Development of a COVID-19 vaccine based on the receptor binding domain displayed on virus-like particles

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Abstract

The recently ermerging disease COVID-19 is caused by the new SARS-CoV-2 virus first detected in the city of Wuhan, China. From there it has been rapidly spreading inside and outside China. With initial death rates around 4%, COVID-19 patients at longer distances from Wuhan showed reduced mortality as was previously observed for the SARS coronavirus. However, the new coronavirus spreads more strongly, as it sheds long before onset of symptoms or may be transmitted by people without symptoms. Rapid development of a protective vaccine against COVID-19 is therefore of paramount importance. Here we demonstrate that recombinantly expressed receptor binding domain (RBD) of the spike protein homologous to SARS binds to ACE2, the viral receptor. Higly repetitive display of RBD on immunologically optimized virus-like particles derived from cucumber mosaic virus resulted in a vaccine candidate (RBD-CuMV$_{TT}$) that induced high levels of specific antibodies in mice which were able to block binding of spike protein to ACE2 and potently neutralized the SARS-CoV-2 virus *in vitro*.

Main

COVID-19 is caused by a novel coronavirus closely related to viruses causing SARS and MERS. As the disease caused by the other two viruses, COVID-19 mainly manifests symptoms in the lung and causes cough and fever[1]. The disease COVID-19 is less severe than SARS and MERS, which is beneficial per se but leads to easier and wider spread of the virus, in particular due to infected individuals with very little symptoms (“spreaders”) and a long incubation time (up to 3 weeks) combined with viral shedding long before disease onset[2]. A vaccine with rapid onset of protection is therefore in high demand for the control of the pandemic that is currently taking its course.

The spike protein of COVID-19 is highly homologous to the spike protein of SARS and both viruses share the same receptor, which is angiotensin converting enzyme 2 (ACE2)[3,4]. The receptor binding domain (RBD) of the SARS spike protein binds to ACE2 and is an important target for neutralizing antibodies[5-7]. By analogy, the RBD of COVID-19 spike protein may be expected to similarly be the target of neutralizing antibodies, blocking the interaction of the virus with its receptor. We have previously shown that antigens displayed on virus-like particles (VLP) induce high levels of antibodies in all species tested, including humans[8]. More recently, we have developed an immunologically optimized VLP platform based on cucumber mosaic virus. These CuMV$_{TT}$ VLPs (hereafter CuMV$_{TT}$) incorporate a universal T cell epitope derived from tetanus toxin providing pre-existing T cell help. In addition, during the production process these VLPs package bacterial RNA which is a ligand for toll-like receptor 7/8 and serves as potent adjuvants[9]. Using antigens displayed on these VLPs, it

was possible to induce high levels of specific antibodies in mice, rats, cats, dogs and horses and treat diseases such as atopic dermatitis in dogs or insect bite hypersensitivity in horses[9-11]. To generate a COVID-19 vaccine candidate, we therefore attempted to display the RBD domain on $CuMV_{TT}$ (Fig. 1a). To this end we gene-synthesized the COVID-19 RBD domain and fused it to an Fc molecule for better expression. As expected, the protein bound efficiently to the viral receptor ACE2 as determined by Sandwich ELISA (Fig. 1b). In a next step, the protein was chemically coupled to the surface of $CuMV_{TT}$ using the well established chemical cross-linkers SATA and SMPH (ref. 9). SDS-Page and Western Blotting confirmed efficient coupling of the RBD-fusion molecule to $CuMV_{TT}$, resulting in the vaccine candidate RBD-$CuMV_{TT}$ (Fig. 1c,d).

To test immunogenicity of the vaccine candidate, mice were immunized three times (weekly schedule) with the RBD-fusion molecule alone or conjugated to the surface of $CuMV_{TT}$ formulated in Montanide adjuvants. As shown in Fig. 2a-c, coupling to VLPs dramatically increased the immunogenicity of the RBD. As shown by ELISA on recombinant RBD, RBD-$CuMV_{TT}$ showed strongly increased immunogenicity at all time-points tested (one week after the vaccine injection time-points). To assess the potential for anti-viral activity, we assessed whether the induced antibodies were able to block binding of the RBD protein to the viral receptor ACE2. As shown in Fig. 3, immune sera obtained after two boosts (day 21) were able to strongly inhibit RBD binding to ACE2.

