Polymerized Porin as a Novel Delivery Platform for Coronavirus Vaccine

Zhongqian Yang
Chinese Academy of Medical Sciences & Peking Union Medical College Institute of Medical Biology
Liangqun Hua
Chinese Academy of Medical Sciences & Peking Union Medical College Institute of Medical Biology
Mengli Yang
Chinese Academy of Medical Sciences & Peking Union Medical College Institute of Medical Biology
Weiran Li
Chinese Academy of Medical Sciences & Peking Union Medical College Institute of Medical Biology
Zhaoling Ren
Kunming Medical University Second Hospital
Xiao Zheng
Chinese Academy of Medical Sciences & Peking Union Medical College Institute of Medical Biology
Haoqian Chen
Chinese Academy of Medical Sciences & Peking Union Medical College Institute of Medical Biology
Qiong Long
Chinese Academy of Medical Sciences & Peking Union Medical College Institute of Medical Biology
Hongmei Bai
Chinese Academy of Medical Sciences & Peking Union Medical College Institute of Medical Biology
Weiwei Hang (hww_imbcams@126.com)
Chinese Academy of Medical Sciences & Peking Union Medical College Institute of Medical Biology
https://orcid.org/0000-0001-6417-3785
Yanbing Ma
Chinese Academy of Medical Sciences & Peking Union Medical College Institute of Medical Biology

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Abstract

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), seriously threatens human life and health. The correct folding and polymerization of the receptor-binding domain (RBD) protein of coronavirus in *Escherichia coli* may reduce the cost of SARS-CoV-2 vaccines. Here, we designed this nanopore by using the principle of ClyA porin polymerization triggered by the cell membrane. We use surfactants to "pick" the ClyA-RBD nanopore from the bacterial outer membrane in this study. More importantly, the polymerized RBD displayed on ClyA-RBD polymerized porin (RBD-PP) already has some correct spatial structures of virus spikes. The nanostructures of RBD-PP can target lymph nodes and promote antigen uptake and processing by dendritic cells, thereby effectively eliciting the production of anti-SARS-CoV-2 neutralizing antibodies and systemic cellular immune responses and immune memory. We applied ofthis PP-based vaccine platform to make an RBD-based subunit vaccine against SARS-CoV-2, which will provide a foundation for the development of inexpensive coronavirus vaccines. The development of novel vaccine delivery system is an important part of innovative drug research. This novel PP-based vaccine platform is likely to be applied to more fields, including other viral vaccines, bacterial vaccines, tumor vaccines, drug delivery, and disease diagnosis.

Introduction

SARS-CoV-2 infection causes severe coronavirus disease 2019 (COVID-19) [1]. Inactivated vaccines [2], viral vector vaccines [3], subunit vaccines [4], mRNA vaccines [5], and DNA vaccines against SARS-CoV-2 have proven the effectiveness of different SARS-CoV-2 vaccines[6].Several nanoscale-based vaccine formulations have also achieved antiviral effects[7], including a particulate vaccine prepared by the display of recombinant receptor-binding domain (RBD) protein on liposome surfaces [8], an S-RBD nanogel vaccine prepared using crosslinkers [9], nanoparticle vaccines prepared by conjugating ferritin to RBD and HR using the SpyTag/SpyCatcher system [10], and two-component protein nanoparticles multivalently displaying RBD [11]. These nanovaccines can present antigens to the immune system at high densities, and antigen multimerization is important for the activation of low-affinity B cells [12, 13]. Relying on their nanoscale characteristics, nanovaccines induce superior lymph node targeting and uptake capacity of antigen-presenting cells [14]. They also exhibit excellent biocompatibility and safety.

Escherichia coli is a low-cost expression system. If an *E. coli* expression system could be used to manufacture a SARS-CoV-2 vaccine, the cost of vaccine production would be greatly reduced, which would facilitate global mass inoculation with SARS-CoV-2 vaccines. However, *E. coli* lacks a posttranslational modification system similar to those of higher organisms, so it cannot guarantee the folding of a protein to the correct conformation [15]. This has severely limited the application of the *E. coli* expression system to vaccine development. In addition, RBD exists in trimeric form in live SARS-CoV-2 [16], and it has been proven that RBD has better effects in multimeric form than in monomeric form [11, 17]. If the expression of the properly folded and polymerized RBD in *E. coli* for vaccine production can be optimized, the production cost of the SARS-CoV-2 vaccine will be greatly reduced.

ClyA is a porin on the outer membrane of *E. coli*. The monomer ClyA will polymerize on the outer membrane to form pores approximately 10-15 nm in size. If the ClyA self-assembled pore structure can directly carry exogenous proteins and form a nanoscale structure, it will be an ideal vaccine platform highly conducive to targeting lymph nodes, enabling antigen presentation and reducing systemic toxicity. In particular, the polymerization properties of ClyA are particularly suitable for presenting antigens that need to be polymerized, especially the RBD of SARS-CoV-2 here. Inspired by these properties, we wanted to construct a ClyA-RBD chimera and use the characteristics of ClyA's own polymerization to complete the polymerization of RBD, thereby constructing a polymerized coronavirus vaccine.

Although the polymeric nanopore structure of ClyA has been reported[18], there has not been any research to realize the use of ClyA nanopores as a vaccine delivery system based on antigen polymerization. Only the ClyA monomer with the correct conformation in the periplasm will be inserted into the outer membrane, thereby triggering the conformational change in the ClyA monomer[18, 19]. The ClyA monomer will move and adjust on the membrane after its conformational change, and multiple monomers will assemble into a polymer[18]. These conformational changes and assembly processes are not chaotic—they follow fixed rules for membrane insertion and assembly—and finally, ClyA will be assembled on the outer membrane into a structure with the C-terminus exposed outside the cell. Since the polymerization of the bacterial porin backbone of ClyA must be completed on the cell membrane, this pore structure is difficult to maintain after separation from the cell membrane[20, 21], which limits the development of ClyA nanopores as an independent delivery system. However, a large number of studies have also proven that surfactants can trigger the conformational rearrangement of ClyA monomers and the formation of nanopores[19, 22]. We took advantage of this feature of ClyA to complete the self-assembly of the ClyA-RBD monomer from the membrane support to the surfactant support under the action of surfactants. In short, we used surfactants to "pick" the ClyA-RBD nanopore from the bacterial outer membrane.

Due to the importance of RBD polymerization in the development of coronavirus vaccines, in this study, we tried to develop and utilize ClyA nanopores as an RBD vaccine delivery system. We fused the ClyA protein, which is highly polymerized in *E. coli*, with RBD and achieved the correct folding and polymerization of RBD by exploiting the ability of ClyA to form nanopore structures on the outer membrane (OM) of *E. coli* [18, 23], finally forming ClyA-RBD polymerized porin (RBD-PP). A high degree of RBD polymerization was achieved in RBD-PP, and furthermore, the resulting nanostructures increased lymph node targeting and antigen uptake and processing by dendritic cells (DCs). *In vivo* immunization with RBD-PP induced the production of sufficient anti-SARS-CoV-2 neutralizing antibodies and T cell responses and simultaneously activated systemic immune memory (Scheme 1). This is the first report of the polymerized porin vaccine vector formed by the assembly of a bacterial protein and is an approach to combating SARS-CoV-2 infection. Our findings provide not only a foundation for research on coronavirus vaccines but also a bacterial nanopore–based platform for vaccine research and development.

