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## Harnessing coronavirus spike proteins' binding affinity to ACE2 receptor through a novel baculovirus surface display system

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### ABSTRACT

Coronavirus disease 2019 (COVID-19) caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a newly emerging infectious disease currently spreading across the world. The spike (S) protein plays a key role in the receptor recognition and cell membrane fusion, making it an important target for developing vaccines, therapeutic antibodies and diagnosis. In this study, we constructed a baculovirus surface display system that efficiently presents both SARS-CoV and SARS-CoV-2 S proteins (including ectodomain, S1 subunit and receptor-binding-domain, RBD) on the surface of recombinant baculoviruses, utilizing transmembrane anchors from gp64 (signal peptide) and vesicular stomatitis virus (VSV). These recombinant baculoviruses were capable of transducing engineered HEK 293T cells overexpressing ACE2 receptors with significantly higher transduction efficiencies, indicating that S proteins displayed on baculovirus surface have antigenicity and can recognize and bind ACE2 receptors. Additionally, the transduction of SARS-CoV-2 S proteins can be inhibited by an antibody against the SARS-CoV-2 RBD. These results demonstrate that this baculovirus surface display system is a promising tool for developing antibodies, vaccines and recombinant protein production.

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### 1. Introduction

Since December 2019, the outbreak of coronavirus disease 2019 (COVID-19) caused by the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has quickly become a public health challenge of global concern, and has since been extensively studied around the world. Among them, the coronavirus spike protein (hereinafter referred to as S protein) is an important target for the development of vaccines, therapeutic antibodies, and diagnosis. The S protein is a trimeric class I fusion protein existing on the surface of the virus [1]. It is responsible for the fusion process between viral membrane and the host cell membrane [2], and also a major target for host immune defense during the viral infection [3]. The membrane of the host cell contains a receptor named angiotensin-converting enzyme 2 (ACE2), which can be recognized and bound specifically by the S protein through its receptor-

binding-domain (RBD) [4,5]. When the S protein binds to the ACE2 receptor, it undergoes a conformational change during which the S1 subunit is shed off and the two heptapeptide repeats of the S2 subunit form a six-helix bundle, therefor shortening the distance between viral and host cell membranes and facilitating the virus-cell fusion [6–8]. Recent studies demonstrated that both SARS-CoV and SARS-CoV-2 invade host cells mainly through the ACE2 receptor [9,10].

The baculovirus-insect cell system has been widely used to express foreign recombinant proteins, and is well-known as a safe, efficient, convenient and economical expression system. The baculovirus surface display system is a specialized eukaryotic surface display technology, which commonly uses *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) envelope glycoprotein gp64 to display the heterologous recombinant protein. In such a system, the target protein is directly fused to the C-terminus of the gp64 protein or fused between the signal peptide (SP) sequence and the transmembrane domain (TM) of the gp64, which help direct and display target proteins onto the surface of recombinant baculoviruses [11–13]. In 2002, Chapple et al. reported an

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alternative strategy that gp64 TM was replaced by the transmembrane domain and cytoplasmic terminal domain (TM&CTD) of VSV-G (vesicular stomatitis virus G) protein for higher display efficiency [14,15]. To date, hundreds of proteins have been successfully displayed with correct folding and proper post-translational modification, and some has been proved to be very effective immunogens [16,17].

This work uses the baculovirus surface display system to display S proteins of SARS-CoV and SARS-CoV-2 (including the S protein fragments that can interact with ACE2 receptor), and the resulting baculoviruses showed significantly higher transduction efficiencies. Further, an antibody against the SARS-CoV-2 RBD was shown to effectively inhibit the transduction efficiency of this display system. Our display system together with an engineered HEK 293T cell line overexpressing ACE2 receptors (hereinafter referred to as ACE2-293T cells), will have a wide range of applications in screening antiviral antibodies and developing new Bac-Mam systems featured by much higher transduction efficiency and protein yield.