The best correlate of protection is viral neutralization. To this end, we generated pseudotyped retroviruses[12] expressing the SARS-CoV-2 spike protein and luciferase for quantification of infection (Fig. 4a). Using these viruses, the neutralizing capacity of the sera from immunized mice was assessed on ACE2-transfected cells (Fig. 4b), directly demonstrating high anti-viral neutralizing activity of the induced antibodies. Hence, the RBD-$CuMV_{TT}$ vaccine candidate is able to induce high levels of SARS-CoV-2 neutralizing antibodies. Furthermore, the $CuMV_{TT}$ based vaccine is based on highly efficient expression systems and chemical conjugation technologies, rendering it an attractive candidate for large scale production under cGMP. Previous studies with a similar VLP-based conjugate vaccine has demonstrated that high levels of specific antibodies can be mounted within a week[13,14] (see also Fig. 2a), offering the additional possibilities to rapidly immunize individuals that have been exposed to infected humans or those that are kept in quarantine. Thus, vaccines based on the SARS-CoV-2 RBD domain displayed on VLPs may have the potential to critically interfere with global spread of the virus.

Methods

Protein expression and purification

The SARS-CoV-2 receptor-binding domain (RBD) and the N-terminal peptidase domain of human ACE2 were expressed using 293F cells (Invitrogen). The SARS-CoV-2 RBD (residues Arg319-Phe541) with an N-terminal IL-2 signal peptide for secretion and a C-terminal Fc tag for purification was inserted into pFUSE-mIgG1-Fc2 vector (Invitrogen). The construct was transformed into bacterial DH5α competent cells, and the extracted plasmid was then transfected into 293F cells at a density of 3×10^6 cells/ml using PEI (Invitrogen). The cell culture supernatant containing the secreted RBD was harvested 96 h after infection, concentrated and buffer-exchanged to HBS (10 mM HEPES, pH 7.2, 150 mM NaCl). RBD was captured by protein A resin (GE Healthcare) and eluted with Gly-HCl buffer pH 2.2. Fractions containing RBD were collected and neutralized to pH 7.0 with 1M Tris. For ELISA coating, ACE2 was cleaved from the Fc part using thrombin as described in the manufacturer's manual.

The human ACE2 (residues Ser19-Ser741) with an N-terminal IL-2 signal peptide for secretion and a C-terminal 6×His tag for purification was inserted into pFUSE-vector (Invitrogen). The human ACE2 was expressed by essentially the same protocol used for the SARS-CoV-2 RBD. ACE2 was captured by Ni-NTA resin (GE Healthcare) and eluted with 500 mM imidazole in HBS buffer. RBD was then purified by gel filtration chromatography using the Superdex 200 column (GE Healthcare) pre-equilibrated with HBS buffer. Fractions containing ACE2 were collected.

Serum competitive ELISA

The antibody competitive binding activities of the serum were assayed by ELISA. ACE2 (1ug/ml) was incubated in 96-well plate overnight at 4 °C. After incubation, the plate was blocked with 2% BSA for 2h at 37°C and then washed five times with PBS containing 0.05% Tween 20. BSA was used as negative control followed by the addition of a mixture of 40-fold diluted serum and RBD-mFc (0.15ug/ml) followed by incubation for 30 min with gentle shaking at 37°C. Plates were washed five times with PBS containing 0.05% Tween 20 (PBT) followed by 100 μl of horseradish peroxidase/anti-mFc antibody conjugate (diluted 1:5000 in PBT buffer), incubated 30 min with gentle shaking. Plates were washed five times PBT buffer and developed with 100 μl of freshly prepared 3,3',5,5'-Tetramethylbenzidine (TMB) substrate. Reaction was stopped with 100 μl of 1.0 M H_3PO_4 and read spectrophotometrically at 450 nm in a microtiter plate reader.