Materials And Methods

Ethics and biosafety statement

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Institute of Medical Biology, Chinese Academy of Medical Sciences (ethics number: DWSP202007002), and every effort was made to minimize animal suffering. All work with infectious SARS-CoV-2 was performed with approval under Biosafety Level 3 (BSL3) conditions by the Institutional Biosafety Committee of the Institute of Medical Biology. The BSL3 facilities have been designed to conform to the safety requirements recommended by the China National Accreditation Service for Conformity Assessment (CNAS) and the National Health Commission of the People's Republic of China CNAS. Experiments with infectious virus were performed in a certified Class IIB biosafety cabinet in BSL3 [24].

Mice, bacterial Strains, cell Lines, and virus

Specific pathogen-free BALB/c female mice (6-8 weeks old) were obtained from the Experimental Animal Center of the Institute of Medical Biology, Chinese Academy of Medical Sciences. All mice used in this study were in a healthy state and were raised in SPF animal facilities of the Institute of Medical Biology of the Chinese Academy of Medical Sciences, with free access to water and a standard chow diet. *E. coli* strain BL21 was cultured in Luria-Bertani (LB) medium at 37°C. The African green monkey kidney cell line Vero E6 (KCB 92017YJ) was obtained from the Conservation Genetics CAS Kunming Cell Bank (Kunming, China). These cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (Gibco) at 37°C in the presence of 5% CO_2. The clinical isolates of SARS-CoV-2 wild-type and SARS-CoV-2 B.1.617.2 (Delta) variant was propagated in Vero E6 cells, and amplified SARS-CoV-2 was confirmed via qRT-PCR, sequencing, transmission electron microscopy (TEM), and titration via plaque assay (10^6 plaque-forming units/mL) [25].

Preparation of RBD-PP

The plasmid pThioHisA was purchased from Invitrogen, Inc. The RBD (Asn331-Val524, YP_009724390) was ligated to the C-terminus of the ClyA protein (AAL55667), with GS as the linker. The DNA fragment encoding the fusion protein ClyA-RBD was optimized and subcloned into pThioHisA using the restriction endonucleases *BamHI* and *SalI*, and the recombinant plasmid expressing the ClyA protein (AAL55667) was prepared in the same manner. Positive plasmids were confirmed by restriction digestion analysis and sequencing. The recombinant plasmids were transformed into *E. coli* BL21 and then inoculated into LB medium. When the optical density at 600 nm (OD_{600}) of the bacterial culture reached 0.4-0.6, the bacterial culture was added to 1 mM isopropyl-β-d-1-thiogalactopyranoside (Solarbio) to induce the expression of the recombinant fusion protein at 30°C overnight. A total of 2 mM EDTA·2Na (Sigma) was added to the sample the next day; after 2 h of incubation, the sample was centrifuged at 14,000 × g at 4°C for 30 min to collect bacterial cells. The cells were sonicated 5 times (power: 300 W, sonication time: 20 s, interval: 20 s) and then centrifuged at 6000 × g and 4°C for 10 min to remove unbroken bacteria. The supernatant was centrifuged at 100,000 × g, and the pellet was resuspended in Hanks's buffered saline solution (HBSS) (Servicebio). N-lauryl sarcosyl (Sigma-Aldrich) was added to a final concentration of 2% and incubated at room temperature for 30 min, followed by centrifugation at 15,000 × g for 30 min to induce

precipitation as a component of the bacterial outer membrane. The precipitate was resuspended in phosphate-buffered saline (PBS) at 5 mg/ml and then incubated with 1% Triton X-100 or DDM at 15°C for 2 h to extract ClyA-RBD nanopores from the bacterial outer membrane. The outer membrane was gradually replaced by surfactant insertion, and then ClyA-RBD nanopores were collected by ultracentrifugation at 130,000 × g for 30 min. The precipitate was resuspended in PBS, stirred overnight at 37°C, and then purified by gradient centrifugation with OptiPrep™ (STEMCELL) medium. The concentrations of OptiPrep™ in HBSS were 45%, 35%, 30%, 25%, 20%, 15%, and 10% for the gradient layers from bottom to top. Samples were collected by centrifugation at 130,000 × g for 4 h. The samples were stained with a Fast Silver Stain Kit (Beyotime). The particle sizes of the samples were analyzed using dynamic light scattering (DLS) (Zetasizer Nano ZS, Malvern Instruments). The protein concentrations of the boiled RBD-PP or PP were detected using a Bradford kit (Sangon Biotech) under denaturing conditions.

Endotoxin removal

Endotoxin was removed using Pierce™ High Capacity Endotoxin Removal Spin Columns (Thermo). The sample (1 mL) was added to a buffer (pH 7.2) containing 10 mM sodium phosphate, 0.15 M sodium chloride, and 0.05% sodium azide and incubated at 22°C with gentle end-over-end mixing for 1 h. The column was centrifuged at 500 × g for 1 min to collect the sample. Endotoxin levels were measured using the Thermo Scientific Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo).

Western blot

Samples were run on a 12% sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) gel and transferred onto polyvinylidene fluoride membranes. The membranes were blocked in 5% milk for 45 min at room temperature. The anti-SARS-CoV-2-S1 rabbit monoclonal antibody (Sino Biological) and the goat anti-rabbit immunoglobulin G (IgG)-horseradish peroxidase (HRP) (Sino Biological) were used as primary antibody (1:2000 dilution) and secondary antibody (1:10,000 dilution), respectively, for the identification of proteins expressed in PP samples. The sera of immunized mice and goat anti-mouse IgG-HRP (Sino Biological) were used as the primary (1:1000 dilution) and secondary antibodies (1:10,000 dilution), respectively, for the identification of SARS-CoV-2 spike recombinant RBD protein (Sino Biological) and SARS-CoV-2 S1 protein (Sino Biological). Blots were visualized by using Clarity™ Western ECL Substrate (Bio-Rad).

Polymerization analysis

The PP and RBD-PP samples were denatured through boiling at 95°C for 5 min. Nondenatured samples did not undergo the above treatment. The samples were separated by 15% SDS-PAGE and stained with the Fast Silver Stain Kit (Beyotime).

Transmission electron microscopy (TEM)

TEM was performed as previously described [26]. The samples were added to a carbon-coated copper grid and negatively stained for 30 s with 2% (wt/vol) uranyl acetate, and TEM images were collected

using a transmission electron microscope (Hitachi).

