Immunization with NDV LaSota expressing the spike proteins protects mice from challenge with a**
353 **mouse-adapted SARS-CoV-2**
354 To assess *in vivo* activity of S-specific antibodies induced by the NDV constructs as well as potential cell-
355 mediated protection, we took advantage of a mouse – adapted SARS-CoV-2 strain that replicates
356 efficiently in BALB/c mice (1, 26). The immunized mice were challenged with 10^4 PFU of the mouse-
357 adapted SARS-CoV-2 at day 11 after the boost, and viral titers in the lungs at day 4 post –challenge were
358 measured. Mice receiving WT NDV and PBS exhibited high viral titers in the lung, while all the groups
359 given NDV expressing the S or S-F showed no detectible viral load in the lung (Fig. 4A). The lungs of
360 infected mice were fixed in 10% neutral buffered formalin for IHC staining using an anti-SARS-CoV-2
361 NP antibody. The IHC staining showed that the SARS-CoV-2 NP protein was largely detected in the
362 lungs of mice that received NDV_LS WT or PBS. The SARS-CoV-2 NP was absent in the lungs of mice
363 vaccinated with the three NDV constructs expressing the S or S-F protein (Fig. 4B). These data
364 demonstrated that the all three NDV vector vaccines could efficiently prevent SARS-CoV-2 infection in a
365 mouse model.
366
367 **Discussion**
368 The consequences of the ongoing COVID-19 pandemic since the end of 2019 are disheartening. With the
369 high transmissibility of the culprit, SARS-CoV-2, and the lack of substantial pre-existing immunity of
370 humans to this virus, many people have succumbed to COVID-19, especially the elderly and people with
371 underlying health conditions. With both therapeutic and prophylactic countermeasures (29-31) still under
372 rapid development, no currently available treatment appears to be effective enough for an over-burdened
373 health care system with limited resources. A vaccine is needed to prevent or at least attenuate the
374 symptoms of COVID-19. As many vaccine candidates are being tested in pre-clinical or clinical studies, a

375 vaccine for cost effective production in low- and middle-income countries has not yet been developed and
376 is still very much in need. Also, the vaccination of small numbers of high-income populations who can
377 afford the vaccine would not efficiently prevent the spreading of the disease in the global population. In
378 this report, we describe promising viral vector vaccine candidates based on NDV expressing the major
379 antigen of SARS-CoV-2. The NDV vectors were engineered to express either the wild type S or a pre-
380 fusion spike with improved membrane anchoring (S-F). These NDV vector vaccines showed robust
381 growth in embryonated chicken eggs despite the fact that a large transgene is inserted into the NDV
382 genome. Importantly, the spike protein is successfully expressed in infected cells, and the S-F construct
383 exhibited superior incorporation into NDV particles, which could potentially be used as an inactivated
384 virus vaccine as well.
385
386 In a proof of principle study, mice receiving live NDV vector vaccines twice intramuscularly have
387 developed high levels of spike-specific antibodies that are neutralizing. Mice given the NDV vector
388 expressing S or S-F were protected equally well against the challenge of a mouse-adapted SARS-CoV-2
389 strain showing no detectable infectious virus or viral antigens in the lungs, while high viral titers were
390 observed in the lungs of mice given the WT NDV expressing no transgenes or PBS. In this study, we did
391 not see significant dose-dependent antibody responses, which was similar to what was observed for
392 different doses (100 µg and 250 µg) of mRNA vaccine in a human trial (2). It could be that we did not
393 measure the peak antibody responses due to the problem of having to transfer mice to the University of
394 North Carolina for the challenge study, or an antibody response ceiling was reached with the low dose of
395 10 µg concentrated virus in mice. In the present study, cellular immunity was not measured, however, this
396 will be of interest to investigate in the future studies. Nevertheless, this study strongly supports that the
397 NDV vector vaccines are promising, as they are expressing immunogenic spike proteins of SARS-CoV-2
398 inducing high levels of protective antibodies. Unlike other viral vectors that humans might be exposed to,
399 the NDV vector would deliver the spike antigen more efficiently without encountering pre-existing
400 immune responses in humans. Importantly, NDV vector vaccines are not only cost-effective with respect
401 to large scale manufacturing but can also be produced under BSL-2 conditions using influenza virus
402 vaccine production technology. In summary, NDV vector SARS-CoV-2 vaccines are a safe and
403 immunogenic alternative to other SARS-CoV-2 vaccines that can be produced using existing
404 infrastructure in a cost-effective way.
405
406 **Contributors**
407 Conceptualization and design, P.P.; methodology, P.P., W.S., S.R.L, S.M., Y.L, S.S., J.O, F.A., A.G.S,
408 F.K., R.S.B; investigation and data analysis, P.P., W.S., S.R.L, S.M., Y.L, S.S., J.O, F.A., A.S, K.H.D,

A.G.S, F.K., R.S.B; first draft of manuscript, P.P. and W.S.; manuscript review and editing, all authors; funding, P.P. and anonymous philanthropist.

