Electronic Supplementary Material (ESI) for Chemical Communications.
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Supporting Information

Protein vaccine with Alum/c-GAMP/poly(I:C) rapidly boosts robust immunity against SARS-CoV-2 and variants of concern

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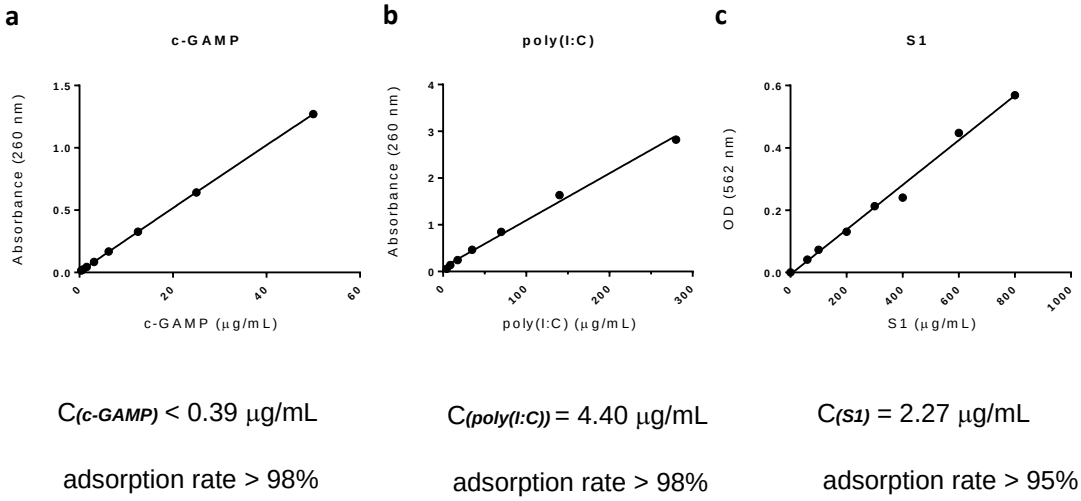

Figure S1. Adsorption of c-GAMP, poly(I:C) and S1 onto Alum. The UV absorbance of (a) c-GAMP, and (b) poly(I:C) were measured at 260 nm at different concentrations. (c) The optical density of the complexes formed using the bicinchoninic acid (BCA) method was measured at 562 nm for different concentrations of S1. Respective adsorption rates were calculated by bringing the absorbance of each component in the supernatant into the corresponding linear regression equations.

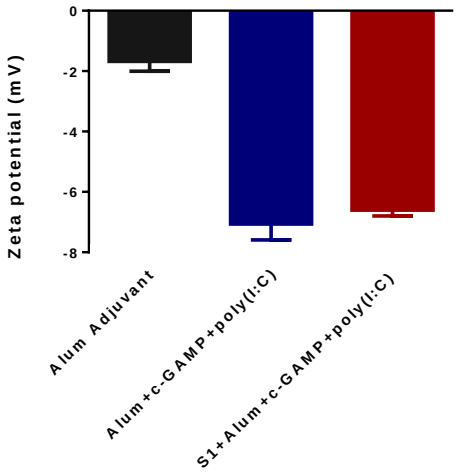

Figure S2. Zeta potential of Alum adjuvant, the ternary adjuvant system and SARS-CoV-2 vaccine candidate.

