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Supporting Information

Construction and immunogenic studies of a Fc fusion receptor binding domain (RBD) of spike protein as a subunit vaccine against SARS-Cov-2 infection

I. General methods

The ELISA data were collected by a ELISA plate reader (PerkinElmer, Waltham, MA, United States). RBD-mFc and RBD-His were purchased from Sino Biological. The inhibitory effect was obtained by a flow cytometry (BD Biosciences, San Diego, CA, USA). IFN-γ and IL-4 spot-forming cells responding to RBD-mFc were determined by a ELISPOT Reader (Bio Reader 4000 Pro-X). Cytopathic effect was observed under a light microscope.

II. RBD-mFc formulation

The recombinant RBD (mFc Tag) of SARS-CoV-2 S protein consists of 457 amino acids and predicts a molecular mass of 51.5 kDa. Briefly, the RBD-mFc was expressed in HEK293 cells transfected with the plasmids following the manufacturer's protocol. After transfected for 72 h, the cell supernatants were harvested. The purity of RBD-mFc was determined by SDS-PAGE (> 95 %).

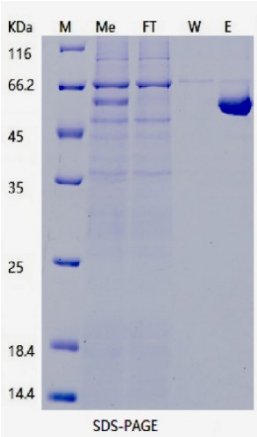

Figure S1. RBD-mFc characterization. Gel electrophoresis of the RBD-mFc protein stained by Coomassie blue. Lane M: Protein marker; Lane Me: Culture medium; Lane FT: Flow through; Lane W: Wash; Lane E: Eluted fractions (RBD-mFc).

III. Animal vaccination and sample collection.

Twenty female C57BL/6J mice (18-22 g) used in this study were purchased from Laboratory animal center of South Medical University (Guangzhou, China) and approved by Guangzhou University of Traditional Chinese Medicine Animal Care and use Committee. All these mice were immunized intramuscularly and maintained under specific pathogen-free conditions (License number: SYXK (Guangzhou) 2019-0144). Sera were collected at 28 and 42 days, and the experiments were finished on day 42. Sera were kept at -80℃.

IV. Enzyme-linked immunosorbent assay

Dissolved RBD-His protein in 0.1 M carbonate buffer (pH 9.6) to obtain the solution of 1 μg/ml, then, added the solution to 96-well microtiter plates 100 μl/well, incubated over night at 4℃. The next day, incubated the plate for 1 h at 37℃ and blocking with 2% non-fat milk (250 μl/well) at 37◦C for 1 h. After washing with PBS-T (PBS + 0.1% Tween), serially diluted mouse sera from 1:300 to 1:1656100 were added to each well

and then the plate was incubated at 37◦C, 1 h. After washed for three times, bound Abs were reacted with HRP-conjugated goat anti-mouse IgG (1:2000), HRP-IgM (1:5000), HRP-IgG1 (1:2000), HRP-IgG3 (1:2000), HRP-IgG2c (1:2000), HRP-IgG2b (1:2000) at 37°C for 1 h. Followed by three washes, 3,3',5'5'-tetramethylbenzidine (TMB) was added and incubated for 20 min in the darkness, then stopped the reaction with 100 μl H_2SO_4 (0.5 M). The absorbance was acquired at 450 nm.

Table S1. IgM antibody levels in pooled antisera at 1:300 dilution collected from mice at 28 and 42 days (Figure 2a).

	1st boost (OD value)			2nd boost (OD value)		
	Mean	**SD**	**N**	**Mean**	**SD**	**N**
Control	0.168	0.006	3	0.116	0.028	3
RBD-mFc	0.541	0.027	3	0.365	0.023	3
RBD-mFc/Al	0.785	0.144	3	0.658	0.015	3
RBD-mFc/FA	0.746	0.027	3	0.512	0.043	3

Table S2. IgG antibody levels in pooled antisera at 1:300 dilution collected from mice at 28 and 42 days (Figure 2b).

