

# A single dose of an adenovirus-vectored vaccine provides complete protection of the upper and lower respiratory tracts against SARS-CoV-2 challenge

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# Abstract

The unprecedented coronavirus disease 2019 (COVID-19) epidemic has created a worldwide public health emergency, and there is an urgent need to develop an effective vaccine to control this severe infectious disease. Here, we found that a single vaccination with a replication-defective human type 5 adenovirus encoding the SARS-CoV-2 spike protein (Ad5-nCoV) protected mice completely against SARS-CoV-2 infection in the upper and lower respiratory tracts. Additionally, a single vaccination with Ad5-nCoV protected ferrets from SARS-CoV-2 infection in the upper respiratory tract. This study suggested that a combination of intramuscular and mucosal vaccination maybe provide a desirable protective efficacy and different Ad5-nCoV delivery modes are worth further investigation in human clinical trials.

## Main Text

The global coronavirus disease 2019 (COVID-19) pandemic has made the development of a vaccine a top global priority. There are more than 100 vaccine development projects in the WHO draft landscape of COVID-19 candidate vaccines, including viral vector-based vaccines, mRNA and DNA vaccines, subunit vaccines, nanoparticle-based vaccines and inactivated-whole virus vaccines<sup>1</sup>. Some COVID-19 vaccine candidates, including that based on inactivated vaccine, a chimpanzee adenovirus-vectored vaccine and a DNA vaccine, can significantly inhibit virus replication and protect non-human primates from SARS-CoV-2 pneumonia<sup>2-4</sup>. However, it may be easier for these vaccine candidates to protect against lower respiratory tract disease than against upper respiratory tract disease in challenge tests<sup>2-4</sup>.

We chose a replication-defective human adenovirus type 5-based COVID-19 vaccine (Ad5-nCoV) that leverages our prior experience with the Ad5-vectored Ebola vaccine<sup>5-7</sup>. Ad5-nCoV encoding the full spike of SARS-CoV-2 has shown its safety and immunogenicity profile with one vaccination in a phase 1 human clinical trial<sup>8</sup>. Considering that SARS-CoV-2 is a serious respiratory disease, we evaluated the protective efficacy of the mucosal vaccination route, in addition to the normal intramuscular vaccination route. We examined the induction of antibodies, T cell responses and protective efficacy of Ad5-nCoV against SARS-CoV-2 challenge following intramuscular and intranasal immunizations in wild-type BALB/c mice and ferrets.

Antigen design was guided by prior knowledge, and the full spike (S) protein was selected as the immunogen based on the Wuhan-Hu-1 strain (YP\_009724390). The S gene (14-1273 aa) was optimized for increased antigen expression in mammalian cells, and transgene expression was verified by western blot. The Ad5 encoding full-length S led by the tissue plasminogen activator (tPA) signal peptide (Ad5-nCoV) was confirmed as a vaccine candidate (Fig. 1a, 1b).

BALB/c mouse is an ideal animal model for SARS-CoV-2 infection both in the upper and lower respiratory tracts. The immune response induced by Ad5-nCoV was first evaluated in BALB/c mice. Six- to eight-week-old female BALB/c mice (n = 10 per group) received a single immunization of  $5 \times 10^9$  virus particles (VP) (high dose),  $5 \times 10^8$  VP (middle dose), or  $5 \times 10^7$  VP (low dose) of Ad5-nCoV or  $5 \times 10^9$  VP of the control

vaccine (Ad5 vector) by the intramuscular (IM) or intranasal (IN) route at week 0. S-specific IgG antibody, anti-SARS-CoV-2 specific neutralizing antibody (NAb), IgA and cellular immune responses were detected in each group. ELISA IgG titres peaked at day 28 in the IM groups and then slightly decreased afterwards, while those in the IN groups remained at a steady peak from week 4 to week 8 (Fig. 1c, 1f). There were higher IgG titres in the high-dose IN groups than in the IM groups ( $P < 0.0001$  at week 6, and  $P = 0.0001$  at week 8) and no difference between the middle- or low-dose IM and IN groups ( $P > 0.05$ ) at weeks 6 and 8 post immunization (Extended Data Fig. 1a). NABs were detected by a virus-specific microneutralization assay, and NAb titres reached peaks at week 6 or week 8 in the IN or IM group, respectively (Fig. 1d, 1g). NAb titres were significantly higher in the high-dose IN groups than in the IM groups ( $P < 0.0001$  at week 4 and week 6, and  $P = 0.0021$  at week 8) from week 4 to week 8, while there was no difference in the middle-dose group at weeks 6 and 8 and no difference in the low-dose group at any time point (Extended Data Fig. 1b). The trend of SARS-CoV-2 pseudovirus NAb (PNAb) titres was similar to that of NAb titres (Fig. 1e, 1h; Extended Data Fig. 1c), and a relatively good correlation was shown among IgG titres, NAb titres and PNAbs titres at week 6 and week 8 post immunization (Extended Data Fig. 2). S-specific IgG in the trachea-lung wash was detected in both the IM and IN groups at week 2 (Extended Data Fig. 3b) and week 10 (Extended Data Fig. 4a), but S-specific IgA was found only in the IN groups (Extended Data Fig. 3c, 4b). NABs and PNAbs were also detected in the trachea-lung washes in both high-dose groups but not in either low-dose group (Extended Data Fig. 4c, 4d). Both the middle-dose IM and IN groups exhibited significant induction of IFN $\gamma$ , TNF $\alpha$  and IL-2 responses in splenic CD8 $^+$  T cells and CD4 $^+$  T cells at week 2, with a higher level in the IM group than in the IN group (Fig. 1i, 1j). Dose-dependent cellular immune responses were found in the IM groups but not in the IN groups at week 10 (Extended Data Fig. 4e, 4f).