Declaration of Interests

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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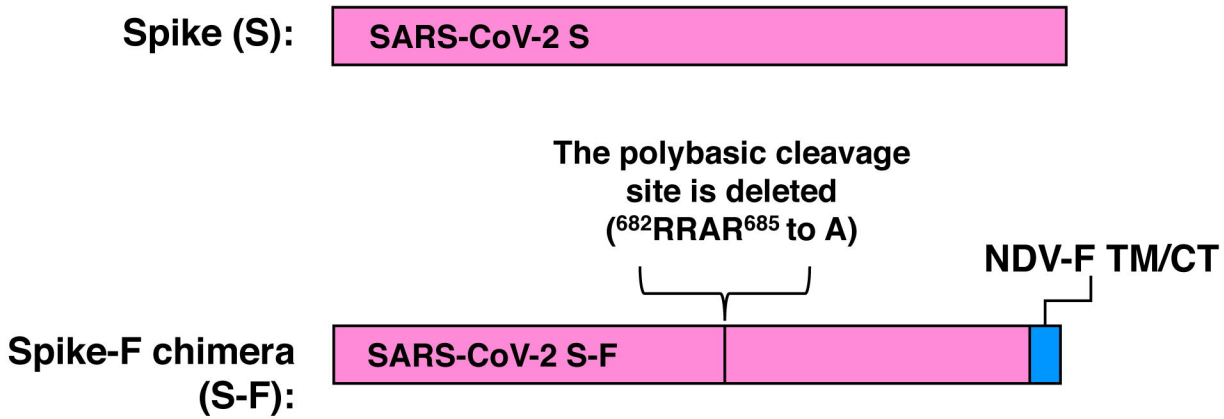
540
541
542 **Figure legends**

543 **Figure 1. NDV vectors expressing the spike protein of SARS-CoV-2**
544 (A) Two forms of spike proteins expressed by NDV. Spike (S) has the wild type amino acid sequence.
545 The Spike-F chimera (S-F) consists of the ectodomain of S without the polybasic cleavage site and the
546 transmembrane domain (TM) and cytoplasmic tail (CT) of the F protein from NDV. (B) Illustration of
547 genome structures of wild type NDV LaSota (WT NDV_LS), NDV expressing the S or S-F in the wild
548 type LaSota backbone (NDV_LS_S or NDV_LS_S-F) or NDV expressing the S-F in the L289A mutant
549 backbone (NDV_LS/L289A_S-F). The L289A mutation supports the HN-independent fusion of the F
550 protein. (C) Titers of NDV vectors grown in embryonated chicken eggs. The rescued viruses were grown
551 in 10-day old embryonated chicken eggs for 2 or 3 days at 37 °C at limiting dilutions. The peak titers of
552 each virus were determined by immunofluorescence assay (IFA).
553
554 **Figure 2. Expression of spike protein in infected cells and NDV particles**
555 (A) Expression of the S and S-F protein in infected cells. Vero E6 cells were infected with three NDV
556 vectors encoding the S or S-F for 16 to 18 hours. A WT NDV control was included. The next day, cells
557 were fixed with methanol-free paraformaldehyde. Surface proteins were stained with anti-NDV rabbit
558 serum or a spike receptor-binding domain (RBD)-specific monoclonal antibody CR3022. (B)
559 Incorporation of S and S-F into NDV particles. Three NDV vectors expressing the S or S-F including the
560 NDV_LS_S (green), NDV_LS_S-F (red) and NDV_LS/L289A_S-F (blue) were concentrated through a
561 20% sucrose cushion. Two clones were shown for NDV_LS_S and NDV_LS_S-F. The concentrated WT
562 NDV expressing no transgenes was used as a control. Two micrograms of each concentrated virus were
563 resolved on a 4-20% SDS-PAGE, the spike protein and NDV HN protein were detected by western blot
564 using an anti-spike 2B3E5 mouse monoclonal antibody and an anti-HN 8H2 mouse monoclonal antibody.
565
566 **Figure 3. NDV vector vaccines elicit high titers of binding and neutralizing antibodies in mice**
567 (A) Vaccination groups and regimen. A prime-boost vaccination regimen was used with a three-week
568 interval. Mice were bled pre-boost and 8 days after the boost. Mice were challenged with a mouse-
569 adapted SARS-CoV-2 MA strain 11 days after the boost. A total of ten groups of mice were used in a
570 vaccination and challenge study. Group 1 (10 µg) and 2 (50 µg) received the WT NDV; Group 3 (10 µg)
571 and 4 (50 µg) received the NDV_LS_S; Group 5 (10 µg) and 6 (50 µg) received NDV_LS_S-F; Group 7
572 (10 µg) and 8 (50 µg) received NDV_LS/L289A_S-F; Group 9 received PBS as negative controls. An
573 age-matched healthy control group 10 was provided upon challenge. (B) Spike-specific serum IgG titers
574 measured by ELISAs. Sera from animals at 3 weeks after-prime (patterned bars) and 8 days after-boost
575 (solid bars) were isolated. Serum IgG was measured against a recombinant trimeric spike protein by
576 ELISAs. The endpoint titers were calculated as the readout for ELISAs. (C) Neutralization titers of serum

