Supporting Information

for Small, DOI: 10.1002/smll.202004237

Severe Acute Respiratory Syndrome Coronavirus-2 Spike Protein Nanogel as a Pro-Antigen Strategy with Enhanced Protective Immune Responses

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Supporting Information

**SARS-CoV-2 Spike Protein Nanogel as a Pro-antigen Strategy with Enhanced Protective Immune Responses**

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Materials and methods

1. Materials

Anti-His antibody (ZSGB Bio)
Anti-spike antibody (Sino Biological 40150-R007)
Anti-spike antibody (Beijing Qianzhao Xinye Biology Science and Technology Co., Ltd)
Crosslinker 1 (Thermo PG82081)
Crosslinker 2 (Xi’an Ruixi Bio)
Anti-hACE2 antibody (Sino Biological, 10108-T24)
ACE2-hFc (Sino Biological, 10108-H02H)
SARS S1 subunit (Sino Biological 40150-V08B1)
HRP-conjugated goat anti-mouse IgG (ZSGB Bio)
HRP-conjugated goat anti-rabbit secondary antibody (ZSGB Bio)
HRP-conjugated mouse anti-human IgG-Fc (Sino Biological, 10702-MM01T-H)
AF546 conjugated goat anti-rabbit IgG (H+L) secondary antibody (Invitrogen, A11010).
Cy5-NHS (Lumiprobe, 43320)
Aluminum hydroxide adjuvant (Imject™ Alum Adjuvant, Thermo Fisher 77161)
TMB (Solarbio, PR1200)
Spike-PV-Luc (GENEWIZ)
Spike-PV-GFP (GENEWIZ)

2. Methods

Construction, expression, and purification of S-RBD The coding sequence of the SARS-CoV-2 receptor binding domain (S-RBD, aa319-541) was codon optimized and cloned into the yeast expression vector pPICZαA between XhoI (5') and NotI (3') with a C-terminal polyhistidine tag. The expression plasmid was linearized by Scal digestion and transformed into Pihcia pastoris strain. Transformants were selected for zeocin resistance, and secretion of S-RBD by the transformants was screened and assessed. High expression clones were used for subsequent scale-up expression according to previous reports. After fermentation, the supernatant was collected. S-RBD was first purified from the supernatant using a HisTrap Column (GE) and then further purified by size exclusion chromatography. The integrity and purity of S-RBD were confirmed by SDS-PAGE and western blotting.

SDS-PAGE and western blotting Recombinant S-RBD protein was characterized by SDS-PAGE and western blotting. Western blotting was conducted using anti-His and anti-spike
antibodies at 1:1000 dilution and HRP-conjugated goat anti-rabbit secondary antibody at 1:1000 dilution. Images were captured using the Bio-Rad Chemidoc system.

**Measurement of binding affinity between S-RBD and ACE2** The measurements were conducted using the ForteBio Octet RED96 system. S-RBD was first biotinylated with biotin-NHS. Streptavidin tips were loaded with 10 µM biotinylated S-RBD and then titrated with various concentrations of ACE2. The dissociation constant was obtained by fitting the association and dissociation curves.

**Expression and purification of S-RBD targeting the nanobody.** An S-RBD targeting nanobody (H11-D4) from the literature\(^2\) was cloned into an E. coli expression vector with a 6 histidine tag. After expression in *E. coli* strain BL21(DE3), the nanobody was purified with HisTrap Column (GE) and the buffer was changed to PBS. The protein was stored at –80 ºC until use.

**Preparation of S-RBD nanogels** S-RBD nanogels were prepared by directly mixing the protein with the crosslinkers. Crosslinker equivalents were set at 10, 20, and 50. The reactions were incubated at 30ºC for 1 h with continuous shaking. The reaction mixtures were passed through a PD-10 column to remove excess crosslinkers. The products were stored at -80 ºC until use for immunization. For confocal imaging or *in vivo* lymph node targeting experiments, S-RBD was pre-labeled with ~0.5 equivalent cy5. Briefly, 10 uM S-RBD was dissolved in PBS buffer and then 10 uM Cy5-NHS was added to the system. The reaction was let proceed at 37 ºC with continuous shaking of 800 rpm for 1 hour. The reaction mixture was then flowed through a PD-10 column (GE) to remove excess amount of Cy5-NHS. The protein and cy5 concentrations were then determined by measuring the absorbance at 280 nm and 662 nm with Nanodrop. Cy5-labeled S-RBD was then used as starting material to prepare Cy5-labeled nanogels with the same methods described above. Cy5-labeled S-RBD nanogels were analyzed by SDS-PAGE under reducing (DTT containing loading buffer) or non-reducing (non-DTT loading buffer) conditions. The gel was assessed by both cy5 fluorescence imaging and Commissie brilliant blue staining.