ClyA-RBD monomer preparation, binding analysis with hACE2 and neutralizing antibodies

As mentioned earlier [23], the osmotic shock method was used to obtain periplasmic components, and the ClyA-RBD monomer was purified. ClyA-RBD monomer and RBD-PP (3 μg/ml) were used to coat 96-well flat-bottomed plates (Corning), which were then incubated at 4°C overnight. After washing the plates with PBS containing 0.1% Tween 20 (PBST) three times, the plates were blocked with 1% bovine serum albumin (BSA) (Sigma) in PBST for 1 h at room temperature, added to serially diluted His-tagged hACE2 (Sino Biological) and incubated at 37°C for 1 h, added to HRP-conjugated His antibody (Sino Biological, 0.2 μg/ml) at room temperature for 1 h, and added to 3,3',5,5'-tetramethylbenzidine (TMB) (Solarbio) to observe the reaction. In the binding analysis of neutralizing antibodies, the SARS-CoV-2 spike neutralizing antibodies MM43, MM57, R001, R004, D001, and D002 (Sino Biological, 0.1ug/ml) were used as the primary antibodies, and HRP-conjugated antibodies against human IgG, mouse IgG, and rabbit IgG (Sino Biological, 1:10,000) were used as the secondary antibodies. After the reaction was terminated with H_2SO_4, a microplate reader (Thermo) was used to measure the OD450.

Live imaging in vivo

Label RBD-PP and RBD with Cy7-NHS (Amersham Biosciences). Excess free dye was removed by a Sephadex G-25 PD 10 desalting column (GE Healthcare). The protein and Cy7 concentrations were determined by measuring the absorbance at 280 nm and 747 nm. Cy7-labeled RBD or RBD-PP (10 μg) or the same amount of free Cy7 was administered to mice via intramuscular injection. Twelve hours after the injection, the mouse's heart, liver, spleen, lung, kidney and right lymph node were collected and imaged with an In Vivo FX PRO imaging system (Bruker) with an excitation filter at 760 nm and an emission filter at 790 nm. Molecular imaging software was used to determine the radiant efficiency and relative fluorescence intensity.

BMDCs uptake in vitro

Bone marrow–derived dendritic cells (BMDCs) isolated from murine femurs and tibias were cultured in plates as previously described[27]. To each well, either RBD-PP or RBD was added at 0.05 mg/ml, followed by incubation at 37°C in 5% CO_2 for 4 h. The incubated BMDCs were stained with the anti-mouse CD11c monoclonal antibody (BioLegend) at 4°C for 30 min. After the excess antibody was removed by centrifugation, BMDCs were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 2% BSA (Sigma), incubated with rabbit anti-SARS-CoV-2-S1 monoclonal antibody (Sino Biological, 1:200 dilution) at 37°C for 2 h, incubated with goat anti-rabbit IgG-FITC (Proteintech, 1:50 dilution) at 37°C for 1 h, and stained with 4',6-diamidino-2-phenylindole (DAPI) (Meilunbio). The stained BMDCs were imaged using an ImageXpress Micro Confocal System (Molecular Devices).

Antigen Uptake and Processing and Presentation Analysis In Vivo

After 10 μg of Cy7-labeled RBD or RBD-PP was injected subcutaneously into mice for 12 h, the lymph nodes of the mice were collected and treated with 0.5 mg/mL collagenase I (Sigma) at 37°C for 1 h. Lymphocytes in the lymph nodes were isolated by passing through a 70-μm cell strainer (FALCON) and centrifuged at 500 × g for 5 min. The collected cells were stained with anti-mouse CD11c, CD86, CD80 and MHC-II (BioLegend) monoclonal antibodies, incubated at 4°C for 30 min, and centrifuged to remove excess antibodies. The cells were analyzed by a CytoFLEX flow cytometer (Beckman Coulter), and data analysis was performed using FlowJo software.

Germinal center responses

BALB/c mice were immunized subcutaneously with vaccine formulations containing 50 μg of RBD-PP and RBD mixed with alum, and alum mixed with PBS as the control. Ten days after injection, cell suspensions prepared from lymph nodes were stained with anti-mouse CD3, CD4, CXCR5, PD-1, CD19, B220, CD95 and GL7 (BioLegend) monoclonal antibodies and analyzed by a CytoFLEX flow cytometer (Beckman Coulter).

Humoral immune response analysis

RBD-PP (50 μg, 5 μg, 0.5 μg), PP (50 μg, 5 μg, 0.5 μg) and ClyA-RBD monomer (RBD, 50 μg, 5 μg, 0.5 μg) were used to immunize 6- to 8-week-old female BALB/c mice on days 0, 14, and 28 by subcutaneous injection with alum (Thermo) as the adjuvant. Sera were collected from the mice on days 7, 21, and 35. Blocked enzyme-linked immunosorbent assay (ELISA) plates precoated with SARS-CoV-2 RBD protein (Sino Biological) and ClyA peptides at 2 μg/ml overnight at 4°C were given diluted serum samples that were sequentially diluted twofold with blocking buffer starting at 1:100 and incubated at 37°C for 1 h. Then, they were treated with biotin-conjugated anti-mouse IgG (Invitrogen, 1:5000 dilution), anti-mouse IgG1 (BioLegend, 1:8000 dilution), and anti-mouse IgG2a (BioLegend, 1:8000 dilution) and incubated at room temperature for 1 h. Next, streptavidin-HRP (BioLegend, 1:3000 dilution) was added and incubated at room temperature for 45 min. Finally, TMB (Solarbio) was added to observe the reaction. After the reaction was terminated with H_2SO_4, a microplate reader (Thermo) was used to measure the OD450. The endpoint titer was defined as the highest reciprocal dilution of serum to give an absorbance greater than 2.1-fold of the background values [17].

Evaluation of antibody blocking S1/ACE2 binding

Diluted serum samples were added to ELISA plates, each well containing 40 ng/ml SARS-CoV-2 RBD protein (Sino Biological), and incubated at 37°C for 1 h. Next, His-tagged hACE2 (Sino Biological, 2 μg/ml) was added and incubated at 37°C for 1 h; HRP-conjugated His antibody (Sino Biological, 0.2 μg/ml) was added and incubated at room temperature for 1 h; and last, TMB (Solarbio) was added to observe the reaction. After the reaction was terminated with H_2SO_4, a microplate reader was used to measure the OD450.

Neutralization assays

The neutralization effect of antisera on SARS-CoV-2 infection was evaluated as previously described [28] in a biosafety level 3 facility. Vero E6 cells were seeded in 96-well plates and grown overnight. Mouse sera were serially diluted twofold with DMEM starting at 1:16. One hundred $TCID_{50}$ (50% tissue culture infectious dose) of SARS-CoV-2 wild-type (WT) virus and SARS-CoV-2 B.1.617.2 (Delta) variant were preincubated with an equal volume of diluted serum and incubated at 37°C for 1 h. The incubated mixture was then added to Vero E6 cells. The cytopathic effect (CPE) was recorded under a microscope on the 3rd day after infection. The neutralization titer NT_{50} was defined as the reciprocal of the serum dilution at which the SARS-CoV-2 infectivity was reduced by 50%.