## 2. Materials and methods

### 2.1. Cells and reagents

The insect cell line used in this study was *Spodoptera frugiperda* 9 (Sf9). Sf9 cells were cultured in shaker flasks (CORNING) at 27 °C with Sf-900 II serum-free medium (Sf-900 II SFM; Thermo Fisher), and suspension cultures were kept at a 27 °C incubator while shaking at 120 rpm. Recombinant baculoviruses were generated and amplified in Sf9 cells. Mammalian cells used for transduction were human embryonic kidney cells 293T (HEK 293T), which were cultured at 37 °C with 5% CO<sub>2</sub> in DMEM medium (Thermo Fisher), supplemented with 10% FBS (Thermo Fisher). ACE2-293T cells were obtained from the Lab of Phenotypic Screening, SIAIS, ShanghaiTech University.

### 2.2. Plasmids construction

DNA sequence of the SARS-CoV S protein was obtained from GenBank database (MW560955), as well as the SARS-CoV-2 S protein (MW560963). The plasmids expressing ectodomains (ECDs) of SARS-CoV and SARS-CoV-2 S proteins, the S1 subunits and the RBD regions were constructed separately using the pFast-Bac Dual vector of the Bac-to-Bac baculovirus expression system (Thermo Fisher). We attempted two strategies to display S proteins on baculovirus surface, one was to place proteins of interest between the GP64 signal peptide 1 (SP1, 1–20 aa) and the GP64 mature domain (MD, 21–512 aa), and the other was to place proteins of interest between the GP64 signal peptide 2 (SP2, 1–34 aa) and the VSV-G TM&CTD (441–511 aa) (Fig. 1).

All DNA sequences were amplified by PCR. The recombinant vectors were constructed using pFastBac Dual vector and under the regulation of baculovirus polyhedrin (polh) promoter. A general subcloning procedure is as follows: first, GP64 SP1 was inserted into pFastBac Dual vector using BamHI/EcoRI sites. Then, GP64 MD was inserted using NotI/XbaI sites. Finally, the S proteins were inserted using the EcoRI/NotI sites to construct the SP1–S–MD–pFastBac Dual vector. Also, a FLAG tag was introduced between GP64 SP1 and the S proteins for subsequent analysis. The same protocol was used to construct SP2–S–VSV G–pFastBac Dual vectors. Ligation was performed using the In Vitro Recombination Cloning Kit (Vazyme). All recombinant plasmids were verified by sequencing.

Notably, SARS-CoV S-ECD and SARS-CoV-2 S-ECD sequences were subcloned into both SP1–S–MD–pFastBac Dual and SP2–S–VSV

G–pFastBac Dual, while SARS-CoV S1&RBD and SARS-CoV-2 S1&RBD were only subcloned into the SP2–S–VSV G–pFastBac Dual.

To determine whether the recombinant S proteins were displayed on baculovirus surface properly (i.e. the recombinant baculoviruses were capable of transducing mammalian cells more efficiently), a CMV-EGFP-WPRE expression cassette was inserted into SP2–S–VSV G–pFastBac Dual vectors.

### 2.3. Generation and expression of recombinant baculoviruses

Recombinant plasmids derived from pFastBac Dual were used to generate corresponding baculoviruses. These plasmids were first introduced into the DH10EMBaY (Y indicates for eYFP, which can be used as virus performance maker) competent cells (from the MultiBac system) [19] to generate recombinant bacmids. After blue/white colonies selection, recombinant bacmids were isolated from single white clones and then be used to transfer Sf9 cells.

Sf9 cells were seeded in 6-well plates (CORNING) and transfected with FuGENE HD Transfection Reagent (Promega) in Sf-900 II serum-free medium. After incubation for 72 h at 27 °C, recombinant baculoviruses were harvested from the supernatant of the transfection cell culture and amplified with 2–3 rounds of infections.