	1st boost (OD value)			2nd boost (OD value)		
	Mean	**SD**	**N**	**Mean**	**SD**	**N**
Control	0.099	0.002	3	0.228	0.013	3
RBD-mFc	0.680	0.069	3	2.379	0.109	3
RBD-mFc/Al	1.941	0.017	3	3.293	0.105	3
RBD-mFc/FA	5.135	0.010	3	5.893	0.054	3

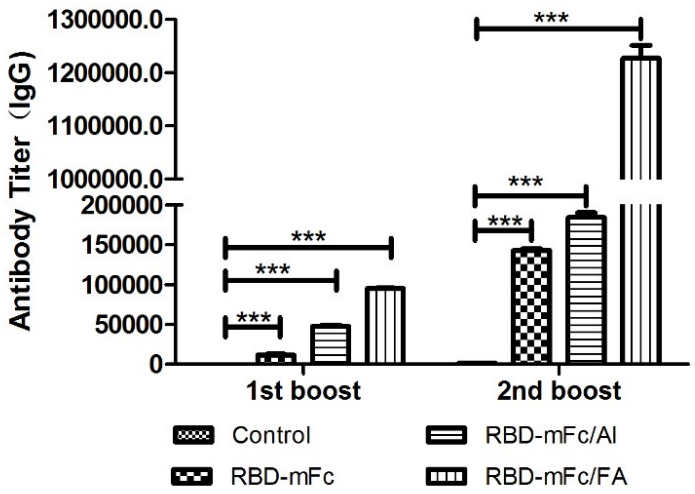

Figure S2. The antibody titers of IgG in mice pooled antisera on day 28 and 42.

Table S3. IgG antibody titers in pooled antisera collected from mice at 28 and 42 days (Figure S2).

	1st boost			2nd boost		
	Mean	**SD**	**N**	**Mean**	**SD**	**N**
Control	13	4	3	1267	716	3
RBD-mFc	11759	1866	3	142947	3757	3
RBD-mFc/Al	47770	2387	3	184303	9990	3
RBD-mFc/FA	95166	1095	3	1227000	24540	3

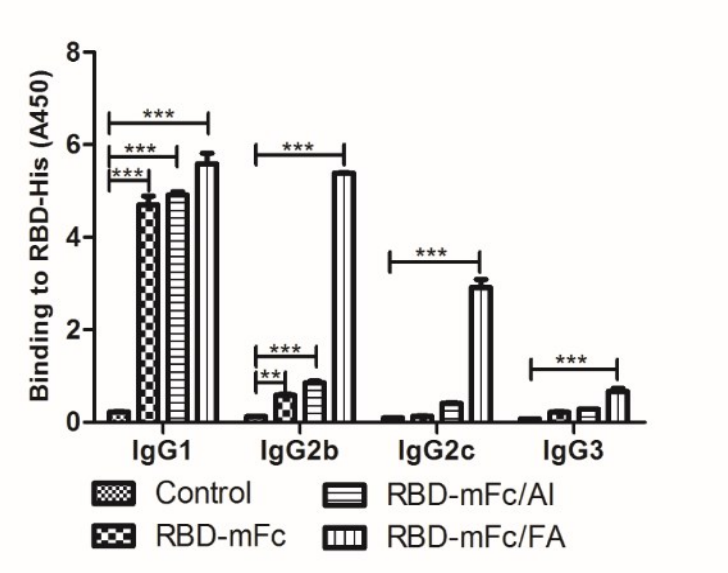

Figure S3. The antibody levels of IgG isotype in mice antisera on day 42 was determined by an ELISA assay using RBD-His as the capture antigen. Each sample was 1:300 diluted. Error bars represent the SD of the mean of the absorbance at 450 nm.

Table S4. The antibody levels of IgG subclasses in mice antisera on day 42 was determined by an ELISA assay using RBD-His as the capture antigen. Each sample was 1:300 diluted. (Figure S3).

	IgG1 (OD value)			IgG2b (OD value)		
	Mean	**SD**	**N**	**Mean**	**SD**	**N**
Control	**0.225**	**0.011**	**2**	**0.123**	**0.005**	**2**
RBD-mFc	**4.701**	**0.270**	**2**	**0.58**	**0.041**	**2**
RBD-mFc/Al	**4.919**	**0.088**	**2**	**0.856**	**0.043**	**2**
RBD-mFc/FA	**5.593**	**0.314**	**2**	**5.391**	**0.008**	**2**
	IgG2c (OD value)			**IgG3 (OD value)**		
	Mean	**SD**	**N**	**Mean**	**SD**	**N**
Control	**0.1**	**0.002**	**2**	**0.077**	**0.003**	**2**

RBD-mFc	**0.132**	**0.008**	**2**	**0.214**	**0.025**	**2**
RBD-mFc/Al	**0.412**	**0.020**	**2**	**0.281**	**0.009**	**2**
RBD-mFc/FA	**2.914**	**0.246**	**2**	**0.672**	**0.097**	**2**