Cellular immune response analysis

Fourteen days after the last immunization, mice were sacrificed. The spleens were harvested, and splenocyte suspensions were isolated through a 70 μm strainer. To measure the percentages of proliferating T cells in response to antigenic restimulation, splenocytes (1×10^6/well) were cultured in 96-well plates and stimulated with SARS-CoV-2 RBD protein (2 μg/ml, Sino Biological). After incubation at 37°C in 5% CO_2 for 48 h, the splenocytes and cell culture media were collected by centrifugation at 500 × g. The cells were stained with anti-mouse CD3, CD4 and CD8 monoclonal antibodies (BioLegend) and analyzed via flow cytometry. The cell culture media were used to determine cytokine levels with mouse GzmB, IFN-γ, and IL-2 ELISA kits (Invitrogen) according to the manufacturer's instructions. Intracellular cytokine staining was performed to analyze SARS-CoV-2-specific $CD4^+$ T helper cell and polyfunctional $CD8^+$ T cells populations. Splenocytes (1×10^6/well) were stimulated with SARS-CoV-2 RBD protein (2 μg/ml) for 1 h, and then 5 μg/ml brefeldin A (BioLegend) was added and incubated for 4-6 h to block the intracellular secretion of cytokines. These splenocytes were washed with PBS and stained with anti-mouse CD3, CD4 and CD8 monoclonal antibodies (BioLegend) at 4°C for 30 min. After staining of surface markers, splenocytes were performed with a fixation/permeabilization kit (BD Biosciences) according to the manufacturer's protocol for anti-mouse IFN-γ, TNF-α, IL-2, and IL-4 monoclonal antibody (BioLegend) staining. The stained splenocytes were analyzed in a CytoFLEX flow cytometer (Beckman Coulter).

Analysis of memory T cells

Fourteen days after the last immunization, mice were sacrificed. The spleens were harvested, and splenocyte suspensions were isolated through a 70 μm strainer. For the analysis of memory T cells, splenocytes were stained with anti-mouse CD3, CD4, CD8, CD44 and CD62L monoclonal antibodies (BioLegend) at 4°C for 30 min. The stained splenocytes were analyzed in a CytoFLEX flow cytometer (Beckman Coulter).

Enzyme-linked immunospot assay (ELISpot)

After splenocytes (3×10^5/well) were stimulated with SARS-CoV-2 RBD protein (2 μg/ml) for 20 h, antigen-specific T cell responses were evaluated using the mouse IFN-γ $ELISpot^{PLUS}$ kit (Mabtech) and

mouse IL-4 precoated ELISpot kit (Dakewe Biotech) according to the manufacturer's instructions. The spots were counted using an ELISpot reader (AID).

Safety evaluation

The body temperature and body weight of the mice were monitored and recorded once every 2 days during the immunization period. Seven days after the third immunization, the kidneys, hearts, livers, and spleens of the immunized mice were collected, and parts of the collected samples were taken for pathological analysis. These samples were fixed with 10% formalin for 24 h and sectioned for hematoxylin and eosin (H&E) staining. H&E images were acquired using a phase-contrast inverted fluorescence microscope (TS2, Nikon). To further evaluate the safety of RBD-PP, 50 μg/50 μl of RBD-PP or 50 μl of PBS was subcutaneously injected into BALB/c mice, and the IL-6 and IFN-γ levels in serum samples at 2 and 24 h after injection were detected using mouse IL-6 and IFN-γ ELISA kits (Invitrogen). The serum levels of alanine aminotransferase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP) and urea nitrogen (BUN) were detected using an automated analyzer (Chemray-800) on 6h and 7 days after injection.

Statistical analysis

Statistical analyses were performed with Prism 8.0 (GraphPad software). Specific methods used to assess the statistical significance of differences between groups are indicated in the legend. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, ns, no significance.

Results

ClyA porin carrying RBD forms RBD-PP

We expressed the fusion protein of a bacterial porin, ClyA, with SARS-CoV-2 RBD in *E. coli* and achieved highly ordered assemblies of the fusion protein ClyA-RBD by utilizing the self-assembly function of ClyA on the outer membrane. We extracted the bacterial outer membrane and gradually inserted surfactants to replace the bacterial outer membrane while maintaining the nanopore structure of ClyA. We obtained RBD-modified nanopores, called ClyA-RBD nanopores, which finally polymerized into the stable RBD-PP (Figure 1A). The endotoxin level of purified RBD-PP was < 0.25 EU/ml. The correct folding of the protein is very important for vaccine design. The modeling results show that ClyA and RBD fold separately without interfering with each otherand form their respective correct protein conformations, and the RBD and the RBM in it are completely exposed [29] (Figure 1B). Compared with bacterial whole cells, the ClyA polymerized porin (PP) and ClyA-RBD polymerized porin (RBD-PP) we harvested after purification exhibited single protein bands (Figure 1C). The S1 antibody against SARS-CoV-2 was used to verify that the obtained RBD-PP contained RBD protein (Figure 1D). The nanopores formed on the bacterial outer membranes that overexpressed ClyA-RBD, and the analysis of the particle size of the ClyA-RBD outer membrane (RBD-OM) by DLS revealed a peak at ~384.9 nm. The existence of these nanopores can be observed on the bacterial membrane by TEM. After surfactant insertion, the outer membrane is gradually

replaced, and the self-assembly of ClyA is realized [19, 30]; hence, we observed two DLS peaks, one at approximately 13.14 nm and one at approximately 324.9 nm, eventually forming the stable RBD-PP, which was approximately 13.9 nm in the DLS data (Figure 1E). When ClyA completes self-assembly, it carries RBD to form a polymerized pore structure (Figure 1F red part). We observed a possible and very few single-pore structures under TEM, and the morphology of RBD-PP was different from that of PP. It can be seen that RBDs may polymerize together. This single nanopore structure intermediate finally forms a stable polymerized porin. It can be seen from the TEM that RBD-PP is larger and the edges become rougher than PP, which may be due to the RBD polymerization display (Figure 1F). DLS analysis also proved that the particle size of RBD-PP is larger than that of PP (Figure 1G). To prove the polymeric state of RBD-PP, we performed SDS-PAGE on denatured or nondenatured samples. The results confirmed the polymeric structures of both RBD-PP and PP (Figure 1H). Most importantly, polymerized RBD-PP shows stronger binding with hACE2 than the RBD monomer (Figure 1I). This shows that RBD-PP exposes a high degree of RBD polymerization on the surface, and also proves that RBD polymerization has a stronger binding ability to hACE2 than RBD monomer. We speculate that the enhanced binding ability of RBD-PP with hACE2 is due to the formation of a natural spatial conformation closer to that of the viral spike after RBD polymerization. Because the RBD expressed in *E. coli* lacks glycosylation modification, the spatial conformation of the RBD expressed in *E. coli* is particularly important, which is related to whether this RBD can induce neutralizing antibodies against the virus. To analyze the conformation of the polymerized RBD displayed on RBD-PP, we used 6 kinds of neutralizing antibodies that have been shown to recognize the spatial epitope of the virus to recognize RBD-PP. The experimental results proved that 4/6 antibodies can recognize RBD-PP, whereas these antibodies have almost no ability to recognize RBD monomers (Figure 1J). This result indicates that the polymerization display of RBD on RBD-PP may form a spatial conformation similar to that of natural virus spikes that is not formed by RBD monomers lacking glycosylation. Therefore, it is suggested that the polymerized RBD is closer to the natural conformation of the virus.