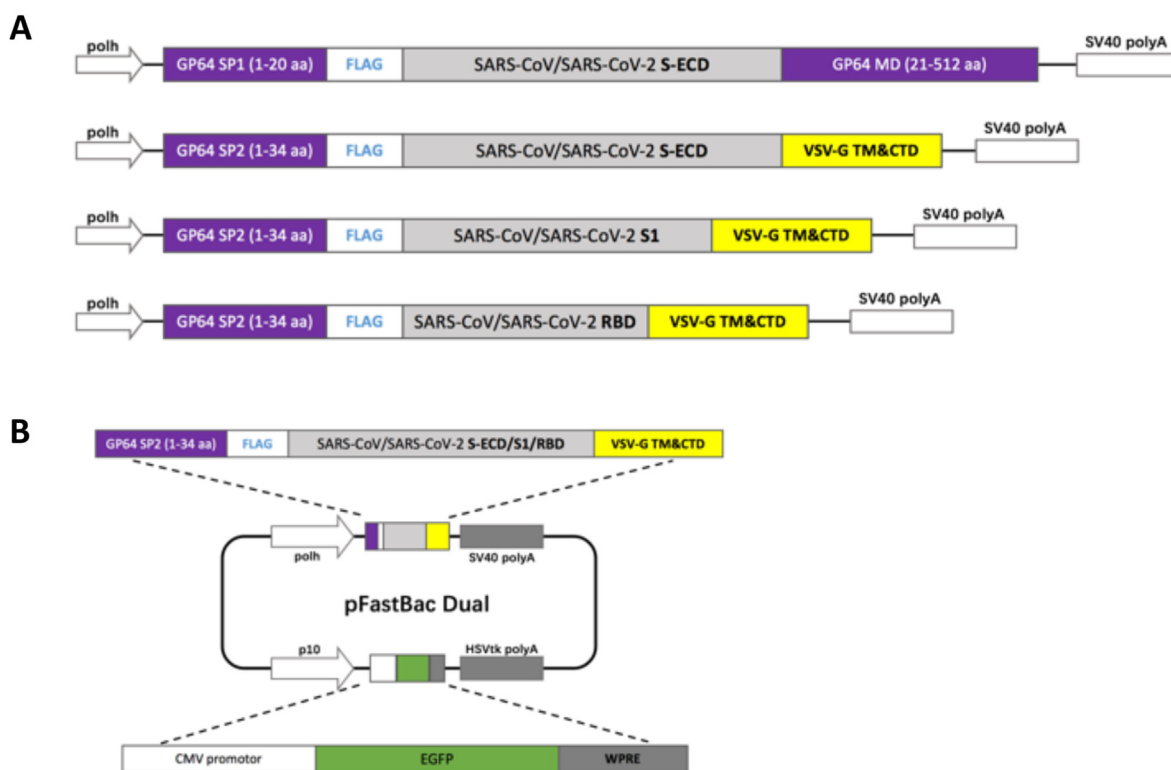
### 2.4. Western blot analysis

Infected Sf9 cell culture was harvested 72 h after infection, cell culture was centrifuged at 500 g for 5 min to separate supernatant (containing budded baculoviruses) and cell pellet. Then, the supernatant was centrifuged at 120,000 g for 2 h to pellet the virus particles. After the centrifugation, the supernatant was discarded and the virus pellets were resuspended with phosphate buffered saline (PBS, BioVision) buffer. Both samples from infected cells and baculoviruses were analyzed by western blot (anti-FLAG).

Samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Life technology), followed by electroblotting to a polyvinylidene difluoride (PVDF, Merck Millipore) membrane. The membrane was blocked in Tris-buffered saline (TBS, Sangon) with 0.1% Tween20 (Sigma) (abbreviated as TBST) supplemented with 5% non-fat milk for 2 h at room temperature (RT), and then incubated with HRP-conjugated Flag Tag Mouse Monoclonal Antibody (Proteintech, 1:10000 v/v dilution) at 4 °C overnight. After washing three times in TBST, the anti-FLAG protein band was developed with Clarity Western ECL Substrate (Bio-Rad), then detected and imaged by a Bio-Rad Imaging System. The target band was analyzed by Image Lab.