Seven of ten vaccinated mice in every group were inoculated intranasally with a mouse-adapted SARS-CoV-2 virus (HRB26M) at a dose of  $10^{3.6}$  plaque-forming units (PFU) per mouse at week 10 post immunization. Four and three out of seven mice in every dose group were sacrificed for viral load quantification in the lungs and turbinates at 3 and 5 days post inoculation (dpi), respectively. No virus was detected in the lungs or turbinates in all the IN vaccinated groups at 3 and 5 dpi by quantitative polymerase chain reaction (qPCR) and PFU assays, while all the IN control group animals were infected with a mean viral load of  $1.2 \times 10^4$  PFU/g in the turbinates and  $5.6 \times 10^5$  PFU/g in the lungs at 3 dpi (Fig. 2). No virus was detected in the lungs in all the IM-vaccinated groups at 3 and 5 dpi, while all the IM control group animals' lungs were infected with a mean viral load of  $3.3 \times 10^6$  PFU/g at 3 dpi. Virus was detected in the turbinates of some mice of the IM group by PFU assays and qPCR, with a significant reduction in the three vaccinated groups compared with the load of the IM control animals (Extended Data Fig. 5). These data demonstrated that a single low dose of Ad5-nCoV can completely protect both the upper respiratory tract and lungs of mice from infection and it seemed that the infection prevention in the upper respiratory tract is more difficult than in the lower respiratory tract from the different protection via different vaccination mode in mice.

The ferret is a mammalian model in which SARS-CoV-2 can replicate in its upper respiratory tract, but not in the lungs<sup>9</sup>. We next evaluated the protective efficacy of the mucosal vaccination and IM vaccination of

Ad5-nCoV in the upper respiratory tracts of ferrets, Eighteen ferrets were equally grouped into the IM vaccination group ( $5 \times 10^{10}$  VP), the mucosal vaccination group (simultaneous oral delivery with  $5 \times 10^{10}$  VP and IN delivery with  $5 \times 10^{10}$  VP for one ferret) and the control group and challenged on week 4 after a single vaccination of Ad5-nCoV. All vaccinated ferrets produced S-specific serum IgG antibodies and NAbs at week 4 post vaccination, which were not detected in control animals, with no difference between the two vaccination groups (Fig. 3a, 3b). We also observed cellular immune responses in 5 out of 6 ferrets in the IM group and 3 out of 6 ferrets in the mucosal vaccination group by IFN $\gamma$  ELISpot assays at week 4 (Fig. 3c). These animals were challenged intranasally with  $10^5$  PFU of SARS-CoV-2 at week 4 post vaccination, and nasal washes were collected every two days for viral load analysis of SARS-CoV-2 by qPCR and PFU assays. No virus was detected in the nose washes of mucosal vaccination group animals by the qPCR and PFU assays from 2 to 8 dpi, in contrast to all the infected control animals (Fig. 3d, 3e). Virus was detected in the nose washes of 3/6, 2/6, 0/6 and 0/6 of the IM-vaccinated animals by PFU assay at 2, 4, 6, and 8 dpi, respectively, and a significant reduction in viral load was found at each day between the IM vaccination group and the control group animals (Fig. 3d, 3e).

We have demonstrated for the first time, to our knowledge, that complete protection for the upper and lower respiratory tracts against SARS-CoV-2 infection can be achieved using a single mucosal inoculation of Ad5-nCoV in mice and ferrets. A single intramuscular inoculation of Ad5-nCoV can protect the lungs of mice from SARS-CoV-2 infection and significantly reduce viral replication in the upper respiratory tract of mice and ferrets. The challenges administered to these animals with a high dose of virus via the intranasal route do not reflect realistic human exposure. Whether a lower challenge dose would result in more efficient protection of the upper respiratory tract by IM vaccination remains to be determined. Although additional work is needed to fully unravel the protective mechanisms associated with the different vaccination delivery modes, we speculate that S-specific mucosal antibodies or mucosal tissue-resident T cells play important roles in protection<sup>10</sup>. Mucosal vaccination, which induces pathogen-specific mucosal immunity in addition to systemic immunity, can provide a first line of protection at the SARS-CoV-2 port of entry. Combining the rapid systemic immune response of intramuscular injection with the local mucosal immunity of the mucosal vaccination may be more effective than a single inoculation model. This work gives us a hint that the different routes of vaccination should be considered in human clinical trials for the development of the SARS-CoV-2 vaccine.

## Methods

### Cells lines and viruses

HEK293 cells (Human embryonic kidney, ATCC), Vero E6 cells (African green monkey kidney, ATCC) and ACE2-293T cells (ACE2-expressing cell line, constructed by hygromycin B screening) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Thermo Scientific, USA) supplemented with 10% foetal bovine serum (FBS) (Thermo Scientific, USA), penicillin (100 units/mL) and streptomycin (100  $\mu$ g/mL) (complete medium) at 37 °C in 5% CO<sub>2</sub>. SARS-CoV-2/human/CHN/Beijing\_IME-BJ01/2020 (Genbank No.