RBD-PP targets lymph nodes and enhances antigen processing

Antigen lymph node delivery mediated by the vaccine delivery system can enhance the immune response ability of the antigen, and in principle, a nanostructure can enter the lymph node efficiently. As expected, we found that RBD-PP can easily enter the lymph nodes due to its size of approximately 13.9 nm. It can be seen by fluorescence tracer *in vivo* that RBD-PP can enter lymph nodes more efficiently than RBD protein at 12 h after subcutaneous injection (Figure 2A). Compared with free dyes, RBD-PP reduces liver fluorescence and highly targets lymph nodes while reducing systemic toxicity (Figure 2B). In addition, we observed that compared to the free RBD form, the vaccine form of RBD-PP greatly improved the uptake of RBD by BMDCs (Figure 2C). To collect lymph nodes, we used flow cytometry to analyze the uptake and processing of antigens by DCs, and the results showed that Cy7-labeled RBD-PP was efficiently taken up by DCs ($CD11c^+MHC\text{-}II^+$) (Figure 3D). At the same time, RBD-PP efficiently promoted a significant increase in the proportion of $CD80^+$ (Figure 3E) and $CD86^+$ DCs (Figure 3F). It has been proven that the

vaccine form of RBD-PP effectively promotes lymph node targeting and promotes the uptake and processing of antigens by DC cells, which is critical for improving vaccine efficacy. More importantly, RBD-PP significantly increased the proportion of T follicular helper (Tfh) cells (Figure 2G) and germinal center (GC) B cells (Figure 2H) in lymph nodes of mice after a single immunization. Tfh cells can promote the production and maturation of neutralizing antibodies by helping B cells. These results show the advantage of RBD-PP in inducing an antibody response.

RBD-PP can elicit anti-SARS-CoV-2 neutralizing antibodies

Mice were vaccinated with RBD-PP on days 0, 14, and 28 of the experiment. Serum were collected 7 days after each vaccination. Anti-RBD IgG antibodies were significantly expressed in the serum of mice vaccinated with RBD-PP, and the levels were dose dependent. The 50 μg dose of RBD-PP can induce binding antidody titer of 10^3 after the second immunization, and the binding antibody titer is as high as $10^{4.8}$ after three immunizations. RBD-PP at doses of 5 μg and 0.5 μg also induced significant binding antibody titers after the third immunization, reaching $10^{3.5}$ and$10^{2.7}$, respectively. The RBD monomer can only induce significant binding antibody titer of 10^4 after immunization with a dose of 50 μg three times. This shows that compared to the RBD monomer, RBD-PP can efficiently induce B cell responses (Figure 3A). To show that RBD-PP induced anti-RBD antibodies, the RBD-PP antiserum was used to identify S1 and RBD proteins expressed in HEK293 cells (Figure 3B). The experimental results showed that the antibody induced by RBD-PP can correctly recognize S1 and RBD. In addition, at the 9th week after immunization, RBD-PP can still maintain a high binding antibody titer, but the RBD monomer has dropped significantly (Figure 3C). The IgG antibody subtypes IgG1 (Figure 3D) and IgG2a (Figure 3E) induced by RBD-PP were also stronger than those induced by RBD monomers. We next detected the ability of the RBD-PP antiserum to block S1/ACE2 binding. The experimental results showed that, compared with the RBD monomer, the serum of mice vaccinated with 50 μg or 5 μg RBD-PP can block S1/ACE2 binding (Figure 3F). Most importantly, the anti-RBD monomer antiserum collected after three immunizations with the highest dose (50 μg) did not induce significantly different neutralizing antibodies but showed only an increasing trend of neutralizing antibodies. In contrast, RBD-PP induced obvious neutralizing antibodies after immunization with a dose of 50 μg twice or with doses of 5 μg three times (Figure 3G).

We also compared the ratio between the neutralizing antibody titer and the binding antibody titer of the serum after three immunizations with a dose of 50 μg[11]. The results showed that this titer ratio induced by RBD-PP increased significantly (Figure 3H). This indicates that the increase in the neutralization titer of the RBD monomer is due to the increase in the titer of the binding antibody. Then, in the case of the same binding antibody, RBD-PP can induce stronger neutralizing antibodies, which shows that RBD-PP has a spatial conformation more similar to that of natural viruses. Therefore, RBD-PP induces more neutralizing antibodies, while the RBD monomer can induce only some binding antibodies with no neutralizing effect. Viral mutation is a challenge to vaccine design, so we also tested the neutralizing antibody titers of the RBD-PP antiserum against variant virus. The results showed that the antibodies induced by RBD-PP could also neutralize SARS-CoV-2 B.1.617.2 (Delta) variant (Figure 3I), but their ability

to neutralize virus decreased by 6.4-fold (Figure 3J). This may be related to the mutation of the RBD site[31].

RBD-PP Can elicit a cellular immune response and immune memory

The cellular immune response plays an important role in the fight against SARS-COV-2 infection [32]. Therefore, we examined the ability of RBD-PP to elicit cellular immune responses. Flow cytometry experiments on splenocytes from mice immunized with RBD-PP showed that all splenocytes had $CD4^+$ (Figure 4A) or $CD8^+$ (Figure 4B) cellular responses to the SARS-CoV-2 RBD protein. Both the CD4+ and CD8+ T cell responses to RBD-PP are stronger than those to RBD monomer. In addition, RBD-PP induced the responses of Th1 (Figure 4C) and Th2 (Figure 4D) cells in T helper cell populations in spleen cells, the mixed of Th1 and Th2 immune response may produce a comprehensive immune protection when the virus invades. SARS-CoV-2-specific CTLs play important roles in virus clearance. Therefore, we also analyzed the activation level of CD8+ T cells. Compared with the RBD monomer, RBD-PP efficiently stimulated the activation of antigen-specific multifunctional CD8+ T cells, which was manifested by a significant increase in the proportion of CD8+ T cells released by IFN-γ (Figure 4E), IL-2 (Figure 4F), and TNF-α (Figure 4G).

The splenocyte lymphocytes of mice immunized with RBD-PP secreted IL-2, GzmB, and IFN-γ effector molecules after being stimulated by SARS-CoV-2 RBD proteins (Figure 4H). We isolated lymphocytes from the spleen for ELISpot analysis. The results showed that RBD-PP induced a significant increase in IFN-γ-secreting (Figure 4I) and IL-4-secreting (Figure 4J) T cells in the spleen. These results show that RBD-PP can elicit systemic SARS-CoV-2-specific cellular immune responses. The induction of T cell immune memory is the key to antiviral protection. Therefore, we also determined the ability of the RBD-PP vaccine to induce $CD4^+$ T and $CD8^+$ memory T cells in the spleen using flow cytometry. The results showed that compared with RBD monomer, the RBD-PP vaccine induced higher $CD4^+$ (Figure 4L) and CD8+ (Figure 4M) central memory T ($CD44^{high}CD62L^{high}$, Tcm) cells, consistent with the induction of effector memory T ($CD44^{high}CD62L^{low}$, Tem) cells in $CD4^+$ (Figure 4L) and $CD8^+$ (Figure 4N) cells. The induction of two memory T cells by the RBD-PP vaccine indicates that when the virus invades the body, the immediate antiviral effect will be provoked by Tem, and the activation of protective immunity will be stimulated through the proliferation and differentiation of Tcm. In summary, compared to RBD monomer, RBD-PP were able to induce a stronger T cell immune response.