### 2.5. Cell surface immunofluorescence

Sf9 cells were seeded in a 12-well plate (CORNING) and infected with recombinant baculoviruses at 27 °C for 72 h. The cell suspension removed from the 12-well plate was washed with PBS and then fixed with 4% paraformaldehyde for 15 min at RT. Fixed cells were incubated with anti-SARS-CoV-2-Spike-RBD antibody (expressed with human Fc, from the Lab of Phenotypic Screening, SIAIS, ShanghaiTech University) for 1 h at RT, and then washed three times with PBS. The cells were incubated subsequently with Alexa555 conjugated secondary antibody that recognizes human Fc (1:1000 v/v dilution, Life technology) for 1 h at RT, and washed three times with PBS. Then the cell membrane was counterstained by Alexa Fluor 647 dye (1:500 v/v dilution, Thermo Fisher) as the control. After incubating at RT for 45 min, the cells were washed three times and resuspended with PBS. Finally, the cells were attached to a glass slide using a cytospin (Thermo Fisher) and visualized under a confocal microscope (Leica). Additionally, the



**Fig. 1. Construction of baculovirus expression vectors. (A)** Strategies of constructing expression vectors encoding fusion proteins. **(B)** Schematic representation of expression vectors for displaying spike proteins, including the expression cassette of the eGFP protein as a marker.

Alexa555 fluorescence signal was confirmed by flow cytometry analysis.

## 2.6. Flow cytometry analysis

Recombinant baculoviruses were used for transduction of both HEK293T cells and ACE2-293T cells using a 48-well plate (CORNING) at 37 °C with 5% CO<sub>2</sub> for 12 h. Then the supernatant was discarded and fresh DMEM medium was added to the corresponding wells. After another 24-h cultivation, cells were collected and resuspended with ice-cold FACS buffer (PBS, 0.05% BSA and 2 mM EDTA). To determine if the transduction was successful, the eGFP protein was introduced to the expression system (as previous described in Plasmids Construction). Therefore, cells that were successfully transduced would express eGFP proteins which can be analyzed by a flow cytometer (CytoFLEX S, Beckman Coulter) and be used to measure the transduction efficiency.

## 3. Results

### 3.1. Construction of recombinant baculoviruses displaying spike proteins from SARS-CoV and SARS-CoV-2

The recombinant baculovirus surface display vectors expressing S proteins of SARS-CoV and SARS-CoV-2 were constructed as described in Materials and Methods (2.2). SARS-CoV and SARS-CoV-2 S protein ectodomains (ECDs) were inserted between signal peptide1 (SP1) and mature domain (MD) of gp64 protein individually. The transmembrane and cytoplasmic terminal domain (TM&CTD) of VSV-G protein was previous confirmed as a successful anchor leading to non-polar distribution of displayed peptides, and with higher expression efficiency comparing to gp64 MD [14]. Therefore, various truncations of S proteins from both SARS-CoV