**Dynamic light scattering (DLS).** S-RBD and S-RBD-NG were first diluted in water at a concentration of ~0.1 mg/ml, and DLS was performed with a size analyzer (Zetasizer Nano ZS-90, Malvern, England).

**Transmission electron microscopy** Protein specimens were stained with phosphotungstic acid and TEM images were captured using a JEM-2100F microscope (JEOL).

**Confocal microscopy.** DC2.4 or RAW 264.7 cells were seeded in chamber one day before addition of cy5-labeled S-RBD or S-RBD-NGs. The next day, cells were treated with cy5-
labeled S-RBD or S-RBD-NGs (0.1 nmol) for 1 or 24 hours. Then, cells were washed with PBS, and nuclei were stained with Hoechst 33324. Images were captured with a Zeiss confocal microscope in the channel of Hoechst 33324 and cy5. Quantitative analysis of cell uptake was conducted using Image J software.

In vivo lymph node targeting Cy5-labeled S-RBD or S-RBD-NGs (0.66 nmol) or the same amount of free cy5 were administered to C57BL/6N mice via intramuscular injection. Twenty-four hours later, mice were sacrificed and the inguinal lymph nodes from the same side were collected and imaged on the PE IVIS Lumina living animal imaging system. Fluorescence intensity was measured. To calculate the radiant efficiency, the background intensity from the PBS group was deducted.

LN digestion and flow cytometry LNs were harvested and gently minced using scissors in FACS buffer (PBS containing 2% FBS). The LNs were then digested with 0.5 mg/mL collagenase I (Sigma) and 0.04 mg/mL DNase I (Roche) at 37°C for 1 h. The suspension was washed with cold FACS buffer, disaggregated by passing through a 70-μm cell strainer (Biologix Group) and centrifuged at 500 × g for 5 min. For flow cytometry analysis, single cells were resuspended in an appropriate volume of FACS buffer (1–5 × 106 cells per 100 μL), blocked with anti-FcγR monoclonal antibody (clone 2.4G2) to block nonspecific binding, and labeled with fluorescence-conjugated antibodies: anti-CD11c (N418, BioLegend), anti-I-A/I-E (M5/114.15.2, BioLegend), anti-CD11b (M1/70, eBioscience), anti-CD169 (3D6.112, BioLegend) and anti-F4/80 (BM8, BioLegend). The working concentration of the antibodies was 2.5 μg/mL. An LSRFortessa flow cytometer (BD Biosciences) and FlowJo software (Tree Star) were used for data collection and analysis, respectively.

In vivo immunization. C57BL/6N mice of 6–8 weeks were purchased from Charles River. Each mouse was intramuscularly injected with 50 μg S-RBD or S-RBD NGs with or without 100 μg aluminum hydroxide adjuvant. PBS was used for the control group. For the Pam3CSK4 adjuvant group, 50 μg S-RBD-NGs were directly mixed with 5 nmol Pam3CSK4 before injection. Mice were immunized on days 0, 14, 28, and one week after each immunization serum was collected. All mice were raised in a clean environment and animal experiments were performed under the guidelines of the IACUC of Peking University Health Science Center (No. LA2018033) and were approved by the Ethics Committee of Beijing Institute of Biotechnology (No. IACUC-DWZX-2020-038)

Collection of sera. One week after each immunization, blood was collected via the eye orbital vein. The blood samples were placed in an EP tube for 1 h at room temperature. The samples were then centrifuged for 10 min at 4000 rpm at room temperature. The supernatant
was collected as serum samples and stored at -80 °C until further use.

