Supporting Information

Co-Delivery of SARS-CoV-2 Prefusion-Spike Protein with CBLB502 by Dual-Chambered Ferritin Nanocarrier Potentiates System and Mucosal Immunity

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MATERIALS AND METHODS
1. **Construction and Generation of Dual-Chambered Ferritin Nanocarrier.** The recombinant clone of dual-chambered ferritin nanocarrier comprised several fragments, including gb1, intC, GGGSGGG linker, *H. pylori* ferritin (5-167, WP_000949190.1), GGSG linker and CBLB502 ([Figure S1a](#)). The recombinant clone was inserted to pET28a between *Eco*RI and *Xho*I restriction sites and transformed into BL21 (DE3) plysS for protein expression. Cells were incubated at 37 °C shaker to OD 0.8 in 500 mL LB with 50 μg/mL kanamycin and 0.2 mM IPTG was then used to induce protein expression at 25 °C for 5h. Cells were harvested and resuspended by 30 mL PBS. After sonication by an ultrasonic processor, cell pellet was collected by centrifugation and resolved by 10 mL PBS with 6 M urea. Nanoparticle was recovered from supernatant by sequential dialysis against PBS with 2M, 1M or 0M urea, respectively.

2. **Eukaryotic expression and purification of proteins.** Genetic fusion of pre-S ectodomain (1-1208) to *H. pylori* ferritin (5-167, WP_000949190.1) was inserted into pCDNA3.1+ between *Hind*III and *Bam*HI with SGGG as a linker ([Figure S1a](#)). Recombinant protein was expressed by Expi293F™ cells, a gift of professor Haitao Yang from ShanghaiTech University, which was cultured by cell-wise balance CD 293 medium and transiently transfected by EZ Trans-II from Shanghai Life iLab Biotech Co., LTD. Recombinant protein, pre-S-ferritin, spontaneously formed nanoparticle and was secreted to cell medium, that was enriched by 20% sucrose cushion and then purified by ultracentrifugation over discontinuous sucrose gradient (10% to 50% w/v sucrose in PBS) for 2 h. pre-S-intN with a 6xHis tag at
its C-terminus was inserted into pCDNA3.1+ between HindIII and BamHI (Figure S1a). Recombinant protein was expressed by ExpiCHO™ expression system according to producer’s manual and purified by standard immobilized metal affinity chromatography (IMAC) protocol.

3. **Generation of co-delivery nanoparticles.** *In vitro* trans-splicing was executed to produce antigen/adjuvant co-delivery nanoparticle at room temperature for 4h with 2 mM EDTA. Dose-dependent assay was performed to ascertain the proper concentration of substrates. The intensity of protein band was evaluated by ImageJ and reaction efficiency was calculated by the following formula: \[ \left(1 - \frac{\text{intensity of retained ferritin nanocarrier}}{\text{intensity of initial ferritin nanocarrier}} \right) \times 100 \]. After reaction, target nanoparticles were separated from mixture by gel filtration with a superose™ 6 increase column that was conducted by ClearFirst 3000 system from Shanghai Flash Spectrum Biological Technology Co., Ltd. Three repeats were performed and data was analyzed by GraphPad Prism 8.0. Values were presented with ± SEM.

4. **Nanoparticle characterization.** Samples of gb1-intC-ferritin-CBLB502, pre-S-ferritin and pre-S-ferritin-CBLB502 were observed by a Tecnai G2 Spirit transmission electron microscopy (TEM) at 200 kV. Abovementioned samples were negatively stained by 1% uranyl acetate and imaged with a magnification of 61k.

5. **Mice immunization and ethic statement.** To evaluate the modulatory effects raised by dual-chambered nanocarrier, 5-8 weeks old female C57BL/6J-TgN (hACE2) mice, gifts from professor Xiaohui Zhou’s group, received two
intramuscular (i.m.) injections and a third intranasal (i.n.) injection of 2 μg antigens in different regimens. Mice were divided into 5 groups according to injected antigens, including ferritin, pre-S-int^N-6xHis (pre-S for short), pre-S-ferritin, pre-S-ferritin supplemented with CBLB502 (pre-S-ferritin+CBLB502) and co-delivery nanoparticle (pre-S-ferritin-CBLB502). There were 4-5 mice for each group and injection interval was two weeks. Orbital serums were collected two weeks after injection. The whole process of mice study was permitted by animal ethics committee of Shanghai Public Health Clinical Center (permission number: 2020-A066-03). Mice were raised under SPF conditions and all experiments were done with careful consideration of animal benefits.

6. **Collection of nasal wash (NW) and bronchoalveolar lavage fluid (BALF).**

Three weeks after third injection, mice received euthanasia by neck dislocation. NW and BALF samples were collected by several washes with 300 μL or 600 μL of cold PBS, respectively. The gained samples were stored at -80 °C.