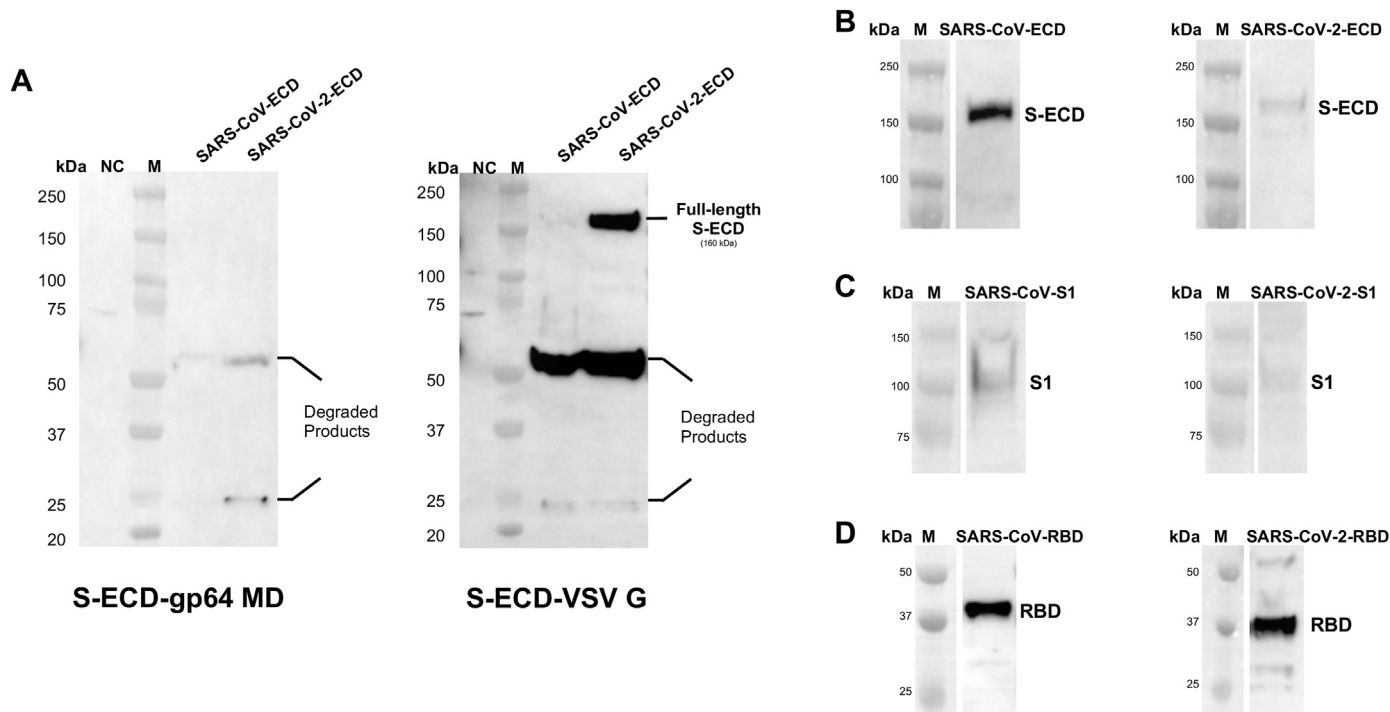
and SARS-CoV-2, including ECD, S1 and RBD were inserted between gp64 SP2 and VSV-G TM&CTD. These constructs were used to generate recombinant AcMNPV bacmids, in which protein encoding genes are under the control of polh promoters (Fig. 1A).

The eGFP protein expression cassette (CMV-EGFP-WPRE) was also inserted into pFastBac Dual vectors downstream of the p10 promoter to determine whether the recombinant baculoviruses enter the mammalian cells successfully. (Fig. 1B).

### 3.2. Comparison of recombinant baculoviruses expressing spike proteins fused with the GP64 mature domain or VSV-G

To investigate whether the S proteins could be successfully displayed in the surface of the virus particles, after transfection and two rounds of infection, the recombinant baculoviruses were collected from the supernatant of infected Sf9 cell culture and verified by Western blot analysis using the anti-FLAG antibody. In results of S-ECD fused with gp64 MD, FLAG signals can be detected in two bands of ~24 kDa and ~55 kDa by western blot in SARS-CoV and SARS-CoV-2, indicating that target proteins were expressed but degraded almost completely (full-length protein should result in a band of ~200 kDa). It is possible that the proteolytic sites are at the N-terminus of the ectodomains of both S proteins. Moreover, the weak bands indicate that the expression level is low. Further, the expression or integration level of the S protein of SARS-CoV may be significantly lower than that of SARS-CoV-2 (Fig. 2A-Left).

In contrast, when S-ECD was fused with VSV-G TM&CTD, Western blot signals can be clearly detected in three bands of ~24 kDa, ~55 kDa and ~160 kDa by in both pelleted baculoviruses displaying the S protein of SARS-CoV or SARS-CoV-2. Notably, the expression levels of both S proteins fused with VSV-G TM&CTD are significantly higher than those fused with gp64 MD. Although the expression level of the full-length S protein of SARS-CoV was still