**Measurement of antibody titers using enzyme-linked immunosorbent assay (ELISA)**

Antibody titers were measured by ELISA. S-RBD protein was diluted in PBS to a concentration of 1 µg/mL and coated on EIA plates at 4°C overnight. The plates were washed once with PBST (0.5% Tween-20). Then, the plates were blocked with 2% BSA in PBS for 2 hours at room temperature. The plates were washed once with PBST. Meanwhile, sera were diluted in PBST-BSA (0.5% Tween and 0.5% BSA in PBS) with serial dilutions. The diluted sera were incubated in the blocked plates for 1 h at room temperature. The plates were then washed with PBST 3 times. HRP-conjugated goat anti-mouse secondary antibody (1:5000 dilution) was added to the plates and incubated at room temperature for 1 h. Then, the plates were washed with PBST 4 times. TMB solution (100 µL) was added to each well in a dark atmosphere and incubated at room temperature. At the desired time, 50 µL of H₂SO₄ (2 N) was added to each well to stop the reaction. Absorbance at 450 nm was measured immediately using a multi-well plate reader. The antibody titers were defined as > 2.1 times the background absorbance.

**Competitive ELISA.** EIA plates were coated with S-RBD and blocked with BSA as described above. The plates were then pre-blocked with 50 µL sera at different dilutions for 30 min. Then, 50 µL ACE-hFc (1 µg/mL) was added and incubated for another 1 h. The plates were then washed with PBST 3 times. HRP-conjugated goat anti-human IgG1-Fc secondary antibody (1:5000 dilution) was added to the plates and incubated at room temperature for 1 h. The coloring procedure was then performed as described above. OD450 values were compared with the control (no serum blocking) to validate the blocking effect of the sera on the interaction between RBD and ACE2.

**Immunofluorescence.** COS7-hACE2 and HEK293T cells were seeded in confocal chambers and cultured overnight. The next day, cells were first fixed with 4% paraformaldehyde for 10 min. Then, the cells were blocked with 2% BSA for one h. After washing with PBST, cells were incubated with anti-hACE2 antibody at a dilution of 1:200 at room temperature for 1 h. Finally, the cells were incubated with AF546 labeled goat-anti-rabbit IgG (H+L) secondary antibody for 1 hour. Cells were washed twice and incubated with Hoechst 33342. Images were captured with a Zeiss confocal microscope in the channel of Hoechst 33324 and AF546.

**Pseudovirus neutralization assay** COS7-hACE2 cells were first seeded in 96-well plates at 1:30 inoculation ratio and cultured for 24 hours. The next day, SARS-CoV-2 spike-based pseudovirus was first incubated with different dilutions of sera on ice for 1 h. The mixture was then added to the cells. The cells were cultured for 24 hours before the medium was
replaced with fresh medium. The cells were then further cultured for another 24 hours. For Spike-PV-Luc, cells were lysed and a luciferase reporter assay was performed to quantify the transduction efficiency. Confocal images were captured for Spike-PV-GFP.

**Data analysis.** Data were collected and analyzed using GraphPad Prism 8 software.

Figure S1. SDS-PAGE and western blot analysis of recombinant S-RBD protein.

![Figure S1](image1)

Figure S2. Intact mass of recombinant S-RBD protein.

![Figure S2](image2)
Figure S3. ELISA test of recombinant ACE2-hFc binding to immobilized S-RBD expressed in yeast.

Figure S4. Measurement of the binding affinity between S-RBD expressed in yeast and ACE2.
Figure S5. Competition of the interaction between S-RBD expressed in yeast and ACE2-hFc by a neutralizing nanobody targeting S-RBD.

Figure S6. Dynamic lighter scattering analysis of the recombinant S-RBD protein.

Figure S7. Confocal images showing the uptake of S-RBD or S-RBD-NG by DC2.4 cells at 1 hour (A) or 24 hours (B). Scale bar: 50 μm.
Figure S8. Confocal images showing the uptake of S-RBD or S-RBD-NG by DC2.4 (A) and RAW 264.7 (C) cells at 1 hour. Scale bar: 50 μm. Quantitative analysis of the cellular uptake by DC2.4 (B) and RAW 264.7 (D) cells. Data are presented as mean ± SEM. n=3.

Figure S9. Representative flow cytometry plots of data presented in Figure 3D. (A) S-RBD; (B) S-RBD-NG.
Figure S10. Competitive ELISA results showing the blocking effect of sera on the interaction between S-RBD and hACE2. Sera were diluted at the indicated folds. Data are presented as mean±SEM. n=3.

Figure S11. Immunofluorescence showing the expression of human ACE2 in COS7-hACE2 cells. Scale bar: 50 μm.
Figure S12. Confocal images showing the uptake of SARS-CoV-S1 or SARS-CoV-S1-NG by RAW 264.7 cells at 1 hour. Scale bar: 50 μm.

References

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