RBD-PP has excellent in vivo safety

Finally, we preliminarily evaluated the animal safety of RBD-PP. We observed no significant body weight loss (Figure 5A) or temperature fluctuation (Figure 5B) in mice vaccinated with 50 μg RBD-PP during the immunization period. At 2 h and 24 h after subcutaneous injection of 50 μg RBD-PP, no significant increase in the serum inflammatory cytokines IFN-γ or IL-6 was observed (Figure 5C). There was no significant tissue lesion (Figure 5D) after the immunization program was completed. In addition, we tested the metabolic biochemical markers in serum 6 h and 7 days after RBD-PP immunization, and the

experimental results showed that RBD-PP immunization did not cause significant liver or kidney damage (Figure 5E). Therefore, we believe that RBD-PP has excellent *in vivo* safety at a 50 μg dose.

Discussion

The keys to successful SARS-CoV-2 vaccination are efficient antigen processing presentation by APC cells, the induction of SARS-CoV-2-neutralizing antibodies, and the activation of T cell responses and lasting immune memory. Although many SARS-CoV-2 vaccines satisfy these prerequisites and have entered clinical trials [6, 33], their production costs and outputs may limit the widespread application of these vaccines in the global pandemic. *E. coli* is the most productive and least costly protein expression system. Vaccines against SARS [34], dengue fever [26], Middle East respiratory syndrome [35], hepatitis E [36], influenza [37], and human papillomavirus [38] have been successfully expressed as nanostructures in *E. coli*. Therefore, the correct folding or assembly of viral antigens into nanostructures in *E. coli* could elicit potential immune protection, thus enabling the successful development of an inexpensive vaccine in *E. coli*.

The RBD of SARS-CoV-2 is the main mediator of the binding between viruses and the host cell receptor ACE2 [39]. It is also the main target of the SARS-CoV-2 vaccine [8, 40–42]. In its natural conformation, the RBD of SARS-CoV-2 shows a trimeric structure [16, 43]. The host immune response caused by this trimeric structure may not be identical to the host immune response caused by the monomeric structure. RBD dimers [17] and multimers [10, 11] have better immunoprotective ability than RBD monomers. Therefore, multimerization of RBD may be a key step in the design of SARS-CoV-2 vaccines. This study also found that RBD-PP has a stronger ability to bind hACE2 than monomers, suggesting the importance of RBD polymers (Figure 1I).

Because of their scale characteristics, nanovaccines can increase the effectiveness of lymph node targeting and antigen presentation [44]. Their unique structural features can enhance immunogenicity by presenting polyvalent antigens, can stabilize antigens, and can induce adjuvant activities [45]. A variety of organic and inorganic nanoparticles have been used in nanovaccines. For example, antigen-loaded nanomaterials, such as gold [46–48], silica [49, 50], magnetosomes [51], poly(lactic-co-glycolic acid) [52], and polyethyleneimine [53], have been successfully used in vaccines against viruses, tumors, bacteria, and inflammatory and immunoregulatory disorders [54]. However, the application of nanovaccines is often limited by their toxicity, bioincompatibility, instability, scale-up shortcomings, and low interbatch reproducibility. Biopolymerizable nanoparticles that rely entirely on biosynthesis are gradually emerging. The available techniques mainly utilize trimer tags, the C-terminal domain of T4 fibritin, three-helix bundles [55], self-assembling proteins (ferritin [56], lumazine synthase [57, 58], encapsulin [59]), and self-assembling peptides [60–62] to carry large proteins or peptides to form regular polymers. Vaccines based on these polymeric structures have induced excellent immunogenic effects in terms of different aspects [57, 63]. Therefore, it is highly important to continue to develop nanovaccine delivery systems with antigen polymerization.

The site of ACE2 binding to the RBD does not have a glycosylation modification, so it is theoretically possible to design RBD-based vaccines in prokaryotic systems [28]. Here, we used the polymerization function of ClyA to achieve the regular polymerization of RBD in *E. coli*, which seemed to simulate an RBD polymeric structure conformation close to that of the natural virus, thus avoiding the incorrect folding of *E. coli*–expressed RBD. ClyA protein naturally polymerizes in *E. coli* OMVs [18, 23]. The ClyA protein is also widely used in genetic engineering for the translocation of heterologous proteins to modify OMVs [64, 65]. OMVs have many toxic proteins, so there is a safety issue with using OMVs as a vaccine vector. If this type of self-assembled ClyA nanopore can directly carry heterologous proteins and form nanometer-scale structures, the self-assembled ClyA nanopore (approximately 14 nm) would be an ideal vaccine platform because it can easily target lymph nodes and reduce toxicity to other organs (Figure 2A) [44, 66], stimulating stronger antigen presentation. More importantly, the polymerization of ClyA is particularly suitable for the presentation of antigens that need to be polymerized, especially the RBD of SARS-CoV-2. No technology utilizing ClyA nanopores as a vaccine vector has been developed before.

We developed ClyA-RBD, a protein formed by the fusion of ClyA, a porin that is polymerized in bacteria, with full-length RBD. The fusion protein ClyA-RBD is abundantly expressed in the cytoplasm of *E. coli* and can migrate to the periplasm and the outer membrane [23], where the oxidative environment favors its proper folding [15]. ClyA undergoes a conformational rearrangement triggered by the lipid membrane and assembles into pores on the bacterial outer membrane [20, 21]. RBD forms a polymeric structure with ClyA, and ClyA-RBD nanopores can be obtained from the outer membrane of *E. coli* using surfactants [67]. This is because lipid membranes and surfactants can trigger and maintain the conformational rearrangement of ClyA monomers [21, 30, 68]. We utilized this feature to successfully induce the self-reassembly of the ClyA-RBD monomers from membrane in surfactant.

ClyA is a toxic protein that can punch holes in red blood cells to dissolve red blood cells. Therefore, the use of ClyA as a vaccine delivery system also presents safety concerns. However, it is encouraging that ClyA that has formed a nanopore structure on OMV, lacks hemolytic activity and loses the function of further attacking host cells [69–71]. In addition, the toxicity of the ClyA monomer with high hemolytic activity can also be removed through gene mutation [72] or truncation of the C-terminus [73]. The PP for vaccine delivery here was designed to address its safety issues. We allowed the ClyA monomer to form a complete nanopore on the bacterial membrane, thereby losing hemolytic activity. Afterwards, the bacterial membrane was removed with the help of detergents, thus ensuring that the PP loses hemolytic activity without destroying the nanostructure. In our preliminary safety experiments, we did not see obvious safety concerns, nor did we see obvious hemolytic toxicity in major organs (Figure 5).