**Fig. 2. Anti-FLAG Western blot analyses of recombinant baculoviruses expressing S proteins.** (A) Comparison between recombinant S proteins fused with gp64 mature domain and VSV-G. **Left:** Recombinant S-ECD fused with gp64 mature domain. Degraded products of S proteins were detected (~24 kDa and ~55 kDa). **Right:** Recombinant S-ECD fused with VSV-G TM&CTD. Full-length S-ECD proteins (~160 kDa) were detected and degraded products of S proteins were also detected (~24 kDa and ~55 kDa). (B–D) Anti-FLAG Western blot analyses of recombinant baculoviruses expressing S proteins fused with VSV-G: (B) Expression of S-ECD (~160 kDa) was detected. (C) Expression of S1 (~100 kDa) was detected. (D) Expression of RBD (~40 kDa) was detected. NC: negative control (insect cells infected by baculoviruses without DNA insertions); M: Marker; each gel sample contains ~1 × 10<sup>9</sup> viruses/lane.

much lower than that of SARS-CoV-2, indicating that the full-length S protein of SARS-CoV was expressed but almost degraded completely. The detected full-length S-ECD fusion protein runs at a slightly higher position than their predicted MWs (SARS-CoV: 144 kDa; SARS-CoV-2: 148 kDa), suggesting post-translational modifications such as glycosylation (Fig. 2A-Right).

The Western blot results of those two kinds of fusion proteins (with gp64 MD or VSV-G TM&CTD) were consistent with previously studies [12,14] that fusion proteins containing the entire gp64 were expressed in fairly low level, comparing to constructs utilizing VSV-G TM&CTD for membrane anchorage.

### 3.3. Verification of spike proteins on the surface of recombinant baculoviruses

Based on the results above, we constructed expression plasmids encoding SARS-CoV or SARS-CoV-2 S-ECD, S1 and RBD between gp64 SP2 and VSV-G TM&CTD. Additionally, the eGFP protein was introduced into the expression system as previous described. Worth to mention that we used these plasmids for subsequent experiments. The proper expression of different S protein fragments was verified by Western blot analysis using the anti-FLAG antibody.

As shown in Fig. 2B–D, FLAG signals of S-ECD proteins were detected in bands at ~160 kDa, which is consistent with previous results. FLAG signals of S1 and RBD proteins were detected separately at ~100 kDa and ~40 kDa, which are also a slightly larger than their predicted MWs (S1: 88 kDa; RBD: 38 kDa), suggesting the same post-translational modifications (glycosylation) as in the case of S-ECD. The expression levels of RBD from both SARS-CoV and SARS-CoV-2 were the highest and the most stable among all,

indicating that shorter fusion proteins lead to higher expression levels.