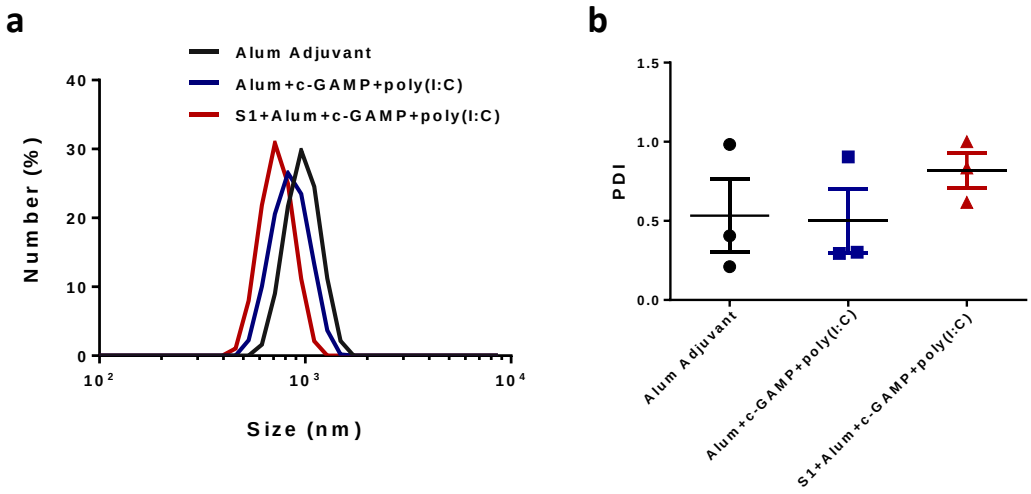

Figure S3. Dynamic light scattering (DLS) analysis of particals. (a) particle size and (b) polymer dispersity index (PDI). Commercial Alum adjuvant, ternary adjuvant system and vaccine formulation were diluted 10 times (10×). Results are shown as the mean ± SEM and are representative of three independent experiments.

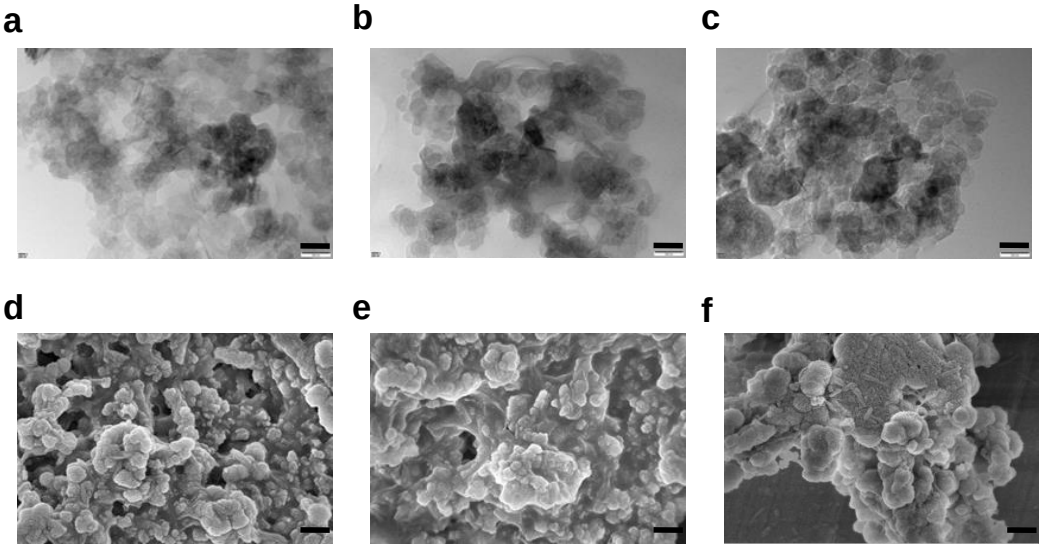

Figure S4. Morphological characterization of particals. Transmission electron microscopy (TEM) of (a) Alum adjuvant, (b) ternary adjuvant system and (c) ternary adjuvant system with added S1 protein. The image scale bars represent 100 nm. Scanning electron microscope (SEM) of (d) Alum adjuvant, (e) ternary adjuvant system, and (f) ternary adjuvant system with S1 protein added. The image scale bars represent 200 nm.

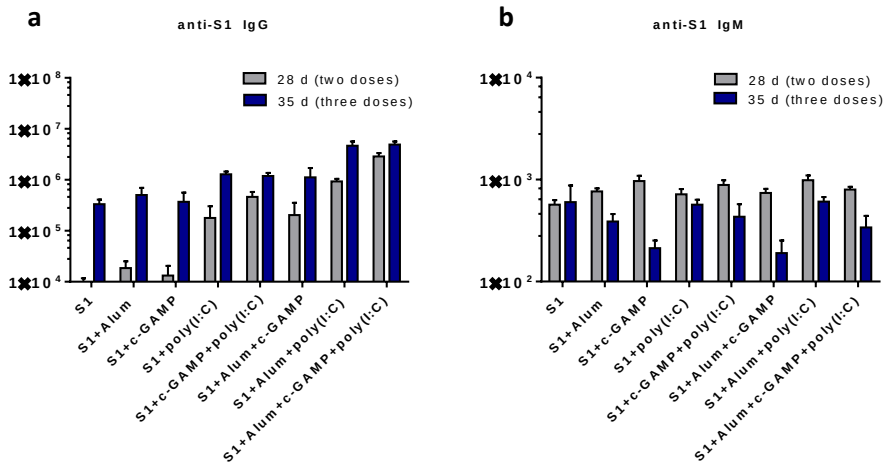

Figure S5. Anti-S1 IgG and IgM antibody titers. (a) S1-specific IgG antibody titers in serum samples from vaccinated mice on 28 d (two doses), and 35 d (three doses). (b) S1-specific IgM antibody titers in serum samples from vaccinated mice on 28 d and 35 d. Results are shown as the mean ± SEM of 5 mice per group and are representative of three independent experiments.