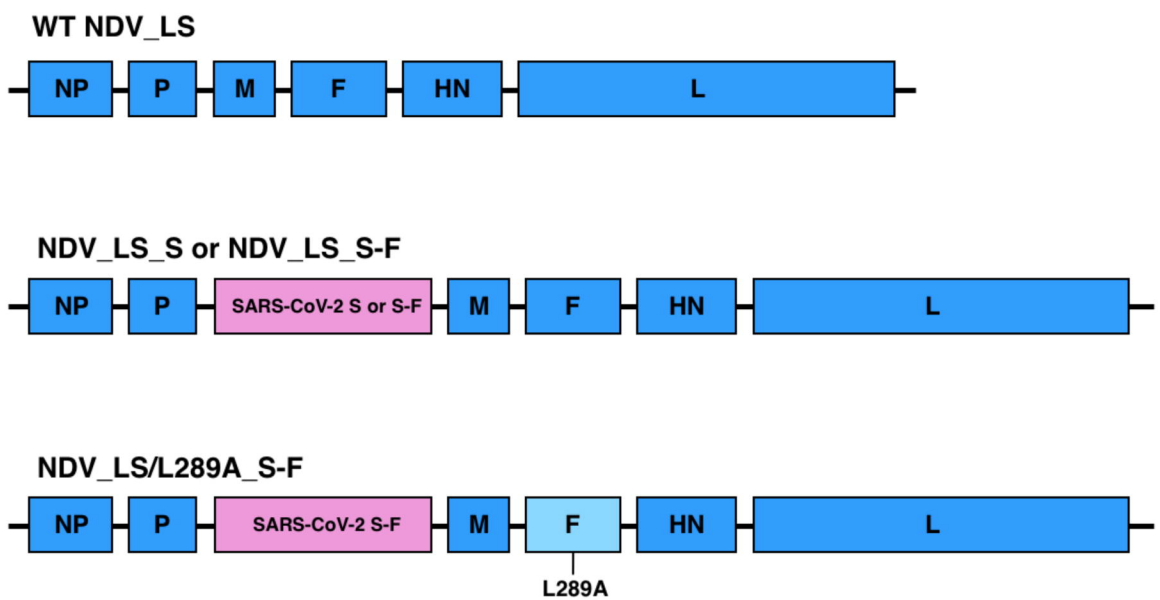
577 antibodies. Sera from 3 weeks after-prime and 8 days after-boost were pooled within each group.
578 Technical duplicates were performed to measure neutralization activities of serum antibodies using a
579 USA-WA1/2020 SARS-CoV-2 strain. The ID50 value was calculated as the readout of the neutralization
580 assay. For the samples (WT NDV and PBS groups) showing no neutralizing activity in the assay, an ID50
581 of 10 was given as the starting dilution of the sera is 1:20 (LoD: limit of detection).
582
583 **Figure 4. NDV vector vaccines protected mice from the SARS-CoV-2 challenge**
584 (A) Viral titers in the lungs. All mice were infected intranasally with 10^4 PFU SARS-CoV-2 MA strain
585 except the healthy control group, which was mock infected with PBS. At day 4 post-challenge, lungs
586 were collected and homogenized in PBS. Viral titers in the lung homogenates were determined by plaque
587 assay. Plaque-forming units (PFU) per lung lobe was calculated. Geometric mean titer was shown for all
588 the groups. LoD: limit of detection. (B) Immunohistochemistry (IHC) staining of lungs. A SARS-CoV-2
589 NP specific antibody was used for IHC to detect viral antigens. Slides were counterstained with
590 hematoxylin. A presentative image was shown for each group. The brown staining indicates the presence
591 of NP protein of SARS-CoV-2.