The keys to the success of this nanopore technique are that (1) the ClyA and RBD components of the ClyA-RBD monomers separately undergo correct folding without forming inclusion bodies, and then the correctly folded oligomers are translocated to the bacterial outer membrane due to the specific secretion of ClyA to the outer membrane, thus ensuring that the correct structures of ClyA and RBD exist on the bacterial outer membrane. (2) The ClyA-RBD polymers on the bacterial outer membrane switch from membrane-triggered to surfactant-triggered[19, 30]. Therefore, stable RBD-PP can be obtained *in vitro*. (3)

The production process of RBD-PP is subject to strict temperature and time constraints. For example, it is critical to control the process of expression in bacteria overnight at 30°C, slow surfactant replacement at 15°C and polymerize into RBD-PP at 37°C.

The RBD-PP vaccine delivery system developed here has the following advantages: (1) ClyA simulates the supporting effect of SARS-CoV-2 S2 on RBD in S1, and the fusion of ClyA and RBD does not interfere with either protein conformation (Figure 1B). Therefore, correct ClyA polymerization also realizes correct RBD polymerization (Figure 1F). (2) Compared with monomeric RBD, the ability of regularly polymerized RBD to bind hACE2 is significantly enhanced, which illustrates the importance of RBD-PP for the efficient polymerization and exposure of RBD (Figure 2I). (3) RBD-PP formed a stable nanostructure of approximately 14 nm (Figure 1F). This size is very conducive to the delivery of antigens to lymph nodes, thus greatly enhancing antigen uptake, processing and presentation (Figure 2). (4) The most important point is that RBD-PP may cause this nonglycosylated modified RBD to display a spatial conformation partially similar to the virus spike structure. (5) RBD-PP has the triple function of inducing humoral immunity, cellular immunity, and immune memory. Neutralizing antibodies play a major role in resisting SARS-CoV-2. It has been proven that neutralizing antibody titers above 100 can effectively resist SARS-CoV-2 infection[28, 74]. However, increasing evidence has also proven the important role of the T cell response and immune memory in the fight against COVID-19 [32, 75]. (6) Our special preparation process not only enables ClyA not only to form polymerized nanostructures but also o inactivates its hemolytic activity, thereby preventing the safety problems of ClyA as a vaccine carrier.

In short, the advantages of RBD-PP are due to its polymerization and display of RBD nanostructures, enabling RBD-PP to exhibit effective immunological activation capabilities, including lymph node targeting (Figure 2A), DC targeting (Figure 2C), induction of Tfh cells (Figure 2G) and GC B cells (Figure 2H). Therefore, the ability of RBD-PP to induce humoral immunity (Figure 3), cellular immunity and immune memory (Figure 4) was stronger than that of the RBD monomer. However, the most important point is that RBD-PP may cause this nonglycosylated modified RBD to adopt a spatial conformation similar to that of the viral spike structure, which is shown by the following evidence: (1) The binding capacity of RBD-PP and hACE2 increased (Figure 1I). (2) Four neutralizing antibodies recognized RBD-PP (Figure 1J). (3) The ratio of neutralizing potency to binding potency increased significantly (Figure 4I). In summary, we have developed a RBD-PP vaccine that achieves high RBD polymerization in *E. coli*, which will provide a design for an inexpensive vaccine against SARS-CoV-2 (Figure 6).

Bacterial PPs have not previously been utilized as vaccine vectors. We developed this PP-based vaccine vector platform and conducted a complete *in vitro* and *in vivo* evaluation. This also suggests that other proteins that can assemble into pores may be modified in the same way to form vaccine vector platforms for wider application. Because of the urgency of COVID-19 vaccine development, we tested the PP platform to make a vaccine against SARS-CoV-2, but different nanopores are likely to be developed in the future and applied as nanoplatforms in this and other fields, including viral vaccines, bacterial vaccines, tumor vaccines, drug delivery, and disease diagnosis.

Conclusions

We designed this nanopore by using the principle of ClyA porin polymerization triggered by the cell membrane and used surfactants to "pick" the ClyA-RBD nanopore from the bacterial outer membrane in this study. This was a great improvement compared to the RBD-BBV vaccine we previously reported. More importantly, the polymerized RBD displayed on RBD-PP already has some correct spatial structures of virus spikes. This RBD-PP promotes antigen lymph node targeting and uptake by DCs and effectively induces the production of anti-SARS-CoV-2 neutralizing antibodies and systemic cellular immune responses and immune memory in mice.

Declarations

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Author contributions

Z.Y., and L.H. performed the experiments, analyzed the data, drafted the manuscript and performed statistical analysis. M.Y. performed viral neutralization experiments. W.L., Z.R., X.Z., H.C., Q.L., and H.B. performed cellular and animal experiments. W.H. designed and performed the experiments, analyzed the data, drafted the manuscript, and performed statistical analyses. Y.M. provided guidance and support for this project and reviewed the manuscript.

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Availability of data and materials

The data used and/or analysed to support the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All animal procedures were approved by the Institutional Animal Care and Use Committee of the Institute of Medical Biology, Chinese Academy of Medical Sciences.

Consent for publication

All authors agreed to publish this manuscript.

Competing interests

The authors declare no competing financial interests.

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Figures

Figure 1

ClyA porin carrying RBD forms RBD-PP. (A) Schematic diagram of the preparation of RBD-PP. (B) Schematic diagram of the ClyA-RBD fusion protein. Red represents the receptor-binding motif (RBM). (C) After SDS-PAGE, the total bacterial protein products and purified PP and RBD-PP samples were analyzed by silver staining. The red arrow labels ClyA , and the blue arrow labels ClyA-RBD. (D) Western blotting

was performed using an anti-SARS-CoV-2 spike S1 antibody. (E) The distributions of particle sizes of bacterial outer membrane overexpressed ClyA-RBD (RBD-OM), the intermediate process of the surfactant being gradually inserted into the outer membrane and self-assembly, and purified RBD-PP. In a representative TEM image of RBD-OM, the small white circles in the white box mark the nanopores on the outer membrane. Bar=100 nm. (F) PyMOL was utilized to visualize homology modeling results; at the top is a schematic representation of the polymeric chains formed by monomers. Red represents RBD, and green represents the linkers. In the middle is the sporadic nanopore structure observed under TEM, bar=10 nm. At the bottom is the final stable RBD-PP and PP electron microscope image, and the bottom left corner is an enlarged photo of the white frame. (G) The top panel shows DLS analysis of RBD-PP and PP, and the bottom panel shows the statistical analysis of particle size (n=3). (H) The denatured or nondenatured samples were analyzed by SDS-PAGE for ClyA and ClyA-RBD. The arrow indicates the position of the denatured monomer and nondenatured polymer. (I) The binding ability of RBD-PP with hACE2 (n=3). (J) Six monoclonal antibodies that can neutralize SARS-CoV-2 were reacted with RBD-PP (n=3). The half-maximal inhibitory concentrations (IC_{50} values) of MM43-, MM57-, R004-, R001-, D001-, and D002-neutralizing antibodies were 1.41 μg/ml, 0.41 μg/ml, 0.234 μg/ml, 0.11 μg/ml, 0.646 μg/ml, and 46.76 μg/ml, respectively. Data are shown as the mean ± SD. Statistical significance was calculated via one-way ANOVA (J), two-way ANOVA (I) or Student's *t*-test (G), giving *p* values, **** $p < 0.0001$, and * $p < 0.05$.