MT291831) and SARS-CoV-2/HRB25/human/2020/CHN (HRB25, GISAID access no. EPI\_ISL\_467430) were respectively isolated from patients and propagated in Vero E6 cells. Mouse-adapted SARS-CoV-2/HRB26/human/2020/CHN (HRB26M, GISAID access no. EPI\_ISL\_459910) was generated by passaging the human patient isolate SARS-CoV-2/HRB26/human/2020/CHN (HRB26, GISAID access no. EPI\_ISL\_459909) in 4-6-week-old (young) female mice for 14 passages and propagated in Vero E6 cells. Virus titers were determined by standard plaque assay on Vero E6 cells, and virus stocks were stored in aliquots at -80 °C until use.

### **Construction of Ad5-nCoV expressing SARS-CoV-2 S**

The full spike protein gene of SARS-CoV-2 based on the Wuhan-Hu-1 strain (NC\_045512.2) was codon optimized by UpGene software, and the signal peptide was substituted with tPA for increased expression in mammalian cells. The gene was synthesized with EcoRI and HindIII upstream and downstream of the open reading frame, respectively, and cloned into the shuttle plasmid of the AdMax adenovirus system (Microbix Biosystem, Canada) by enzyme digestion and ligation. After sequencing identification, the shuttle plasmid with the target gene was cotransfected into HEK293 cells with the backbone plasmid (pBHGloxΔE1, 3Cre) by TurboFect transfection reagent (Thermo Scientific, USA) according to the manufacturer's instructions. The transfected cells were passaged when they were overgrown and collected until Ad-related cytopathic effects were observed. The cells were lysed by three freeze-thaw cycles to release the recombinant viruses. The recombinant adenoviruses were confirmed by target gene sequencing, monocloned by agarose plaque selection, amplified by serial passage on HEK293 cells, and purified by ion exchange chromatography and size exclusion. The number of total VP was measured by ultraviolet spectrophotometer analysis, with one OD<sub>260</sub> equal to approximately  $1.1 \times 10^{12}$  VP, and the infectious units (ifu) were titrated on HEK293 cells using an AdenoX™ Rapid Titer Kit (Clontech, USA) following the manufacturer's instructions.

### **Western blotting**

HEK293 cells in 6-well cell culture clusters were transiently transfected with 2 μg of plasmids expressing different S protein constructs by TurboFect transfection reagent (Thermo Scientific, USA). The culture supernatant was discarded, and cells were rinsed with phosphate-buffered saline pH 7.4 (PBS) and lysed by 150 μL of RIPA Lysis and Extraction Buffer (Thermo Scientific, USA) for each well at 48 h post transfection. The cell lysate was centrifuged, and the supernatant was collected, mixed with NuPAGE LDS sample buffer (Thermo Scientific, USA) and run on a SurePAGE 4-20% Bis-Tris protein gel (GenScript, China) with 20 μL per lane. Protein was transferred to a nitrocellulose membrane by an eBlot L1 transfer system (GenScript, China). Membranes were blocked for 1 h at room temperature (RT) in Tris-HCl-buffered saline pH 7.6 with 0.1% Tween 20 (TBST) containing 5% skim milk and then incubated overnight at 4 °C with a 1:2000 dilution of a polyclonal rabbit anti-SARS-CoV spike antibody (Sino Biological, China). After four washes with TBST, the membranes were incubated for 1 h at RT with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibody (Cell Signaling Technology, USA). The membranes were developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific,

USA), and images were acquired with a Clix ChemiScope imaging system (Clix Science, China). As an internal parameter,  $\beta$ -actin was detected on the same membrane by an HRP-conjugated anti- $\beta$ -actin antibody (Abcam, UK).

## **Animal experiments**

The experiments involving animals were approved by and carried out in accordance with the guidelines of the Institutional Experimental Animal Welfare and Ethics Committee. Specific pathogen-free (SPF) female BALB/c mice aged 6-8 weeks were obtained from Beijing Vital River Laboratory Animal Technologies Co., Ltd. (Beijing, China) and were housed and bred in the animal facility of the Animal Center, Academy of Military Medical Sciences, Beijing. Three- to four-month-old female ferrets purchased from Wuxi Cay Ferret Farm (Wuxi, China) were housed in the animal facility of Harbin Veterinary Research Institute (HVRI) of the Chinese Academy of Agricultural Sciences (CAAS). Mouse and ferret experiments with infectious SARS-CoV-2 were performed at the biosafety level 4 and animal biosafety level 4 facilities in HVRI, which is approved for such use by the Ministry of Agriculture and Rural Affairs of China.

## **Vaccination and challenge of mice**

BALB/c mice ( $n = 10$  per group) were immunized intramuscularly or intranasally with  $5 \times 10^9$  VP (high dose),  $5 \times 10^8$  VP (middle dose) or  $5 \times 10^7$  VP (low dose) of Ad5-nCoV at day 0 or  $5 \times 10^9$  VP of Ad5 vector as a control. Sera were collected for S-specific ELISA, SARS-CoV-2 NAb titration ( $MN_{50}$ ) and SARS-CoV-2 PNAbs titration at different time points. Another three groups of mice immunized with a middle dose of Ad5-nCoV by the IM or IN route or with the control vaccine were euthanized at day 14 post immunization for splenic cellular immune response detection and trachea-lung wash antibody detection. At 10 weeks after vaccination, three of ten vaccinated mice in every group were euthanized for splenic T cell response detection and trachea-lung wash antibody and NAb detection. The remaining mice (seven per group) were challenged intranasally with SARS-CoV-2 HRB26M strain at a dosage of  $10^{3.6}$  PFU in a volume of  $50 \mu\text{L}$ . Four and three out of seven mice in every dose group were sacrificed for viral load quantification in the lungs and turbinates at 3 and 5 dpi, respectively.