Figure 1

A


Spike (S):
SARS-CoV-2 S
The polybasic cleavage site is deleted (^{682}RRAR^{685} to A)
NDV-F TM/CT
Spike-F chimera (S-F):
SARS-CoV-2 S-F

B


WT NDV_LS
NP
P
M
F
HN
L
NDV_LS_S or NDV_LS_S-F
SARS-CoV-2 S or S-F
NDV_LS/L289A_S-F
SARS-CoV-2 S-F
L289A

C

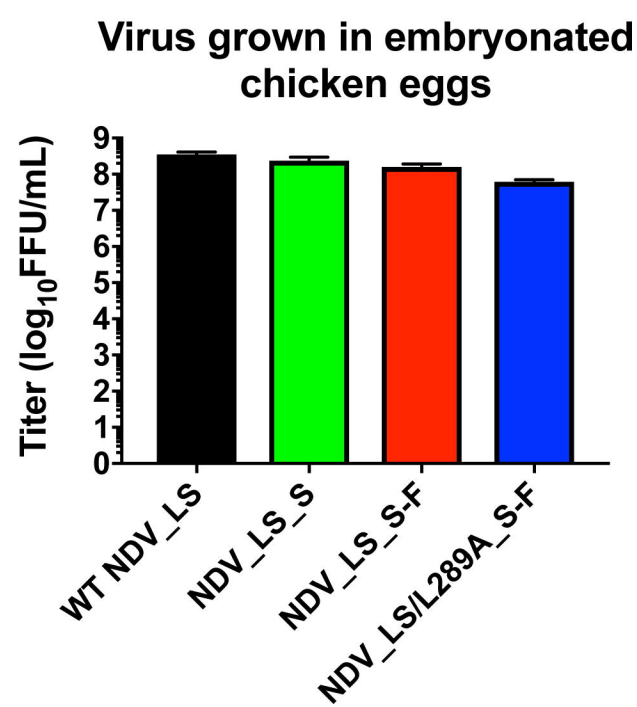
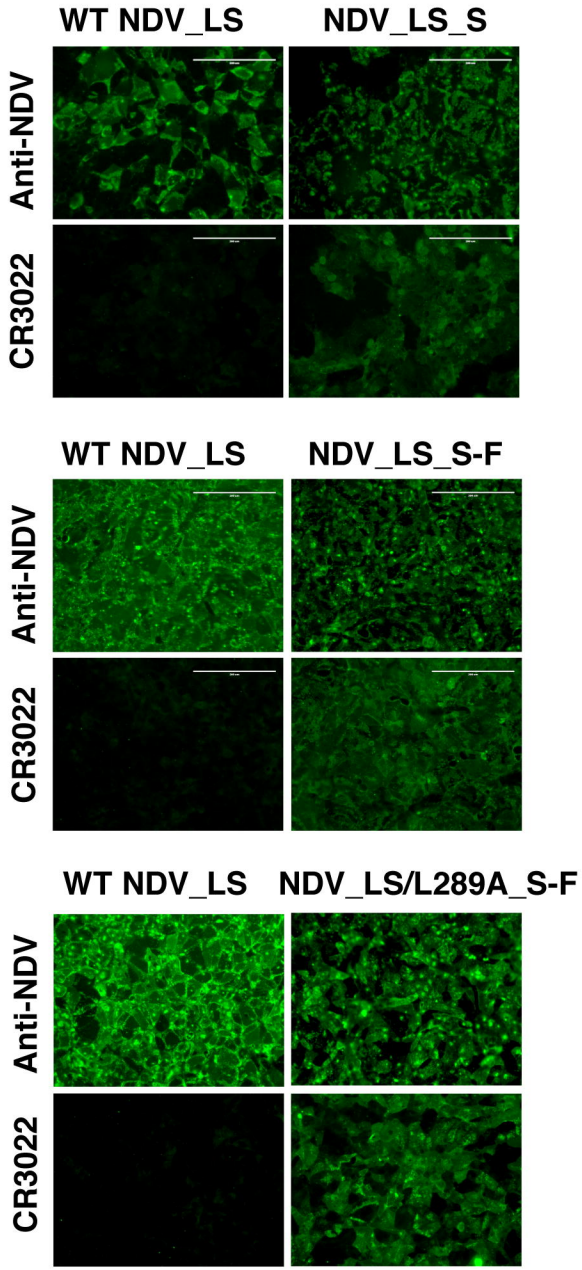
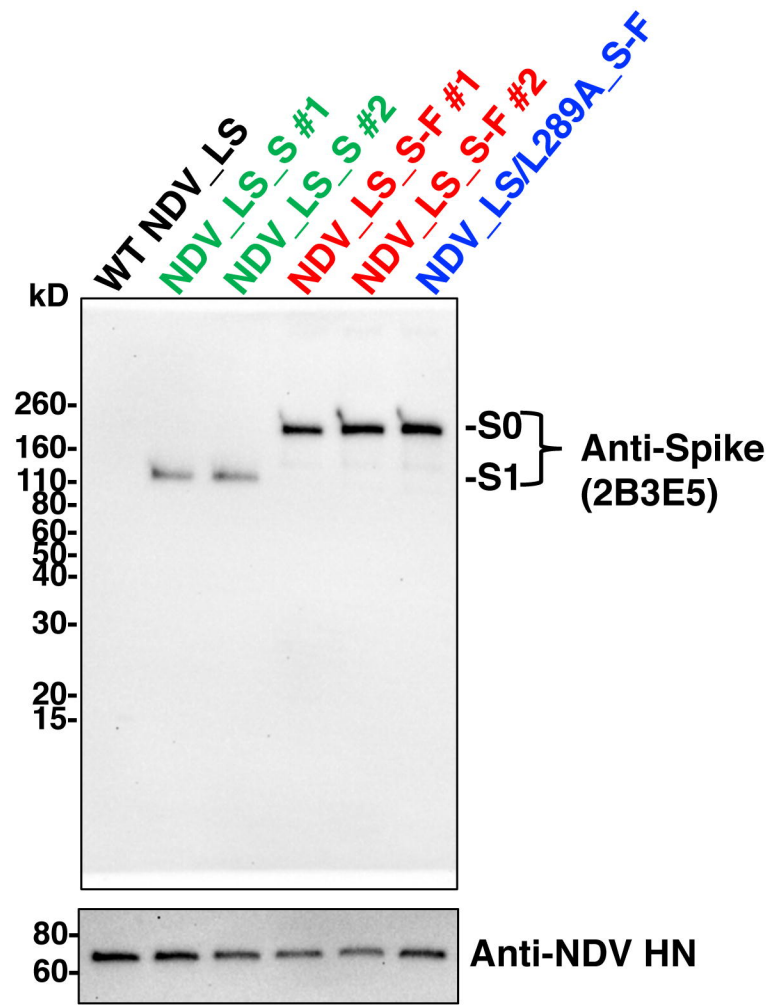