Production of $CuMV_{TT}$ VLPs

The production of $CuMV_{TT}$ was described in detail in Zeltins et al.[9] Briefly, *E coli* C2566 cells (New England Biolabs, Ipswich, Mass) were transformed with the $CuMV_{TT}$ coat protein (CP) gene–containing plasmid pETCuMV*tt*. The expression was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The resulting biomass was collected by low-speed centrifugation and was frozen at −20°C. After thawing on ice, the cells were suspended in the buffer containing 50 mM sodium citrate, 5 mM sodium borate, 5 mM EDTA, and 5 mM 2-Mercaptoethanol (pH 9.0, buffer A) and were disrupted by ultrasonic treatment. Insoluble proteins and cell debris were removed by centrifugation (13,000 rpm, 30 minutes at 5°C, JA-30.50Ti rotor, Beckman, Palo Alto, Calif). The soluble $CuMV_{TT}$ CP protein in clarified lysate was pelleted using saturated ammonium sulfate (1:1, vol/vol) overnight at 4°C. Soluble $CuMV_{TT}$ CP–containing protein solution was separated from the cellular proteins by ultracentrifugation in a sucrose gradient (20%-60% sucrose; ultracentrifugation at 25,000 rpm for 6 hours at 5°C [SW28 rotor, Beckman]). After dialysis $CuMV_{TT}$-containing gradient fractions, VLPs were concentrated using ultracentrifuge (TLA100.3 rotor, Beckman; at 72,000 rpm for 1 hour, +5°C) or by ultrafiltration using Amicon Ultra 15 (100 kDa; Merck-Millipore, Cork, Ireland).

Generation of the RBD-$CuMV_{TT}$ vaccine

The RBD was conjugated to $CuMV_{TT}$ using the cross-linker Succinimidyl 6-(beta-maleimidopropionamido) hexanoate (SMPH) (Thermo Fisher Scientific, 10-molar excess, 60 minutes, 23°C). The coupling reactions were performed with 0.3x molar excess of RBD, 0.3x RBD, or equal molar amount of RBD regarding the $CuMV_{TT}$ (shaking at 23°C for 3 hours at 1200 rpm on DSG Titertek; Flow Laboratories, Irvine, United Kingdom). Unreacted SMPH and RBD proteins were removed using Amicon-Ultra 0.5, 100K (Merck-Millipore, Burlington, Mass). VLP samples were centrifuged for 2 minutes at 14,000 rpm for measurement on ND-1000. Coupling efficiency was calculated by densitometry (as previously described for IL17A-CuMVTT vaccine[9]), with a result of approximately 20% to 30%.

Mice

BALB/c mice at the age of 6 weeks were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd and kept at the animal facility of Anhui Agricultural University. All animal experiments were performed in accordance with the National Animal Protection Guidelines and approved by the Chinese Association for Laboratory Animal Sciences.

Vaccination

Six-week-old naive BALB/c mice (3 per group) were immunized by subcutaneous injection with 50 μg of either RBD-$CuMV_{TT}$ or RBD （$CuMV_{TT}$ injected as control). The boosts of the vaccination were conducted at the 2nd and 3rd week. Serum was collected for ELISA analysis 1 week after every vaccination.

Generation of pseudovirus

Pseudovirus expressing the SARS-CoV-2 spike protein was produced by lentivirus second-generation packing system[15]. Plasmids of pwpxl-luc, HIV-1 PSD and pCMV3 containing the SARS-CoV-2 spike gene were co-transfected into 7 x 10^5 293LT cells using Sinofection (Sino Biological). The medium was replaced with fresh DMEM containing 10% FBS after overnight incubation. Supernatants containing pseudovirus were collected 48 h and 72h after transfection and filtered using a 0.45-μm filter syringe. All filtered supernatants were collected together and stored at −80 °C until use.

Titration of pseudovirus

The 293T-ACE2 cells which stably express ACE2 receptors on the cell membrane were prepared by transfection of ACE2 gene into 293T cells using lentivirus system. Pseudoviruses prepared above were added to the 293T-ACE2 cells (3 × 10^4 cells/well) with 100 μl polybrene (16μg/ml). After 48 h, the infection was monitored using the Luciferase Assay System (Promega). Titer was calculated based on serial dilutions of pseudovirus.

Neutralization assay

The mouse serum samples (2 μl) were diluted to 1:10, 1:40, 1:160, 1:640 and 1:2560 respectively, and then mixed with an equal volume of pseudovirus stock. After incubation at 37°C for 1 h, the mixture was inoculated on the 293T-ACE2 cells (3 x 10^4 cells/well). At the same time, pseudovirus+DMEM medium was set as a positive control and DMEM medium only was set as a negative control. After the cells were incubated for 72 hours, serum neutralization was measured by luciferase activity of infected pseudovirus. A cut-off of >80% was used as to determine neutralizing titer.