7. **Antibody response assays.** Enzyme-linked immunosorbent assay (ELISA) was implemented to investigate anti-spike IgG or IgA responses. In brief, pre-S-int^N protein was applied into 96-well high-binding microplate (100 ng in PBS for each well) at 4 °C overnight. Microplates were sequentially blocked by 5% milk in PBS, incubated with diluted serum, NW or BALF samples and added with anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3 or IgA--horseradish peroxidase (HRP) conjugates in 1% milk, and microplates were washed thrice by PBST (PBS + 0.05% Tween-20) for each step. Then 50 μL TMB was used for color development which was
terminated by 50 μL of 1 M sulfuric acid. Finally, the absorbance of optical density 450 nm was measured. A serial of 10-fold dilution of serum samples was applied to determine the IgG endpoint titers. Serums were 1:10^4 diluted to analyze IgG isotypes and 1:40 diluted to identify antigen-specific IgA responses. NW and BALF samples were 1:5 diluted for IgA response assay and 1:10 diluted for IgG response assay.

8. **Pseudovirus neutralization assays.** To estimate the neutralizing capability of serums, specimens were serially diluted by three-fold after inactivation at 56°C for 30 min, with initial dilution of 1: 40. NW and BALF were diluted by two-fold with initial dilution of 1: 8. Triple repeats of the diluted specimens were incubated with 200 TCID_{50} of SARS-COV-2 S pseudovirus (lentivirus-based pseudovirus with luciferase as reporter gene) at 37 °C for 1h in 96-well microplates. Then 2x10^4 cells were added into microplates for each well. Forty-eight hours after infection, medium was emptied and 100 μL of lysis buffer was added and luciferase signal was detected using ONE-Glo™ Luciferase Assay System from Promega. Neutralizing efficiency were calculated by GraphPad Prism 8.0 with non-linear regression fit. The concentration that is correlated with 50% decrease of luciferase signal is defined as the 50% inhibitory concentrations (IC_{50}).

9. **Cellular response assays.** Spleens of immunized mice were obtained three weeks after the third injection. Enzyme Linked Immunospot Assay (ELISPOT) was conducted to detect IFN-γ or IL-4 levels secreted by splenocytes under stimuli of pre-S-int^N protein. MultiScreen@HTS 96-well microplates were coated with anti-
murine IFN-γ or IL-4 capture antibodies at 4 °C overnight. Each well was added with 5x10^5 splenic cells in 1640 medium with 10% FBS that were stimulated by 2 μg pre-S-intN protein. After 48h, microplates were sequentially incubated with detection antibody, biotin-conjugated anti-IFN-γ or anti-IL-4, and HRP-conjugated streptavidin. TMB color development was stopped by extensive water wash and spot-forming cells were imaged by AT-Spot 3300 from bjatyx Biotech Co., LTD.

10. **Statistical analysis.** Figure 3c was plotted by Origin 2018 with standard error of the mean (± SEM) as error bar and other experiment data were analyzed by GraphPad Prism 8.0. Mean values or mean values with ± SEM were plotted during data process. As there were four to five groups, multiple comparisons of the values for each group were calculated by a one-way analysis of variance (ANOVA) and significant difference between two group was performed by Tukey’s multiple comparisons test. P < 0.05 was regarded as statistically significant and designated by * and P < 0.01 was represented by **.

**Supplementary figures**
**Figure S1.** Construction of recombinant clones and purification of nanoparticles. (a) Clones used in this study were depicted as above. The gb1-int<sup>C</sup> moiety was introduced to ferritin N-terminus that acted as ‘adaptor’ to load cargo fused with complementary int<sup>N</sup> (pre-S-int<sup>N</sup>, for example); CBLB502 was fused to ferritin C-terminus which served as adjuvant. (b) Schematic of coupling pre-S to ferritin-CBLB502. (c) The gb1-int<sup>C</sup>-ferritin-CBLB502 protein aggregated in *E.coli* that was resolved by 6 M urea in PBS. Centrifugation was carried out to remove cell debris and target protein in supernatant was re-natured by step-wise dialysis against 2 M, 1 M and 0 M urea in PBS. Lane 1: protein marker, lane 2: all represented the crude lysis of *E.coli*; lane 3: up meant the supernatant of *E.coli* crude lysis after centrifugation; lane 4: all was designated as re-suspension of pellet by 6 M urea in PBS; lane 5: up was denoted as supernatant of re-suspension in 6 M urea after centrifugation; lane 6-8: up represented the collected specimens after centrifugation. (d) Discontinuous sucrose gradient ultracentrifugation was used to purify pre-S-ferritin. Aliquots of 12 fractions were tested by coomassie brilliant blue (CBB) staining assay.
**Figure S2.** IgG1 and Ig2b titers and neutralizing pseudovirus capability assays of serums. Serums were 10-fold diluted to determine the endpoint titers of IgG isotypes. Since serums showed low reactivity with HRP labeled goat anti-mouse IgG2a or IgG3, further titration assay was not conducted. Positive was defined by OD450 > 0.2. (a) Titration of anti-pre-S IgG1 titers. (b) IgG2b responses were measured. (c-g) Neutralizing capability of individual groups were tested. The 50% reduction of luciferase signal represented inhibitors concentration 50% (IC$_{50}$). Statistical significance was denoted by the following symbols: ns= not significant, *= p < 0.05, **= p < 0.01.