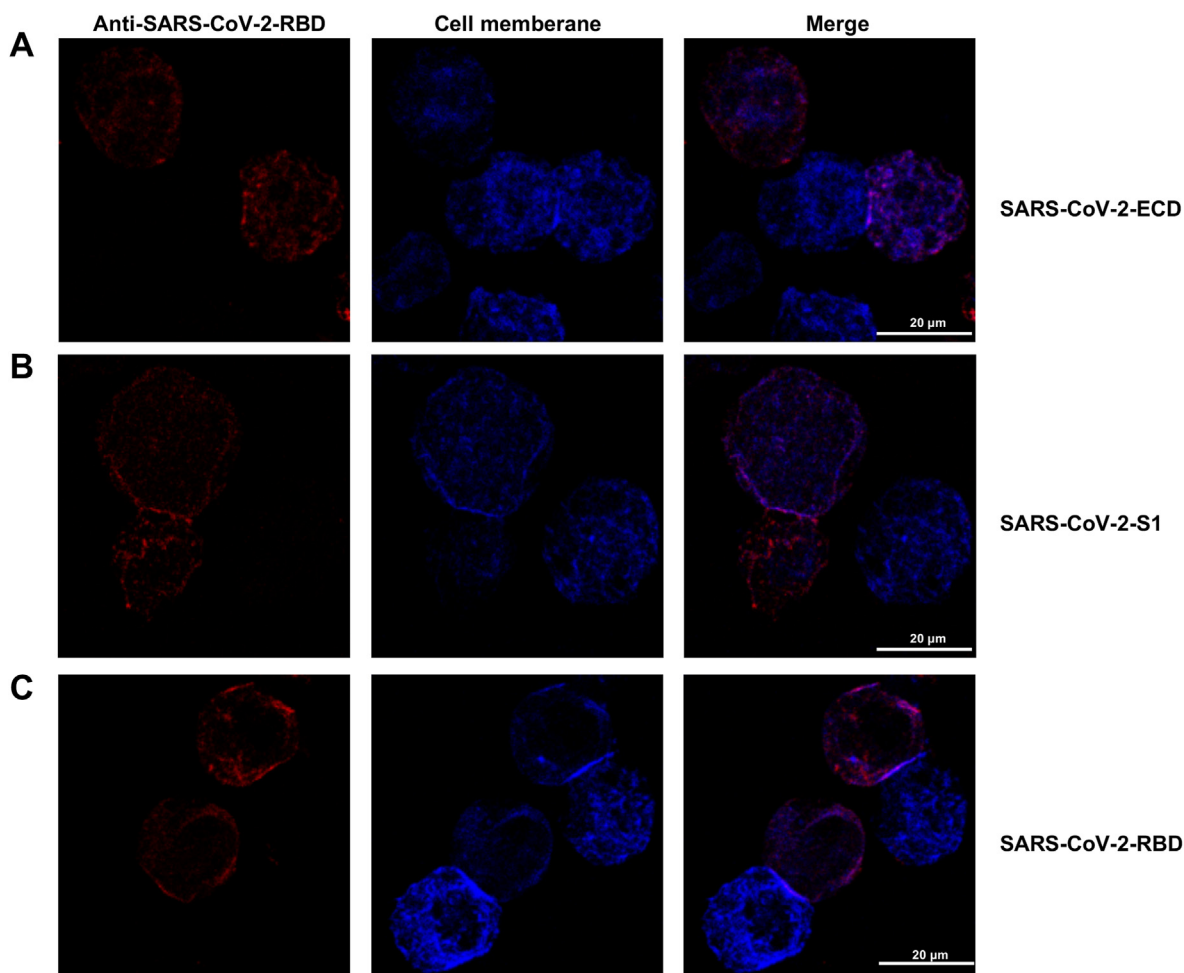
### 3.4. Display of spike proteins on the surface of infected cells

Apart from displaying heterologous proteins on the surface of budded viruses, recombinant baculoviruses can also display foreign proteins on the surface of infected insect cells [18]. With an appropriate signal peptide and a transmembrane domain, such as the full-length gp64 (including SP and MD) or a truncated version (only the SP part) fused with VSV-G, a membrane bounded foreign protein can be expressed and translocated to the surface of infected host cells through recombinant baculoviruses.

To verify if our recombinant baculoviruses can infect insect cells and display the S protein on the surface of Sf9 cells, cell surface immunofluorescence assays were performed. After infection, Sf9 cells were incubated with anti-SARS-CoV-2-Spike-RBD antibody and then a Alexa555 conjugated secondary antibody, which can be detected using 555 nm excitation light (red signals in Fig. 3), and also a cell membrane dye Alexa Fluor 647 for counterstaining (blue signals in Fig. 3). The anti-SARS-CoV-2-Spike-RBD antibody specifically recognizes the RBD region of SARS-CoV-2 S protein. Red signals (corresponding to the S proteins) can be observed in Sf9 cells infected by recombinant baculoviruses displaying SARS-CoV-2-S-ECD (Fig. 3A), S1 (Fig. 3B) and RBD (Fig. 3C), indicating that the ECD, S1 and RBD of SARS-CoV-2 S protein were expressed, translocated to host cell membrane and can be recognized by the antibody effectively.

In addition, the anti-SARS-CoV-2-Spike-RBD antibody was also incubated with Sf9 cells infected by recombinant baculoviruses displaying SARS-CoV-S-ECD, S1 and RBD, but no red signals can be





**Fig. 3.** Detection of S proteins in Sf9 cells infected with recombinant baculoviruses by cell surface immunofluorescence. (A) Sf9 cells infected by baculoviruses encoding SARS-CoV-2-ECD. (B) Sf9 cells infected by baculoviruses encoding SARS-CoV-2-S1. (C) Sf9 cells infected by baculoviruses encoding SARS-CoV-2-RBD (red fluorescent signal represents S proteins, blue fluorescent represents cell membrane, and scale bar = 20 µm). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

observed (Fig. S1), suggesting that the specificity of the anti-SARS-CoV-2-Spike-RBD antibody is very high.