## **Vaccination and challenge of ferrets**

Ferrets (6/group) were randomized by body weight, sex, and age and grouped into the IM vaccination group ( $5 \times 10^{10}$  VP), the mucosal vaccination group (simultaneous oral delivery with  $5 \times 10^{10}$  VP and IN delivery with  $5 \times 10^{10}$  VP for one ferret) and the control group. Blood was collected at week 4 for analysis of antibody responses by ELISA, plaque reduction neutralization assays, and IFN $\gamma$  ELISpot. The immunized animals were challenged intranasally with a dose of  $10^5$  PFU of SARS-CoV-2 HRB25 strain at day 28, and the viral load of the nasal wash was detected by qPCR and PFU assay every two days post infection.

## **Quantitative RT-PCR**

Viral loads were determined by quantitative real-time PCR. Viral RNA was extracted by using a QIAamp vRNA Minikit (Qiagen, Germany). Reverse transcription was performed by using HiScript II Q RT SuperMix for qPCR (Vazyme, China). qPCR was conducted by using an Applied Biosystems QuantStudio 5 Real-Time PCR System (Thermo Scientific, USA) with Premix Ex Taq for probe qPCR (TaRaKa, China). *N* gene-specific primers (forward, 5'-GGG GAA CTT CTC CTG CTA GAA T-3'; reverse, 5'-CAG ACA TTT TGC TCT CAA GCT G-3') and probe (5'-FAM-TTG CTG CTG CTT GAC AGA TT-TAMRA-3') were utilized according to the information provided by the National Institute for Viral Disease Control and Prevention, China (<http://nmcdc.cn/nCoV>). The amount of vRNA for the target SARS-CoV-2 *N* gene was normalized to the standard curve from a plasmid (pBluescript II SK-N, 4,221 bp) containing the full-length cDNA of the SARS-CoV-2 *N* gene. The assay sensitivity was 1000 copies/mL.

## ELISA

For SARS-CoV-2 S-specific IgG assays in mice, 96-well polystyrene high-binding microplates (Corning, USA) were coated with 2 µg/mL recombinant SARS-CoV-2 S protein purified from insect cells (Sino Biological, China) in carbonate-bicarbonate buffer pH 9.6, and the plates were incubated at 4 °C overnight. The plates were then blocked at 37 °C for 1 h with PBS pH 7.4 in 5% skim milk (blocking buffer) and washed with PBST. Serial dilutions of sera or trachea-lung washes of mice in dilution buffer were added to the plates and incubated at RT for 1 h. HRP-conjugated goat anti-mouse IgG (Abcam, UK) or HRP-conjugated goat anti-mouse IgA (Abcam, UK) was added to the plates, and the plates were incubated at RT for 1 h and washed with PBST. The assay was developed for 10 min at RT with 100 µL of TMB substrate solution (Solarbio, China), stopped by the addition of 50 µL of stop solution (Solarbio, China) and then measured at 450 nm/630 nm (SPECTRA MAX 190, Molecular Device, USA). The endpoint titre was defined as the highest reciprocal serum dilution that yielded an absorbance  $\geq 2.1$ -fold over negative control serum values.

A double antigen sandwich ELISA kit (ProtTech, China) was used for SARS-CoV-2 S-specific IgG assays for the ferrets. Briefly, 100 µL of serum was added to an antigen-coated microtitre plate, and the plate was incubated at RT for 30 min and washed with PBST. Then, the plate was incubated with HRP-conjugated antigen at 37 °C for 30 min and washed with PBST. The optical density (OD) was measured at 450 nm after the addition of the substrate solution and the subsequent stop solution. Seropositivity was defined as an OD value  $\geq 0.2$ .

## SARS-CoV-2 neutralization assay

The neutralizing activity of sera from the mice was assessed using a microneutralization (MN) assay. Serial dilutions of heat-inactivated sera were incubated with 100 TCID<sub>50</sub> of SARS-CoV-2 IME-BJ01 strain at 37 °C for 2 h. Antibody-virus complexes were added to pre-plated Vero E6 cell monolayers in 96-well plates and incubated for 48~72 h. The cells were stained with 0.05% crystal violet for 30 min. The OD was measured at 570 nm/630 nm after the addition of the decolorization solution. Neutralization results were analysed by Reed-Muench method to estimate the dilution of sera required for half-maximal

neutralization of infection ( $EC_{50}$  titre). The initial dilution of sera (1:16) was set as the limit of confidence of the assay. Seropositivity was defined as a titre  $\geq 16$ .

The neutralizing activity of heat-inactivated sera from the ferrets was assessed using a plaque reduction neutralization test (PRNT) assay. Serial dilutions of sera were incubated with 50 PFU of the SARS-CoV-2 HRB25 strain at 37 °C for 21 h. Antibody-virus complexes were added to pre-plated Vero E6 cell monolayers in 24-well plates and incubated for 48 h with agarose overlay. Neutralizing antibody titres were calculated as the maximum serum dilution yielding a 50% reduction in the number of plaques relative to that for control serum prepared from uninfected animals. Seropositivity was defined as a titre  $\geq 8$ .