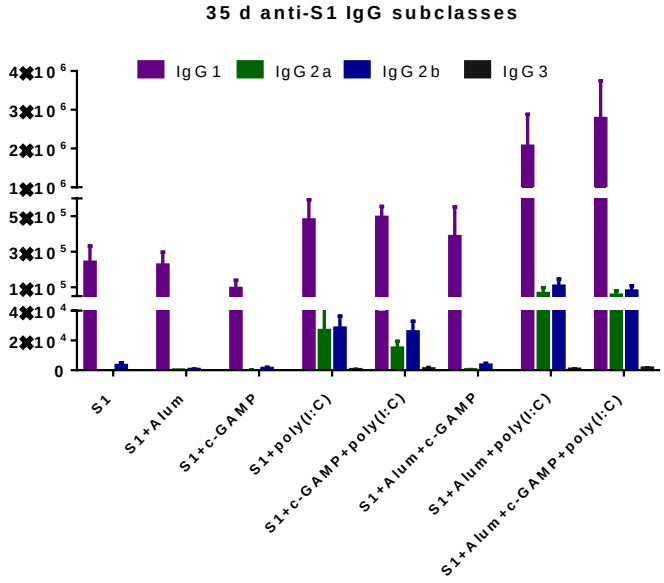

Figure S6. Anti-S1 IgG antibody subclasses on 35 d (three doses). Results are shown as the mean ± SEM of 5 mice per group and are representative of three independent experiments.

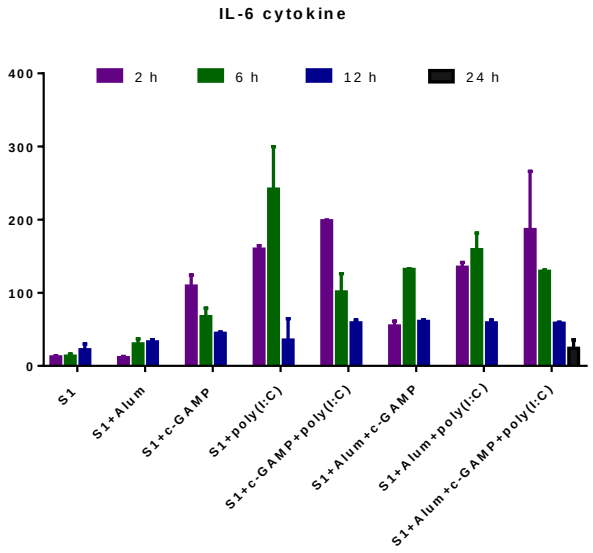

Figure S7. IL-6 secretion in the serum. Mice were treated s.c with different vaccines as indicated. IL-6 cytokine in pooled sera were detected at different time points after a single immunization. Results are shown as the mean ± SEM and are representative of three independent experiments.

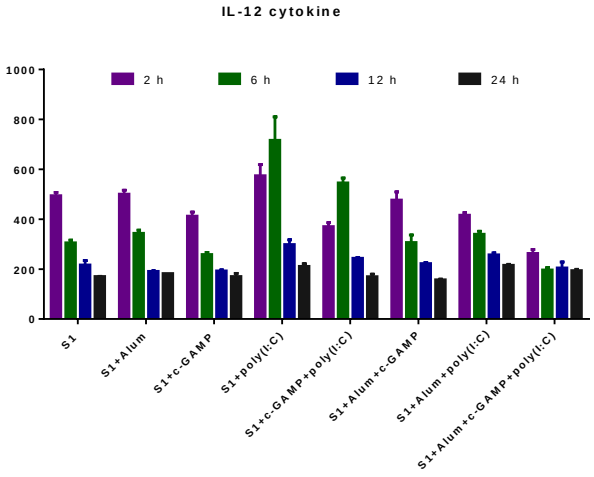

Figure S8. IL-12 secretion in the serum. Mice were treated s.c with different vaccines as indicated. IL-12 inflammatory cytokine in pooled sera were detected at different time points after a single immunization. Results are shown as the mean ± SEM and are representative of three independent experiments.