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Figure 1

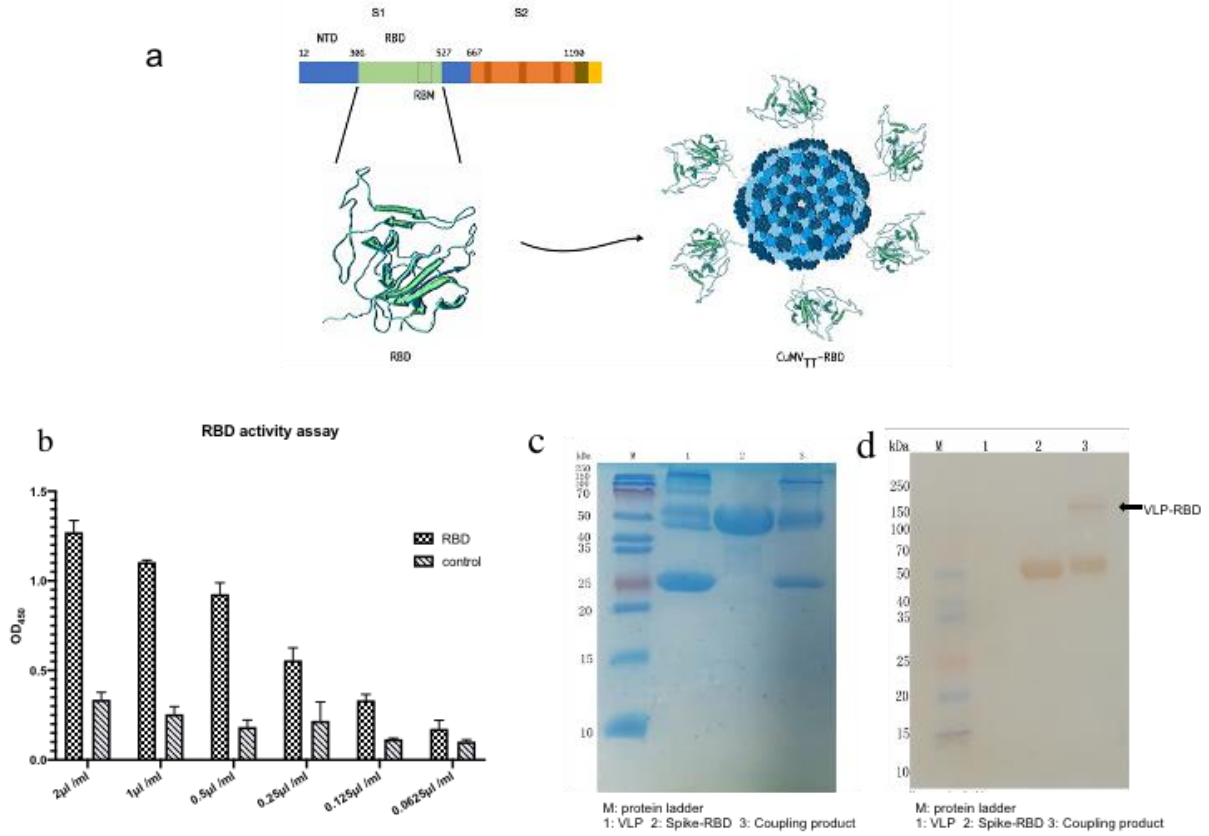

Fig. 1 Production of the SARS-CoV-2 spike-RBD CuMV$_{TT}$ virus-like particle (VLP) vaccine. A) Vaccine design. B) Binding of recombinantly expressed RBD to ACE2 demonstrated by ELISA. C,D) Coupling band (150kd) as shown by C) SDS-PAGE and D) Western blotting of RBD displayed on the surface of CuMV$_{TT}$ VLPs via SATA and SMPH chemical cross-linker.