Virus grown in embryonated chicken eggs
Titer (log_{10}FFU/mL)
0
1
2
3
4
5
6
7
8
9
WT NDV_LS
NDV_LS_S
NDV_LS_S-F
NDV_LS/L289A_S-F

Figure 2

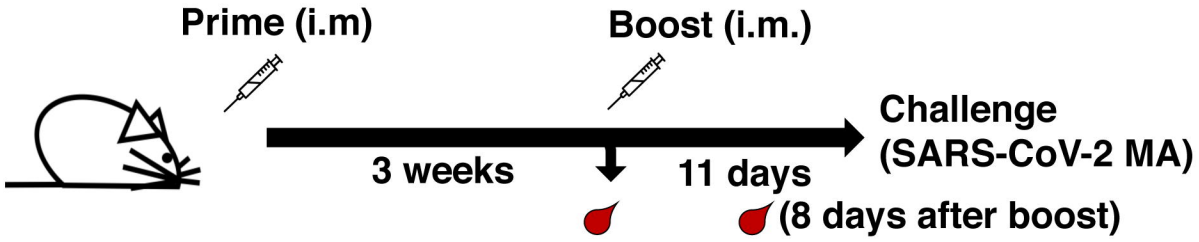
A


WT NDV_LS
NDV_LS_S
Anti-NDV
CR3022
WT NDV_LS
NDV_LS_S-F
Anti-NDV
CR3022
WT NDV_LS
NDV_LS/L289A_S-F
Anti-NDV
CR3022

B

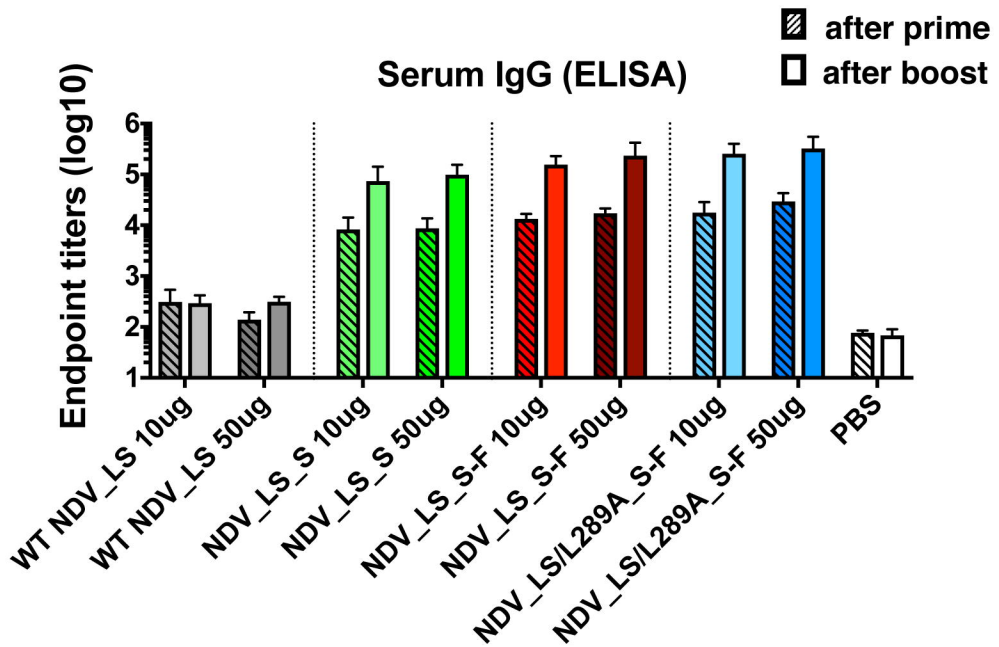

WT NDV_LS
NDV_LS_S #1
NDV_LS_S #2
NDV_LS_S-F #1
NDV_LS_S-F #2
NDV_LS/L289A_S-F
kD
260-
160-
110-
80-
60-
50-
40-
30-
20-
15-
-S0
-S1
Anti-Spike
(2B3E5)
80-
60-
Anti-NDV HN

A



Groups (n=5)	Prime	Boost
1	WT NDV_LS 10ug	WT NDV_LS 10ug
2	WT NDV_LS 50ug	WT NDV_LS 50ug
3	NDV_LS_S 10ug	NDV_LS_S 10ug
4	NDV_LS_S 50ug	NDV_LS_S 50ug
5	NDV_LS_S-F 10ug	NDV_LS_S-F 10ug
6	NDV_LS_S-F 50ug	NDV_LS_S-F 50ug
7	NDV_LS/L289A_S-F 10ug	NDV_LS/L289A_S-F 10ug
8	NDV_LS/L289A_S-F 50ug	NDV_LS/L289A_S-F 50ug
9	PBS	PBS
10	Healthy control	