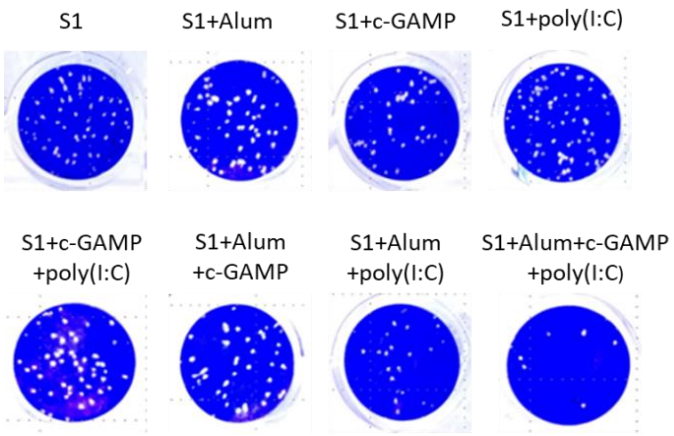

Figure S9. Plaques formed in Vero E6 cells inoculated with live SARS-CoV-2. Neutralizing ability of live SARS-CoV-2 in the presence of 1/400 diluted pooled sera (28 d) from each group was assayed using plaque reduction neutralization test.

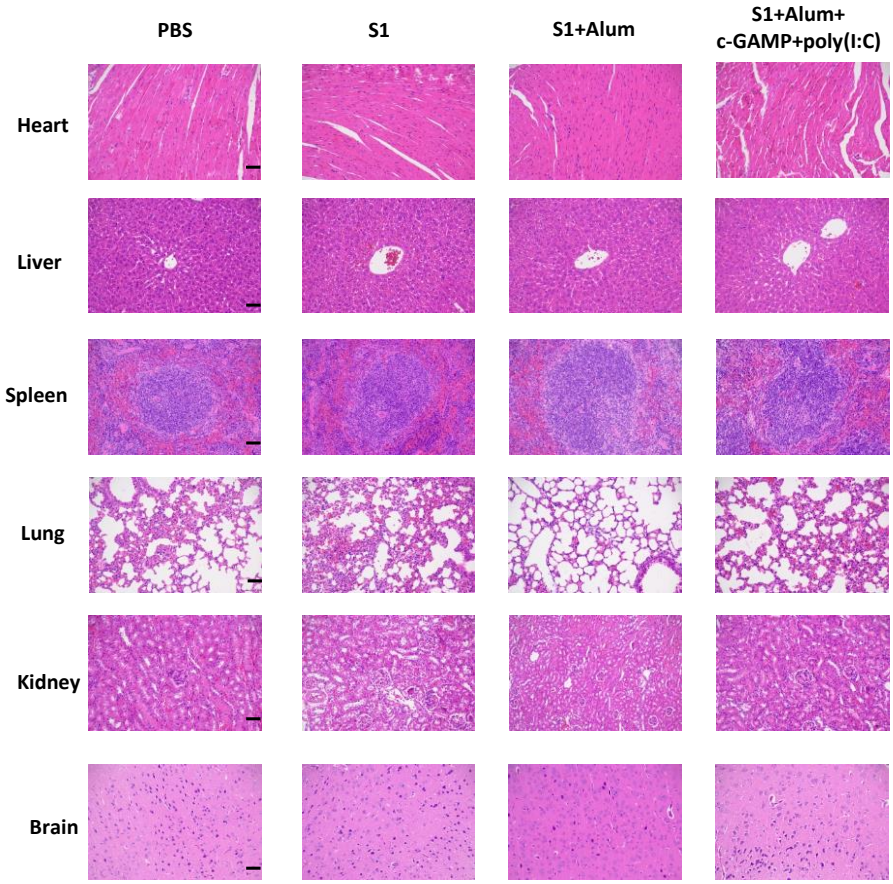

Figure S10. Vaccine biosafety evaluation. Histological sections (H&E staining) of the major organs of immunized mice. Scale bar = 100 μm.

Supplementary methods

Ethics statement

All animals were obtained from Experimental Animal Centre of Huazhong Agriculture University (2020-0084) and were acclimated under standard SPF laboratory conditions. All animal experiments and associated procedures were conducted in accordance with the approved "Guiding Principles in the Care and Use of Animals" and approved by Ethics Review Committee for Life Science Study of Central China Normal University.