Figure 2

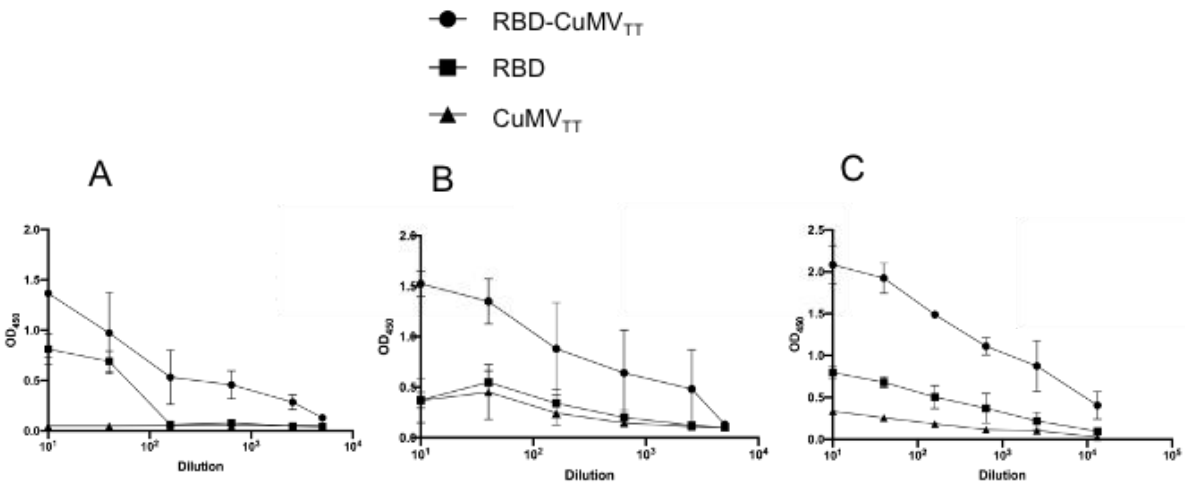

Fig. 2 Vaccination with RBD-CuMV$_{TT}$ induce high specific antibody titers. Mice (3 per group) were immunized with 10 ug of RBD-CuMV$_{TT}$, RBD alone or CuMV$_{TT}$ alone, formulated in Montanide, on days 0, 7 and 14. Serum was harvested on A) day 7, B) day 14 and C) day 21 and tested by ELISA on recombinant RBD. Strong antibody responses could be observed already 1 week after immunization with RBD-CuMV$_{TT}$.

Figure 3

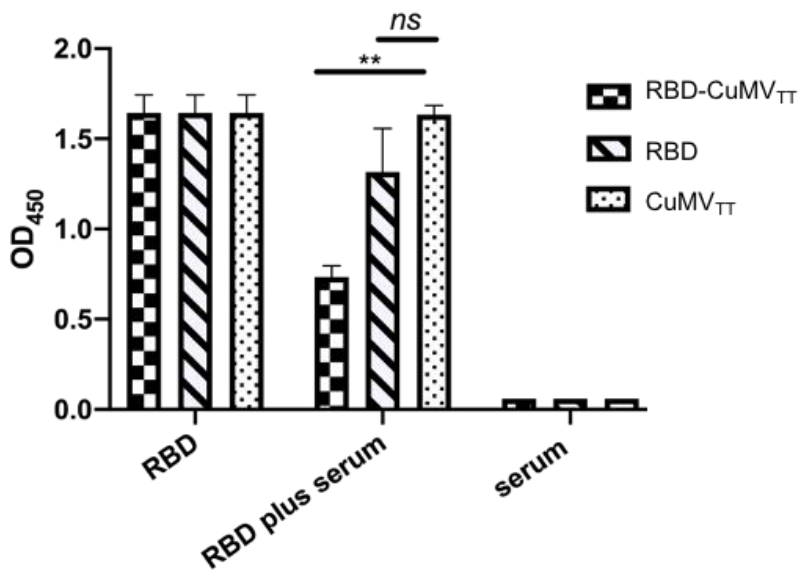

Fig. 3 Immunization with RBD-$CuMV_{TT}$ induces antibodies that block binding of RBD to ACE2. Mouse sera (1:40 dilution, day 21) after the 3rd vaccination were incubated with RBD, then placed on the ACE2 coated plate. RBD binding was determined by ELISA.