### 3.5. Determination of transduction efficiency of ACE2-293T cells

To evaluate the transduction efficiency of S protein displaying recombinant baculoviruses, HEK 293T cells and ACE2-293T cells were transduced with baculoviruses displaying ECD, S1 and RBD of both SARS-CoV and SARS-CoV-2, then corresponding cells were analyzed by flow cytometry. The green fluorescence was only detectable when baculoviruses transduced into the HEK 293T or ACE2-293T cells successfully through FITC channel.

The results showed in Fig. S2A indicate that baculoviruses displaying SARS-CoV-ECD and SARS-CoV-2-ECD both can transduce HEK 293T and ACE2-293T cells. With ACE2-293T cells, the transduction efficiency was about two times higher than HEK 293T cells (SARS-CoV-ECD, HEK 293T: 25.3%, ACE2-293T: 42.1%; SARS-CoV-2-ECD, HEK 293T: 15.1%, ACE2-293T: 30.7%). Moreover, when the anti-SARS-CoV-2-Spike-RBD antibody was added during transduction, the ACE2-293T cells transduction efficiency decreased to 16.5%, which is about the same transduction level as HEK 293T cells, and the transduction efficiency of HEK 293T cells is not significantly different, suggesting that there was no contribution from S protein in the HEK 293T transduction process and the S protein-ACE2-

binding mediated transduction could be blocked by anti-SARS-CoV-2-Spike-RBD antibody. When the displayed S protein was in the truncation form of S1 or RBD, the results was consistent with those of the ECD (Figs. S2B and C). These results confirmed that the S proteins displayed on recombinant baculovirus surface have antigenicity, and the specific recognition and binding mechanism between S proteins and ACE2 receptor may benefit for the transduction process.

## 4. Discussion

Baculovirus surface display technology is one of the most innovative applications of the baculovirus expression system, which has been widely used to produce heterologous proteins in cultured insect cells. Similar to other baculovirus expressing systems, it also features high-level recombinant protein production, proper eukaryotic post-translation modification, large insertion capacity, low cost, and limited host range that makes it safer than many mammalian viruses. Therefore, baculovirus surface display technology has been applied in a wide range of fields, the virion can be used as a substitution of human-infectious virus, and the displayed heterologous proteins can be used as effective vaccines, or be used as specific ligands to enhance the transduction efficiency of mammalian cells.

In this study, we successfully displayed the S proteins (including ectodomain, S1 subunit and RBD region) of SARS-CoV and SARS-CoV-2 on recombinant baculovirus and Sf9 cells. Baculoviruses with S protein also exhibited capability to specifically recognize and interact with ACE2 receptor and enhance the transduction efficiency of ACE2-293T cells, suggesting that the recombinant baculoviruses displaying S proteins of SARS-CoV and SARS-CoV-2 had biological activity and could be applied in screening of antibodies. Considering the low toxicity of baculoviruses, the risk would be much lower than other mammalian viruses. Additionally, the displayed S protein of SARS-CoV-2 can be blocked by a specific antibody against SARS-CoV-2 S protein RBD during the transduction process, indicating that it has antigenicity and could also be used to evaluate the efficiency of candidate antibody. Another promising application of the baculovirus surface display system is that based on the specific affinity between S protein and ACE2 receptor, it can effectively enhance transduction efficiency with the mammalian cells and the recombinant foreign protein production, suggesting a more efficient approach to overproduce foreign proteins in mammalian cells.

The outbreak of SARS-CoV-2 has posed a severe pandemic threat and continues to grow and spread throughout the world and had led to over 460 million confirmed cases and more than 6 million deaths by this time. It is still a heavy burden for the healthcare system and economy in many nations. Our study implies good antigenicity of recombinant S proteins (especially the RBD region), which is a crucial vaccine target, and this baculovirus surface display system provides a practical and safe platform for novel antibody and vaccine research and development.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2022.03.062>.

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