B



C

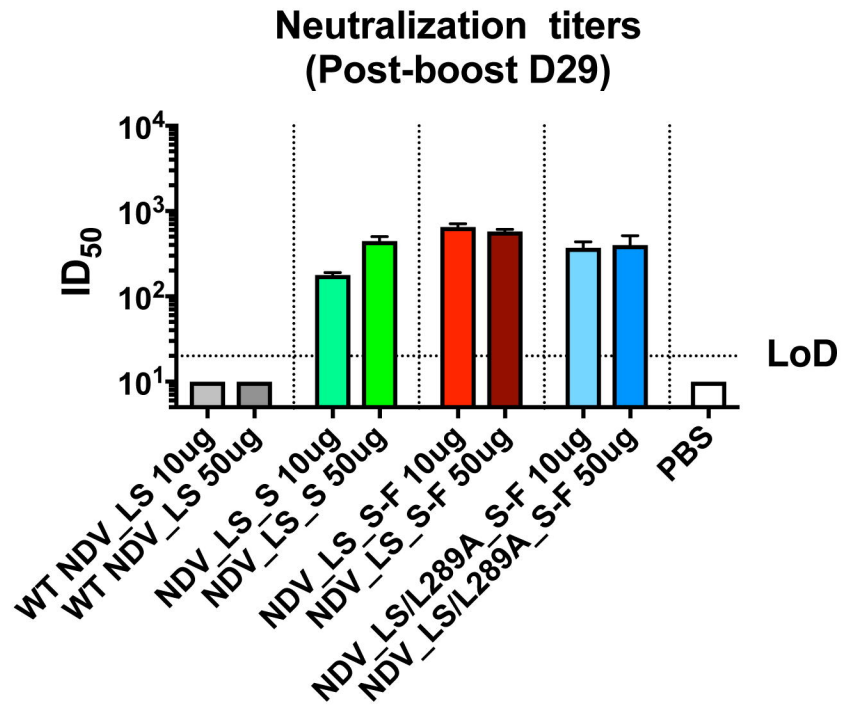
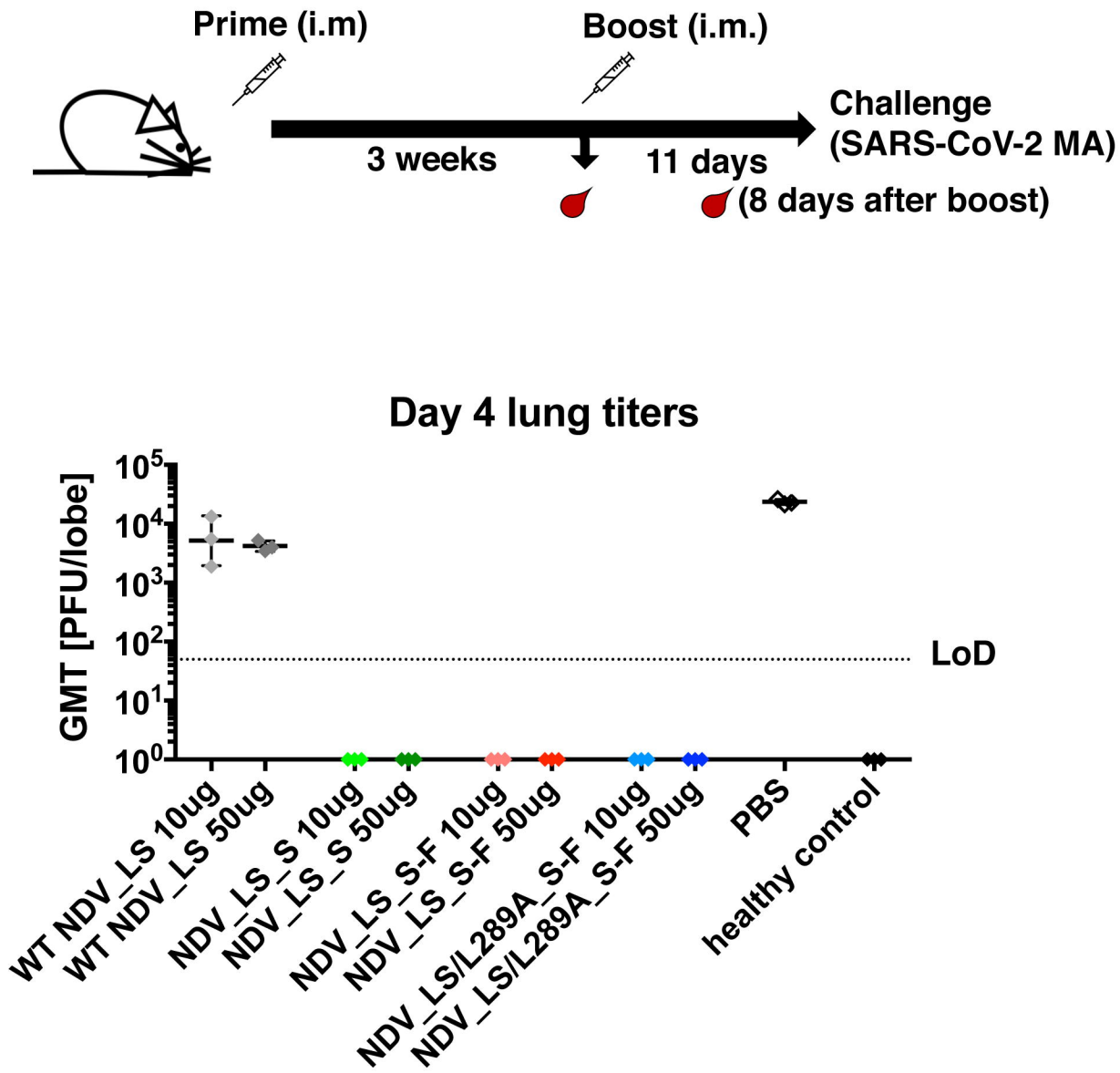