### **SARS-CoV-2 pseudovirus neutralization assay**

SARS-CoV-2 pseudovirus bearing the full-length spike protein of SARS-CoV-2 was produced in an Env-defective, luciferase-expressing HIV-1 backbone.  $7 \times 10^6$  293T cells were aliquoted into a 10-cm plate and co-transfected with 23  $\mu\text{g}$  of pNL4-3.Luc-R<sup>E</sup> and 1  $\mu\text{g}$  of CMV/SARS-CoV-2-S by TurboFect transfection reagent (Thermo Scientific, USA). At 48 h later, the supernatants containing pseudovirus were collected, filtered, aliquoted and frozen at -80 °C. Serial dilutions of heat-inactivated sera were mixed with the titrated pseudovirus, incubated for 60 min at 37 °C and added to ACE2-293T cells in duplicate in 96-well microplate. Cells were lysed 48 h later and luciferase activity was measured.  $EC_{50}$  neutralization titres were calculated for each individual mouse serum sample using Reed-Muench method.

### **ELISpot**

SARS-CoV-2-specific cellular immune responses in ferrets were assessed by a Mabtech Ferret IFN $\gamma$  ELISpot Kit (Mabtech, Sweden) following the manufacturer's instructions. In brief,  $1 \times 10^5$  of peripheral blood mononuclear cells (PBMCs) of vaccinated ferrets were produced by density gradient sedimentation and stimulated with inactivated SARS-CoV-2 in a pre-coated ELISpot plate for 16 h in a 37 °C humidified incubator with 5% CO<sub>2</sub>. The next day, the plate was washed 5 times with PBST, incubated for 1 h at RT with the biotin conjugated detection antibody, washed, incubated for 1 h at RT with streptavidin-HRP, washed, and developed with AEC substrate (BD Pharmingen, San Diego, CA). The plate was washed extensively in deionized water to stop color development and dried in the dark, and the spots were counted in an AID ELISPOT reader (AID GmbH, Strassberg, Germany). The result was expressed as the number of SARS-CoV-2-specific spots per 1 million PBMCs.

### **Intracellular cytokine staining**

Splenocytes of BALB/c mice were prepared by pushing the spleen through a 70- $\mu\text{m}$  cell strainer, followed by red blood cell lysis and several washes. The cells were stimulated for 6 h at 37 °C with or without 1  $\mu\text{g}/\text{mL}$  of overlapping 15-amino-acid peptides covering the S protein and with BD GolgiStop<sup>TM</sup> and BD GolgiPlug<sup>TM</sup> to block cytokine secretion. Following peptide pool stimulation, the splenocytes were washed and stained with a mixture of antibodies against lineage markers, including anti-CD3 PerCP-Cy5.5 (clone

17A2), anti-CD4 Alexa Fluor 700 (clone RM4-5), and anti-CD8 FITC (clone 5H10-1), and the viability dye Near-IR to exclude dead cells from data analysis. After one wash with PBS, the cells were fixed and permeabilized with Cytotfix/Cytoperm (BD Biosciences, USA), washed with Perm/Wash buffer (BD Biosciences, USA), and stained with anti-IFN $\gamma$  PE (clone XMG1.2), anti-TNF PE-Cy7 (clone MP6-XT22) and anti-IL-2 Brilliant Violet 421 (clone JES6-5H4). The cells were washed successively with Perm/Wash buffer and PBS and resuspended in PBS, and data were acquired on a FACS Canto<sup>TM</sup> (BD Biosciences, USA). At least 200,000 events were collected for each sample, and the data were analysed by FACS Diva software. CD8<sup>+</sup> and CD4<sup>+</sup> T cells were gated from single cells (FSC-A vs FSC-H), lymphocytes (FSC-A vs SSC-A) and live CD3<sup>+</sup> T cells (CD3<sup>+</sup> vs Near-IR<sup>-</sup>), successively, and the detection results were defined as the percentage of cytokine-positive cells among CD8<sup>+</sup> or CD4<sup>+</sup> T cells.

## Statistical analysis

The analyses were performed with GraphPad Prism v.8.0.2. Two-tailed nonparametric Mann-Whitney's rank tests were conducted to compare differences between groups. Correlations were assessed by Spearman rank-correlation tests.

## Declarations

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**Author Contributions** W.C., Z.B. and L.H. initiated and coordinated the project. S.W., Z.Z., B.W., Z.Z., Q.G., J.Z. and J.L. produced the vaccine candidate; J.Z., L.F., X.S., Y.C., Y.Y., T.F., P. L. J.X. and C.Y. conducted the immunogenicity evaluation on mice. G.Z., R.L., L. S., Z.W., C. W. and J.W. conducted the immunogenicity evaluation on ferrets and the challenge studies on mice and ferrets. W.C., Z.B., S.W. and L.H. analyzed the data and wrote the manuscript.