Materials

Recombinant spike S1 protein was provided by Jiangsu East-Mab Biomedical TechnologyCo.Ltd (VISC2-S1, His tag). Imject^{TM} Alum Adjuvant was purchased from Thermo Scientific (77161, 40 mg/mL aluminum hydroxide and 40 mg/mL magnesium hydroxide plus inactive stabilizers). The c-GAMP (tlrl-nacga-1) and poly(I:C) (vac-pic) were purchased from InvivoGen.

Adsorption of c-GAMP and poly(I:C) to Alum

Alum adjuvant (600 μL) was vortex mixed with 30 μg of c-GAMP or/and 300 μg of poly(I:C) in 600 μL PBS for 30 min and left to adsorb overnight. The supernatant was collected next day by centrifugation at 10000 rpm for 5 min. The concentrations of c-GAMP and poly(I:C) in the supernatant were determined using a UV spectrophotometer. The supernatant after adsorption of the protein antigen was collected in the same way and the amount of residual protein was determined by the bicinchoninic acid (BCA) method.

Material Characterization

Alum adjuvant, ternary adjuvant system (500 μL/mL Alum adjuvant plus 25 μg/mL c-GAMP and 250 μg/mL poly(I:C) in PBS) or vaccine formulation (500 μL/mL Alum plus 25 μg/mL c-GAMP, 250 μg/mL poly(I:C) and 50 μg/mL S1 in PBS) were characterized in drops on a 400 mesh carbon grid under a FEI Tecnai G2 F30 transmission electron microscope. Again, the above samples were diluted 10 times

and dried by dropping on silicon wafer, plated with gold film and then their morphology was determined under a ZEISS GeminiSEM 300 scanning electron microscope.

Vaccine preparation

Before subcutaneous injection of mice, recombinant spike S1 protein alone or combined with different adjuvants were prepared in 200 μL PBS. Negative group was treated with S1 protein alone (10 μg). In addition, S1+Alum (10 μg S1, 100 μL Alum) was used as a positive control. Same doses of S1 protein (10 μg) was combined with 5 μg c-GAMP (S1+c-GAMP), or 50 μg poly(I:C) (S1+poly(I:C)), or 5 μg c-GAMP plus 50 μg poly(I:C) (S1+c-GAMP+poly(I:C)), or 5 μg c-GAMP adsorbed by Alum adjuvant (S1+Alum+c-GAMP), or 50 μg poly(I:C) adsorbed by Alum adjuvant (S1+Alum+poly(I:C)), or 5 μg c-GAMP and 50 μg poly(I:C) co-adsorbed by Alum adjuvant (S1+Alum+c-GAMP+poly(I:C)).

Immunization of Mice and Sample Collection

Eight groups of mice were vaccinated subcutaneously with different vaccines as indicated two times with two-week intervals. Another 8 groups were vaccinated three times in the same way. Mice serum was collected two weeks after each immunization and splenocytes were collected after the last immunization. Serum was collected by centrifugation (8000 rpm, 4℃) for 15 min, and stored at -30℃.

Enzyme-linked immunosorbent assay of antibody titers

Recombinant S1 protein was used to coat the 96-well plates (Corning 3590) with 10 μg/plate in carbonate buffer (pH 9.6) at 4℃ overnight. Next day, plates were washed three times with PBST (0.05% (v/v) Tween 20 in PBS), and 3% (w/v) BSA in PBS was used to block the ELISA plates at 37℃ for 1 h. After washing three times with PBST, the mice serum, diluted with PBS solution containing 0.1% BSA, were added to plates and incubated at 37℃ for 1 h. Then, plates were washed and incubated with diluted anti-mouse IgG/IgM/IgG1/IgG3/IgG2a/IgG2b HRP-conjugated secondary antibodies at 37℃ for 1 h. Plates were washed five times with PBST, and treated with TMB solution at room temperature in the dark for 5-10 min. Last, the reactions were

stopped with 2M H_2SO_4 and the absorbance (450 nm) was measured by the microplate reader (BioTek, Synergy H1).

Enzyme-linked immunosorbent assay of cytokines

96-well plates were coated with capture antibodies dissolved in the coating buffer and incubated overnight at 4°C. The plates were then washed with PBST and blocked with FBS at rt for 1 h. After blocking, 100 μL/well of standard, sera or control were added and incubated at rt for 2 h. Then, plates were washed and incubated with diluted detection antibody and Sav-HRP reagent at rt for 1 h. After washing steps, the tetramethyl benzidine (TMB) substrate solution was added and protected from light for 30 minutes at rt. The reactions were stopped with 2M H_2SO_4. The absorbance was measured at 450 nm by microplate reader (BioTek, Synergy H1).