Figure 3

A


Prime (i.m)
Boost (i.m.)
Challenge
(SARS-CoV-2 MA)
3 weeks
11 days
(8 days after boost)
Day 4 lung titers
GMT [PFU/lobe]
10^5
10^4
10^3
10^2
10^1
10^0
LoD
WT NDV_LS 10ug
WT NDV_LS 50ug
NDV_LS_S 10ug
NDV_LS_S 50ug
NDV_LS_S-F 10ug
NDV_LS_S-F 50ug
NDV_LS/L289A_S-F 10ug
NDV_LS/L289A_S-F 50ug
PBS
healthy control

B

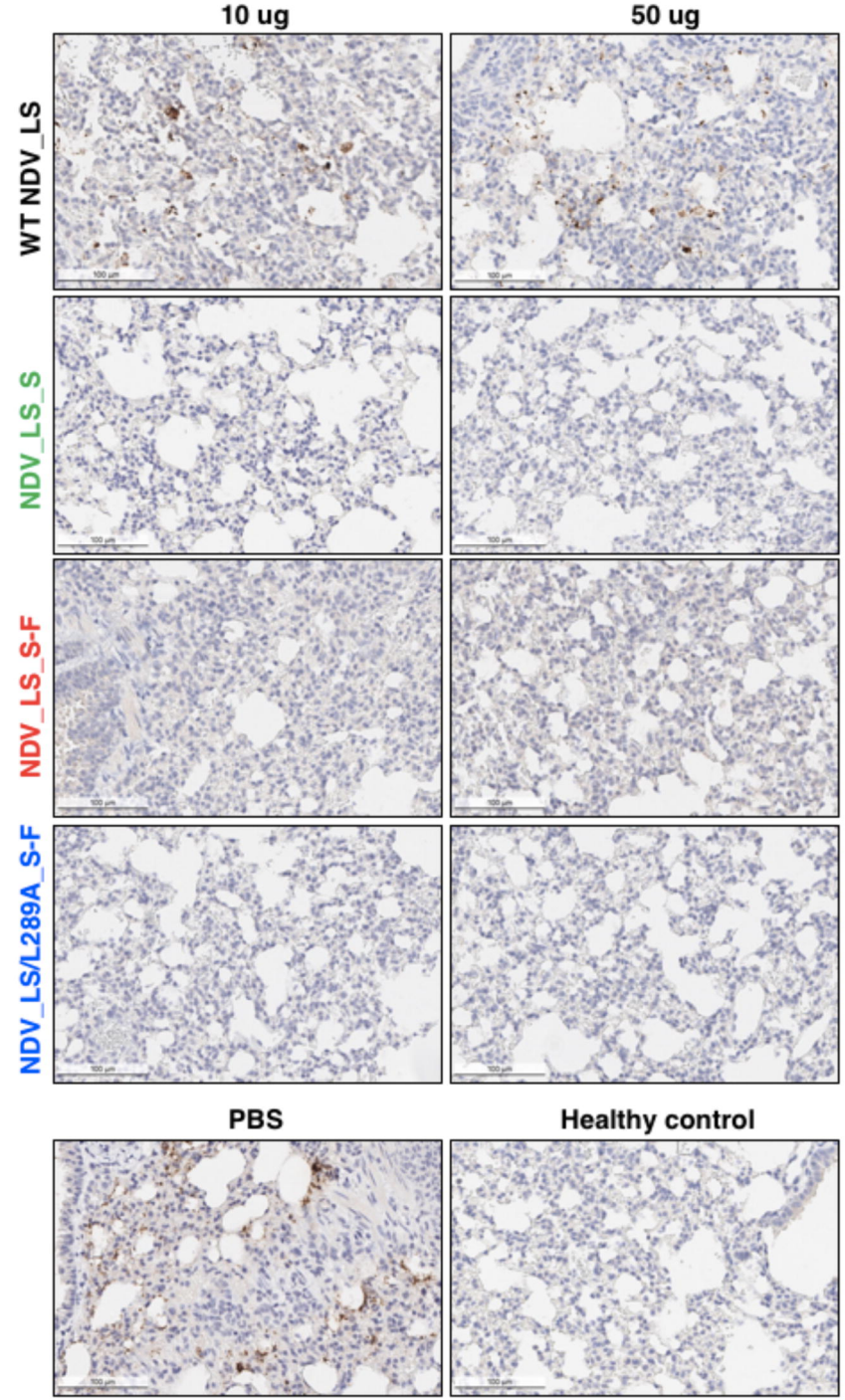

10 ug
50 ug
WT NDV_LS
NDV_LS_S
NDV_LS_S-F
NDV_LS/L289A_S-F
PBS
Healthy control

Figure 4