Figure 2

RBD-PP targets lymph nodes and enhances antigen processing. (A) Twelve hours after the subcutaneous injection of 10 μg Cy7-labeled samples from each group, the heart (H), liver (Li), spleen (S), lung (Lu), kidney (K) and inguinal lymph nodes (LN) were collected and observed by live imaging. (B) The fold change of the fluorescence intensity relative to the free Cy7 control group (n=3). (C) BMDCs uptake. Representative images with green representing the antigen RBD, red representing CD11c, and blue representing DAPI-stained nuclei. (D) Twelve hours after the subcutaneous injection of 10 μg Cy7-labeled samples from each group, flow cytometry analysis of representative graphs of $Cy7^+CD11c^+MHC\text{-}II^+$, (E) $CD80^+CD11c^+MHC\text{-}II^+$, and (F) $CD86^+$ $CD11c^+MHC\text{-}II^+$ cells in lymph nodes (n=3). (G) Ten days after mice were immunized with 50 ug different vaccines, the percentage of T follicular helper (Tfh) cells ($CD3^+CD4^+CXCR5^+PD\text{-}1^+$) and (H) germinal center (GC) B cells ($CD19^+B220^+CD95^+$ $GL7^+$) in lymph nodes were measured by flow cytometry (n=3). Data are shown as the mean ± SD. Statistical significance was calculated via one-way ANOVA (B, D-H), giving *p* values, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Figure 3

RBD-PP can elicit anti-SARS-CoV-2-neutralizing antibodies. Mice were vaccinated with various vaccines on days 0, 14, and 28 of the experiment. Serum was collected 7 days after each vaccination. (A) Detection of anti-RBD IgG titer in mouse serum using ELISA (n=3). (B) The left panel shows S1 and RBD proteins expressed in HEK293 cells. The right panel shows the results of a Western blot using diluted anti-RBD-PP and anti-ClyA serum (1:1000 dilution). (C) The time-course curve of anti-RBD IgG titer elicited with different vaccines (n=3). (D) Detection of anti-RBD IgG1 and (E) IgG2a titer in mouse serum using ELISA (n=3). (F) Antiserum collected after three immunizations with different vaccines to block the binding of S1 and ACE2, denoted by the OD450 (n=10). (G) The neutralizing activity of the immune serum was evaluated with SARS-CoV-2 wild-type (WT) virus, and the 50% neutralization titer (NT_{50}) was determined (n=3). (H) The ratio of NT_{50} titer to endpoint titer after three immunizations with 50 μg dose of vaccines (n=3). (I) The neutralizing activity of the immune serum was evaluated with SARS-CoV-2 B.1.617.2 (Delta) variant, and the NT_{50} was determined (n=3). (J) Serum after three immunizations with RBD-PP 50 μg, comparison of the NT_{50} between SARS-CoV-2 wild-type and SARS-CoV-2 B.1.617.2 (Delta) variant (n=3). The dotted horizontal lines represent the lower limit of detection of the assay. Data are shown as the mean ± SD. Statistical significance was calculated via one-way ANOVA (A, D, E, G, I), two-way ANOVA (C) or Student's *t*-test (F, H, J) , giving *p* values, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Figure 4

RBD-PP can elicit a cellular immune response and immune memory. Mice were immunized three times with 50 μg of different vaccines. Fourteen days after the last immunization, splenocytes were isolated and stimulated with the RBD protein expressed in HEK293 cells. Flow cytometry was used to analyze the ratio of RBD-specific (A) $CD4^+$ T ($CD3^+CD4^+$), (B) $CD8^+$T ($CD3^+CD8^+$), (C) Th1 ($CD3^+CD4^+IFN\text{-}\gamma^+$), and (D) Th2 ($CD3^+CD4^+IL\text{-}4^+$) cells. The ratio of multifunctional $CD8^+$ T cells, which is expressed as $CD8^+$ T cells that release (E) IFN-γ, (F) IL-2, and (G) TNF-α, was detected (n=3). (H) Splenocytes of immunized mice were stimulated with RBD protein expressed in HEK293, and the culture supernatant after stimulation was used to analyze the release of cytokines (n=3). (I) Representative ELISpot images of spots corresponding to and bar graphs of IFN-γ- and (J) IL-4-secreting lymphocytes in RBD-stimulated splenocytes of immunized mice (n=3). (K) Flow cytometric analyses of memory T cells in splenocytes from immunized mice. Bar graphs summarizing the flow cytometry results for $CD4^+$ central memory T cells ($CD3^+CD4^+CD44^{high}CD62L^{high}$) (n=3), (L) $CD4^+$ effector memory T cells ($CD3^+CD4^+CD44^{high}CD62L^{low}$) (n=3), (M) $CD8^+$ central memory T cells ($CD3^+CD8^+CD44^{high}CD62L^{high}$) (n=3), and (N) $CD8^+$ effector memory T cells ($CD3^+CD8^+CD44^{high}CD62L^{low}$) (n=3) in the spleen. Data are shown as the mean ± SD. Statistical significance was calculated via one-way ANOVA (A-N), giving *p* values, **** $p < 0.0001$, *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$.

Figure 5

RBD-PP has excellent *in vivo* safety. (A) Body weight and (B) body temperature during the entire immunization program using 50 μg RBD-PP (n=3). (C) ELISA of the levels of the serum inflammatory cytokines IFN-γ and IL-6 2 h and 24 h after subcutaneous injection of 50 μg RBD-PP (n= 3). (D) Representative images of HE-stained mouse tissue sections after the immunization program were completed. (E) Analysis of metabolic biochemical markers in serum. Six hours and 7 days after the mice were subcutaneously injected with RBD-PP (50 μg), the serum levels of ALT, AST, ALP and BUN were detected (n=3). The gray area refers to the fluctuation range of the normal level. Data are shown as the mean ± SD. Statistical significance was calculated via Student's *t*-test (C, E), ns, no significance.

Figure 6

The design principle of RBD-PP vaccine. The ClyA-RBD monomer assembled into pores on the outer membrane of the bacteria. Consequently, RBD successfully formed a polymeric structure on the membrane in the form of ClyA-RBD. ClyA-RBD nanopores were displaced from the membrane with surfactants to form stable polymerized porin. This highly polymerized RBD-PP was used as a vaccine to induce anti-SARS-CoV-2 neutralizing antibodies and T cell responses in mice.

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