**Competing interests** The authors declare no competing interests.

**Data availability** All raw data are available from the corresponding author on reasonable request.

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## Figures

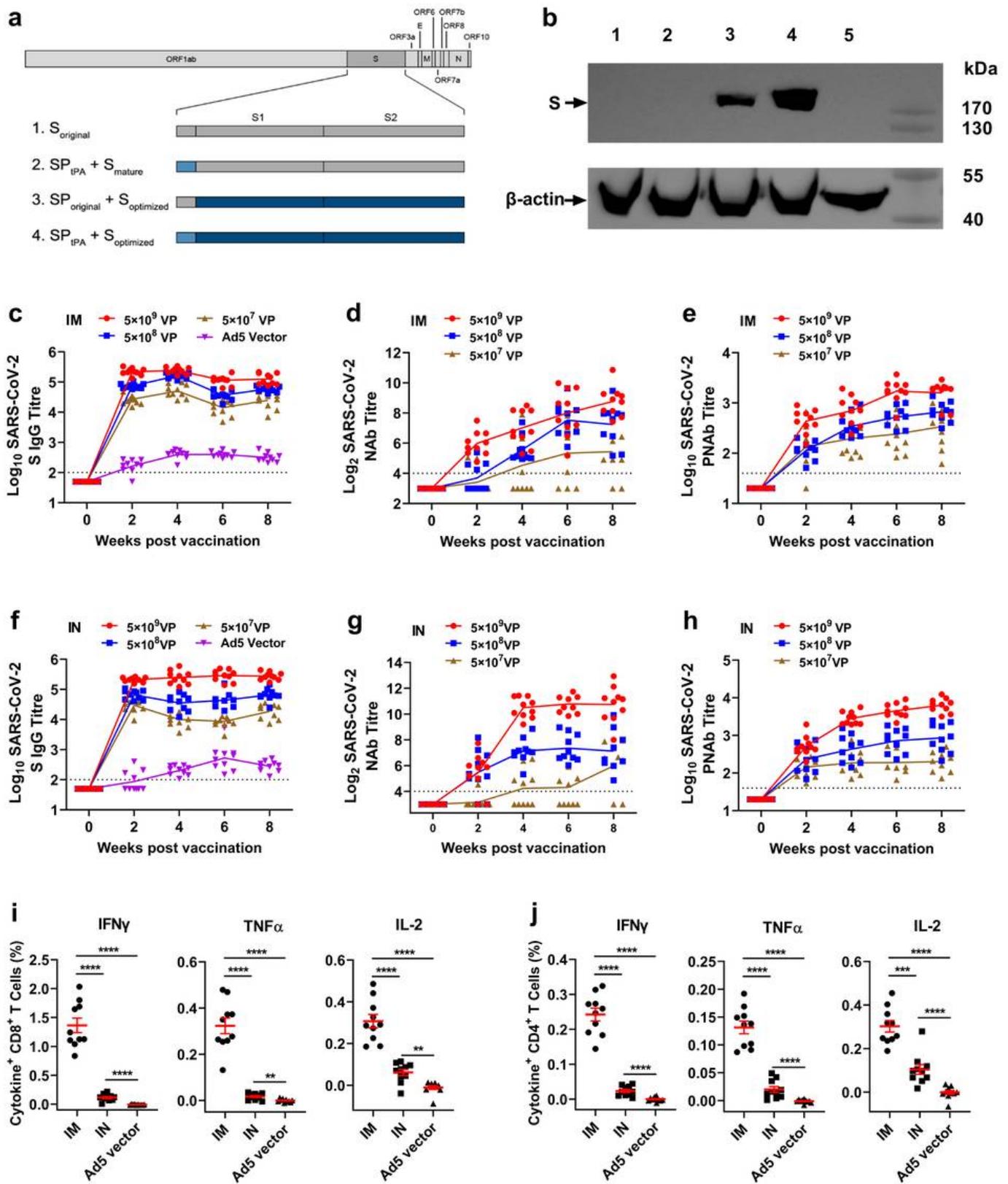
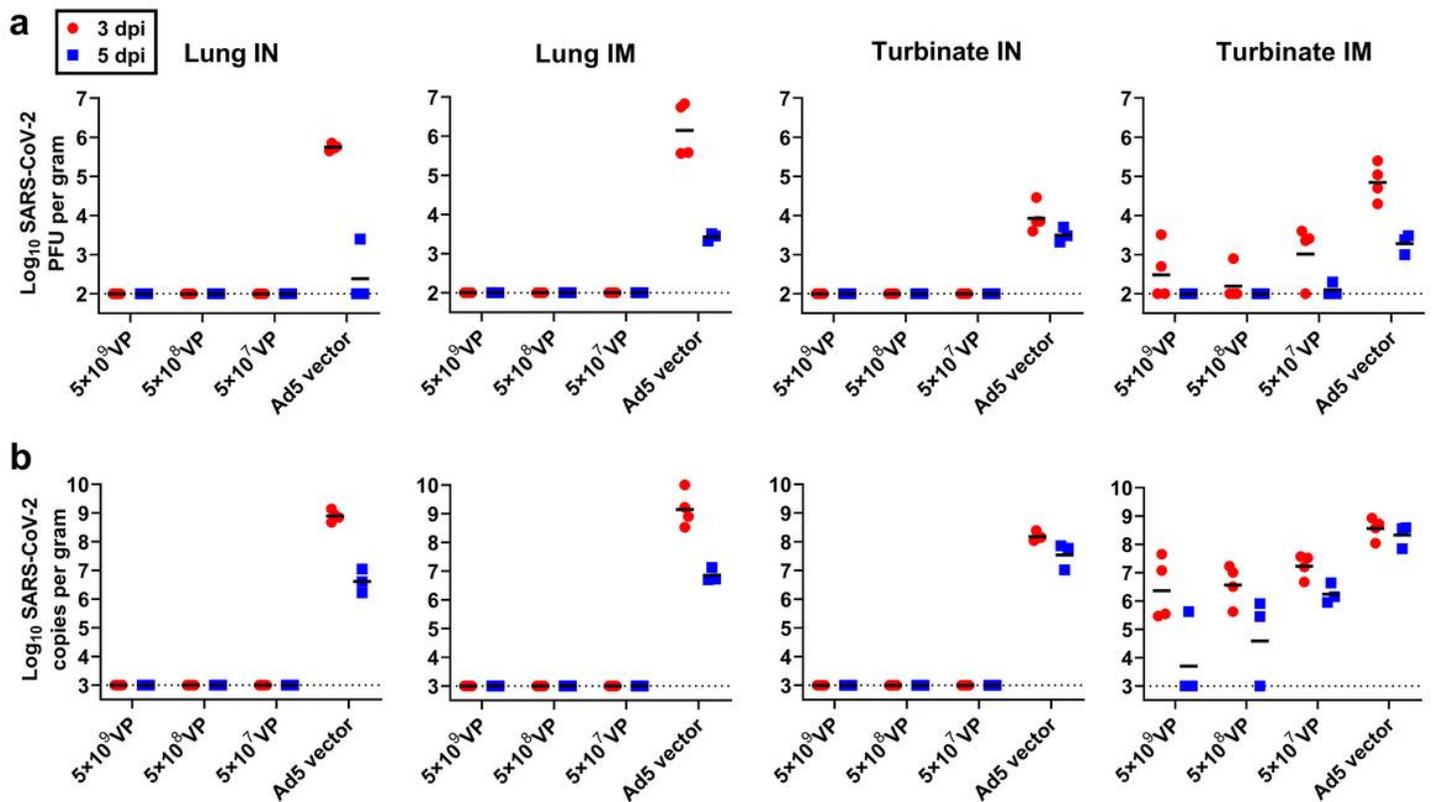