Figure 4

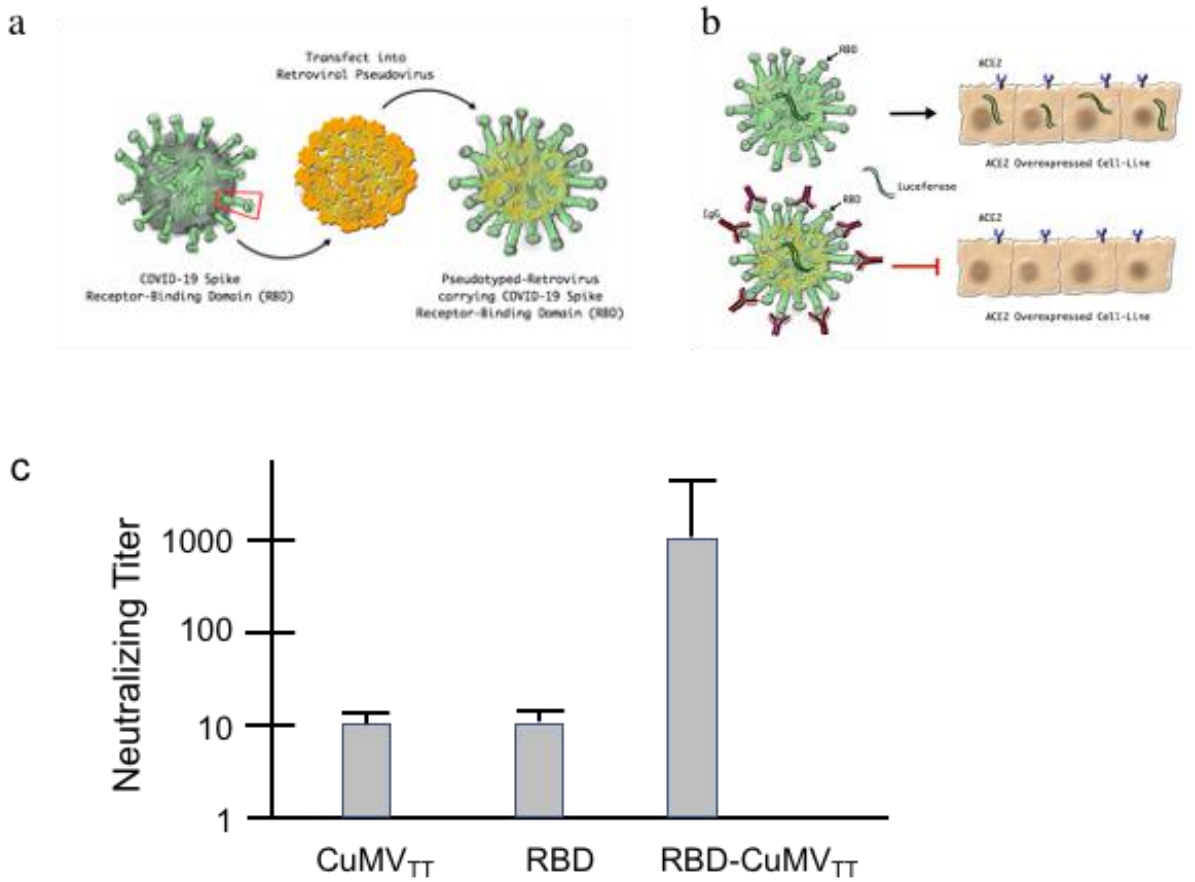
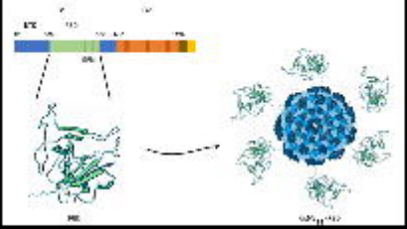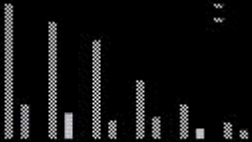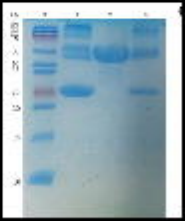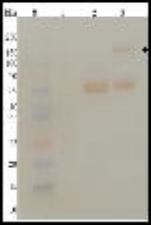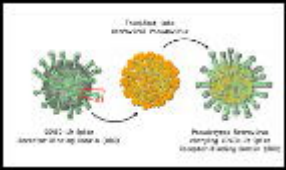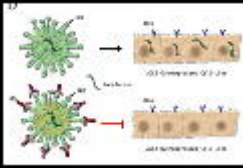
Fig. 4 Serum neutralization assay. Pseudovirus expressing the SARS-CoV-2 spike protein was generated by co-transfection of the plasmids of luciferase-expressing HIV pseudoparticles into HEK 293 T cells using second-generation lentiviral packaging system. A,B）Schematic presentation of the principle of the assay. C) Pseudovirus expressing the SARS-CoV-2 spike protein can infect the cells via the ACE2 receptor, and antibodies induced by RBD-CuMV$_{TT}$ (day 21) potently neutralize the virus.