ELISpot assay

Spleens of untreated and immunized mice were separated, grinded and filtered through a cell strainer. Next, cells were collected by centrifugation and lysed with lysis solution to remove the red blood cells. Splenocytes suspensions were added to ELISpot plates pre-coated with IFN-γ capture antibody at 10^6 cells/100 μL per well. Then, an overlapping peptide library of spike protein (GenScript, RP30020, Spike glycoprotein (P0DTC2), 1Met-1273Thr, 316 peptides (15 mers with 11 aa overlap) spanning the SARS-CoV-2-S S1) was used to stimulate the cells for 24 h. After incubation, the cells were lysed and biotinylated detection antibody and streptavidin-HRP were added. Plates were treated with AEC solution at 37℃ for 30 min. Last, the reactions were terminated by washing the plates with deionized water and spots were counted using the ELISpot reader after natural drying.

Intracellular cytokine staining (ICS) and flow cytometry

Mouse splenocytes were added to 24-well plates at 10^6 cells per well. The cells were stimulated with the overlapping peptide library about 3 h. Next, monensin and brefeldin A were added to block protein transport and the plates were incubated for 12 h at 37℃ in a 5% CO_2 incubator. Cells were collected by centrifugation and stained with anti-CD3, anti-CD4, and anti-CD8 markers on ice for 30 min. After washing, the

cells were fixed and permeabilized, and stained with anti-TNF-α and anti-IFN-γ markers on ice for 30 min. Cells were analyzed with a CytoFLEX S flow cytometer (Beckman Coulter).

Wild-type pseudovirus neutralization assay

The heat-inactivated mice serum, 2-fold serially diluted with Opti-MEM, was added to plates (Corning 3610). The SARS-CoV-2 spike protein pseudovirus (Yeasen Biotech, Cat: 11906ES50) was diluted with Opti-MEM and incubated with serum samples for 1 h at room temperature. The medium was mixed with an equal volume of pseudovirus as the negative control. HEK293T cells overexpressing ACE2 were counted and the cell concentration was adjusted to 3×10^5 cells/mL with complete medium. After co-incubation with serum samples and pseudovirus, 50 μL ACE2-HEK293T cell suspension was added to each well. After incubation for 48 h, the cells were lysed with lysis solution at room temperature for 15 min and luciferase activity was measured by the Luciferase Reporter Gene Assay Kit (Yeasen Biotech, Cat: 11401ES60). Pseudovirus neutralization ID50 titers (pVNT50) were calculated with 50% relative light units (RLU) compared with the virus control.

Variant pseudovirus neutralization assay

B.1.1.7/alpha (GM-0220PV33), B.1.351/beta (GM-0220PV32), P.1/gamma (GM-0220PV47), B.1.617.2/delta (GM-0220PV45), B.1.1.529/omicron (GM-0220PV84) and wild-type pseudovirus (GM-0220PV07) cross-neutralization assay were carried out. Mouse serum was heat-inactivated and serially diluted and co-incubated with pseudovirus at room temperature for 1 h. Next, the mixture was incubated with HEK293T-ACE2 cells (GM-C09233) per well for 48 h. The luminescence was measured using a Bio-lite Luciferase assay system (GM-040501B) and RLUs were read using a Spark multimode microplate reader (Tecan).

Live virus neutralization assay

Vero E6 cells were added to 24-well culture plate at 1.5×10^5 per well and incubated overnight at 37°C in a 5% CO_2 incubator. The heat-inactivated mice serum, 2-fold serially diluted with 2-DMEM was prepared. An equal volume of SARS-CoV-2

working stock was added and the mixture was incubated at 37°C for 1 h. Then, the cell medium was removed from the 24-well culture plate and the serum-virus mixture was added. Cells infected with SARS-CoV-2 virus were applied as positive control. After incubation for 1 h, the serum-virus mixture was removed from Vero E6 cells and PBS was washed. 0.9% carboxymethyl cellulose was then added and incubated for 3 days at 37°C in a 5% CO_2 incubator. At 3 days post-infection, cells were fixed and stained by 0.5% crystal violet and then rinsed with water. Last, plaques were counted after drying. The PRNT for live SARS-CoV-2 virus was finished in a biosafety level 3 facility at Wuhan Institute of Virology.