V. Flow cytometry analysis

To confirm the inhibitory effect of mice antisera on RBD-His binding to receptor ACE2, flow cytometry assay was used. Briefly, human renal epithelial cell lines 293T were transfected with a human ACE2 vector (10 μg pCAG-hACE2/T75) for 48 h, then detached and washed the cells with PBS for three times. The 293T/ACE2 cells were resuspended in PBS buffer, and seeded into 1.5 ml centrifuge tubes (1×10^6 cells/tube). RBD-His was added to de cells to a final concentration of 1 μg/mL in the presence or absence of mice sera at 1:10 dilution, followed by incubation for 30 minutes at room temperature. After rinsed with PBS, the anti-6X His tag antibody-FITC (ab1206) at a dilution of 1:100 was added and incubated in the darkness at 4◦C for 1 h. In the negative control, only 293T/ACE2 cells suspended in PBS without mice sera, RBD-His and anti-6X His tag antibody-FITC was added. In the positive control, RBD-His and anti-6X His tag antibody-FITC without mice serum were added. Washed the cells thoroughly and resuspend the cells with PBS. Cells were then fixed with 1% formaldehyde. Fluorescent intensity of the samples was analyzed by FlowJo v7.6 software.

$$The\ inhibition\ rate = 1 - \frac{experimental\ group\ mean\ FITC-A - Negtive\ control\ mean\ FITC-A}{Positive\ cntrol\ mean\ FITC-A - Negtive\ control\ mean\ FITC-A} \times 100\%$$

Table S5. Mean FITC-A and collected cells of each group. In the negative control, neither mice sera no RBD-His was added while in the positive control, no mice sera was added. (Figure 3).

Name	∑ Mean: FITC-A	Cells
Positive control	591	11230
Control sera	586	10842

RBD-mFc/PBS antisera	310	13395
RBD-mFc/Al antisera	241	13279
RBD-mFc/FA antisera	172	13770
Negative control	163	10782

VI. ELISPOT assay

The cytokines were detected by ELISPOT kits (DAKEWE, Cat#:2210005, Cat#:2210402). The 96-well plates were pre-coated with rat anti-mouse IFN-γ or IL-4. 200 μl RPMI1640 without FBS (Fetal Bovine Serum) was added to each well to activate the monoclonal antibodies. Splenocytes harvested from vaccinated mice were seeded into the wells 5×10^5 cells/well in RPMI 1640 with 10% (v/v) FBS containing 10 μg/ml of synthetic peptides in duplicate. The cells firstly cultured for 16 h at 37◦C and 5% CO_2, then lysed with ddH_2O for 10 min at 4◦C, finally washed the plates with 1 × washing buffer for six times. Biotinylated anti-mouse IFN-γ and IL-4 antibodies (1:100) were added and incubated for 1 h at 37◦C. Washed the wells and then added streptavidin-horseradish peroxidase (1:100), incubated additional 1 h. After washed the plates, added the 3-amino-9-ethylcarbazole 100 μl per well. AEC was added to develop spots in dark for thirty minutes at 37◦C, and then quenched the reaction with ddH_2O and air-dried the plates. The spots of peptide-specific IFN-γ and IL-4 secreting T cells were counted.

Table S6. IFN-γ and IL-4 spot-forming cells responding to RBD-mFc (Figure 4).

	IFN-γ			**IL-4**		
	Mean	**SD**	**N**	**Mean**	**SD**	**N**
Control	11	1.414	2	7	1.414	2
RBD-mFc	12.5	0.707	2	3.5	0.707	2
RBD-mFc/Al	30	7.071	2	51.5	12.02	2

RBD-mFc/FA	18	2.828	2	9.5	3.536	2

VII. Neutralization assay for 2019-nCoV infection

Vero E6 cells (2×10^4 cells/well) were seeded into 96-well plates, cultured at 37◦C and 5 % CO_2 overnight until a monolayer formed. 125μl 100 $TCID_{50}$ (median tissue culture infective dose) of SARS-CoV-2 (Number: NPRC2020.00002) was mixed with serial 4-fold dilutions from 1:4 to 1:1024 of mice antiserum and incubated for 2 h, 37◦C. Serum samples were per-heated at 56◦C 30 min. Subsequently, to the mixture were added Vero E6 cells. In each assay, 100 $TCID_{50}$ SARS-CoV-2 infected cells were positive controls while 0.1 $TCID_{50}$ SARS-CoV-2 infected cells were negative controls, then, Cytopathic effect (CPE) in each well was observed daily and recorded at the 3 day after infected. The neutralizing titers in mice serum vaccinated with RBD-mFc fusion protein that completely suppressed 50% CPE of the wells were calculated by Reed-Muench method.

The formula for the proportional distance (PD) of the endpoint above the dilution giving next below 50% CPE is:

$$PD = \frac{50\% - (50\%\ CPE\ at\ dilution\ next\ below)}{(50\%\ CPE\ next\ above) - (50\%\ CPE\ next\ below)}$$

As the dilutions are increasing on a logarithmic scale, the final reading as shown below:

$$Sum\ (log.\ of\ endpoint) = Logarithm\ of\ lower\ dilution + PD \times \log dilution\ factor$$