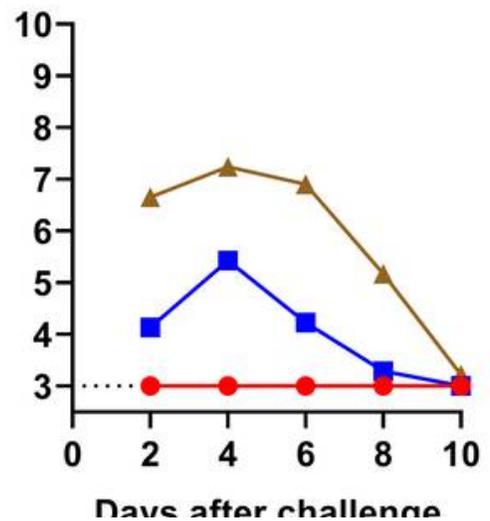
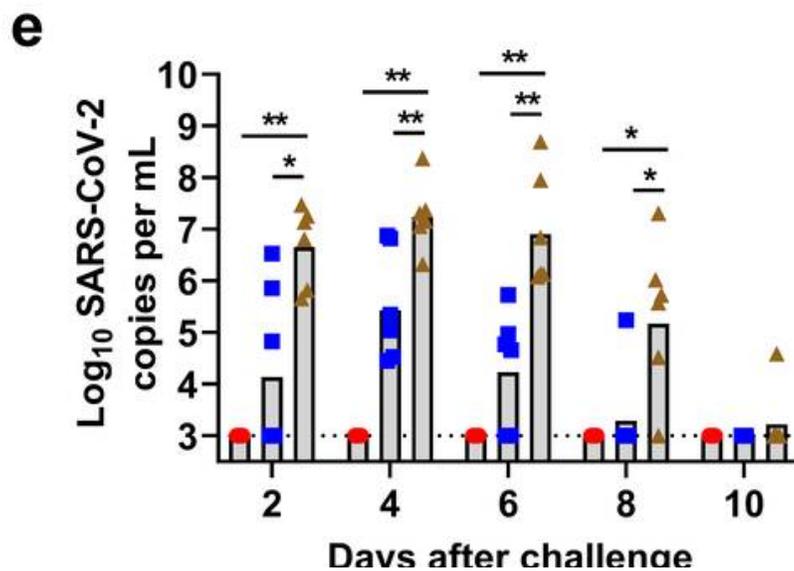
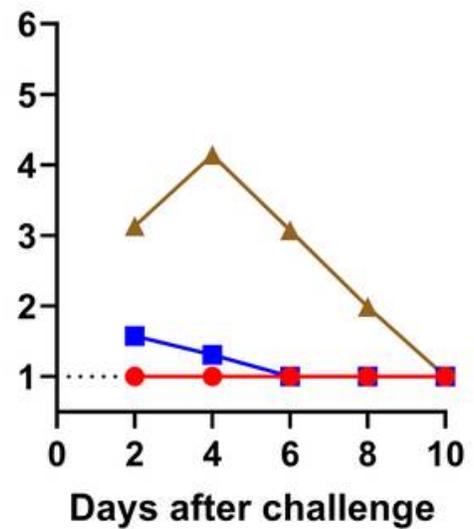
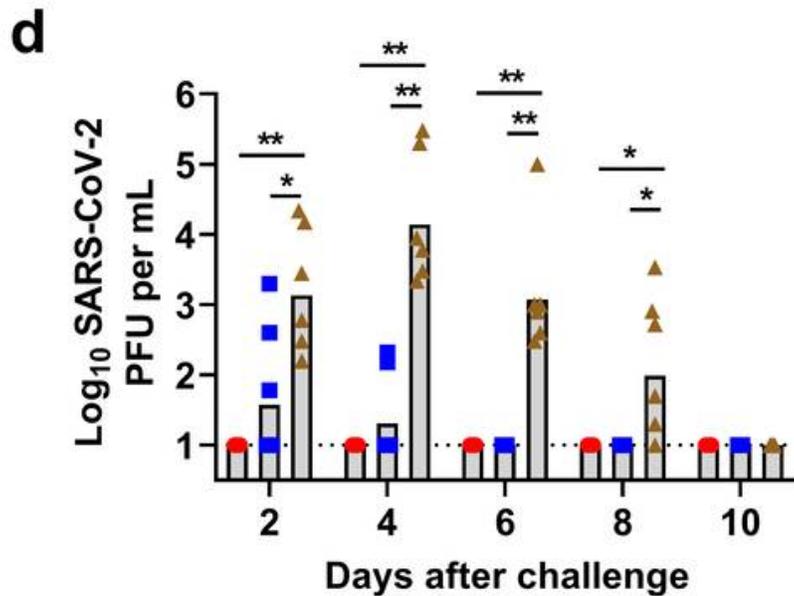
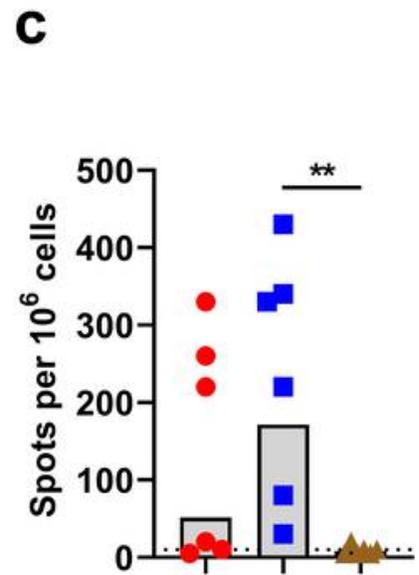
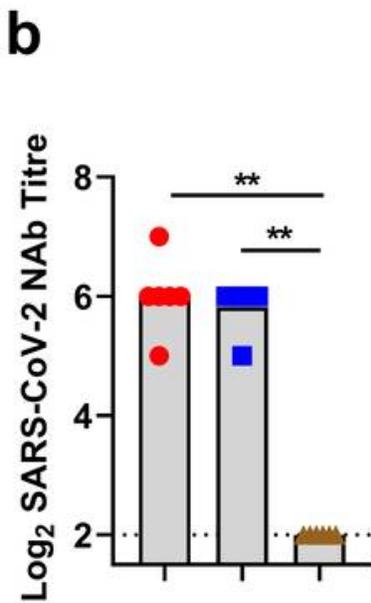
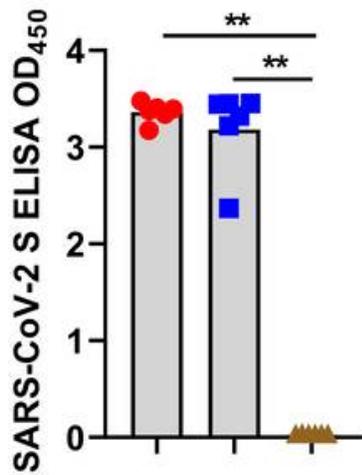
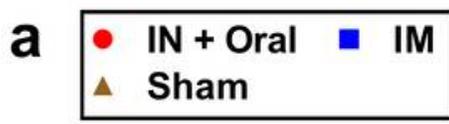
Figure 1

Adenovirus-based vaccine design and immunogenicity in mice. a, Schematic of SARS-CoV-2 S immunogens. b, Western blot of transgene expression from (1) Soriginal, (2) SPtPA add Smature, (3) SOriginal add Soptimized, (4) SPtPA add Soptimized, and (5) an empty plasmid transfected in HEK293 cells. BALB/c mice (n = 10 per group) received a single immunization with different doses of Ad5-nCoV or Ad5 vector by the IM or IN route. c-h, Humoral immune responses were assessed at weeks 0, 2, 4, 6 and 8 following vaccination by S-specific ELISA (c, f), SARS-CoV-2 NAb titration (MN50) (d, g) and SARS-CoV-2 PNAbs titration (e, h). Connecting lines reflect geometric means. i, j, Cellular immune responses were assessed at day 14 following vaccination in the  $5 \times 10^8$  VP dose groups by intracellular cytokine staining assays. Red bars represent the means with SEMs. \*\*=p-value < 0.01, \*\*\*=p-value < 0.001, \*\*\*\*=p-value < 0.0001. S = spike protein, SP = signal peptide, tPA = tissue plasminogen activator. Dotted line = the limit of detection.



**Figure 2**

Protective efficacy of Ad5-nCoV in mice. BALB/c mice (n = 10 per group) received a single immunization with different doses of Ad5-nCoV or Ad5 vector by the IM or IN route, and seven of ten mice were challenged at week 10 with 103.6 PFU of SARS-CoV-2 HRB26M strain in a volume of 50  $\mu$ L by the IN route. Tissue live virus numbers (a) and tissue viral loads (b) were detected at 3 dpi (n=4) and 5 dpi (n=3) in the lungs and turbinates, respectively. Black bars reflect geometric means; 3 dpi = red circles; 5 dpi = blue squares; dotted line = the limit of detection.



### Figure 3

Immunogenicity and protective efficacy of Ad5-nCoV in ferrets. Ferrets (n = 6 per group) received a single immunization by the IM route or two simultaneous doses by the IN and oral routes with  $5 \times 10^{10}$  VP per dose of Ad5-nCoV or received a sham vaccine. a, b, Humoral immune responses were assessed at week 4 following vaccination by S-specific ELISA (a) and NAb titration (PRNT90) (b). c, Cellular immune response was assessed by IFN $\gamma$  ELISpot assays. d, e, Live virus numbers (d) and viral load (e) of SARS-CoV-2 in nasal washes obtained from ferrets after challenge. Bar at the geometric mean, and connecting lines reflect geometric means; \* = p-value < 0.05, \*\* = p-value < 0.01, IN and oral inoculation animals = red circles; IM injection animals = blue squares; control animals = brown triangles; dotted line = the limit of detection.

## Supplementary Files

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