Supporting Information

Peptide-based Subunit Candidate Vaccines against SARS-CoV-2 Delivered by Biodegradable Mesoporous Silica Nanoparticles Induced High Humoral and Cellular Immunity in Mice

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Methods

Analysis of B cell and T cell epitopes by the IEDB

B cell and T cell epitopes derived from SARS-CoV-1 were searched on the IEDB by querying for the organism "SARS-CoV-1 (ID:10002316)" and for the antigen "Spike glycoprotein [P59594]" from "Any" host. For B cell epitopes searching, we limited the positive outcome for B cell assay including ELISA qualitative binding or biological activity neutralization or antigen inhibition qualitative binding. The IEDB's Immunobrowser tool[1] was used for identifying the dominant regions of the "Spike glycoprotein [P59594]" considering residues stretches where the response frequency score (RF) was >0.37. For T cell epitopes searching, we limited the positive outcome for (i) either T cell assay including enzyme-linked immune absorbent spot (ELISPOT) or intracellular cytokine staining (ICS) or ELISA IFN-γ release (ii) or MHC binding assays including cellular MHC/direct/fluorescence qualitative binding or purified MHC/competitive/radioactivity dissociation constant KD (~IC50). And the B cell and T cell epitopes derived from SARS-CoV-2 were also searched on the IEDB by querying for the organism "SARS-CoV-2 (ID:2697049)" and for the antigen "Spike glycoprotein [P0DTC2]" from "Any" host by the same procedure as above.

Comparison of SARS-CoV-1 epitope sequences to SARS-CoV-2

The IEDB's Epitope Analysis Tool "Conservation Across Antigens"[2] was used to compare theSARS-CoV-1 epitope sequences obtained from IEDB following the above procedure to SARS-CoV-2. The protein sequence of surface glycoprotein (NCBI:YP_009724390.1) derived from the Wuhan-Hu-1 virus isolate (GeneBank:MN908947) was used as the template and the sequence

identity threshold was set to be ≥20%.

Prediction of B cell and T cell epitope of spike protein from SARS-CoV-2

The BebiPred 2.0 (Jespersen et al., 2017) algorithm available in IEDB was used for prediction of linear epitopes from SARS-CoV-2 spike proteins (S) (NCBI: YP_009724390.1). A threshold value of 0.5 (corresponding to specificity 0.57 and sensitivity 0.59) was taken into account for potential linear B cell epitopes prediction. By using the SARS-CoV-2 spike glycoprotein structure (PDB ID: 6VSB) as a template, the Discotope 2.0[3] from IEDB was used for structure-based antibody prediction with a threshold value of -3.7 (corresponding to specificity 0.75 and sensitivity 0.47).

A TepiTool "NetMHCpan EL 4.0"[4] from IEDB was used for $CD8^+$ T cell epitope prediction. The set of epitopes associated HLA class I referred to the reference sets provided by Weiskopf, D. et al.[5] which were prepared using the following criteria: (i) the most common specificities in the general population, based on data available from DbMHC and allelefrequencies.net. (ii) representative of commonly shared binding specificities (i.e., supertypes). The specific alleles included were: HLA-A*01:01, HLA-A*02:01, HLA-A*02:03, HLA-A*02:06, HLA-A*03:01, HLA-A*11:01, HLA-A*23:01, HLA-A*24:02, HLA-A*26:01, HLA-A*30:01, HLA-A*30:02, HLA-A*31:01, HLA-A*32:01, HLA-A*33:01, HLA-A*68:01, HLA-A*68:02, HLA-B*07:02, HLA-B*08:01, HLA-B*15:01, HLA-B*35:01, HLA-B*40:01, HLA-B*44:02, HLA-B*44:03, HLA-B*51:01, HLA-B*53:01, HLA-B*57:01, HLA-B*58:01. This reference set for class I should provide population coverage of >97%. The SARS-CoV-2 S protein sequence with a size range of 8-11merswas run against this set of alleles using the NetMHCpan EL 4.0.and epitope peptides with prediction score of >0.3 were selected for immunogenicity analysis using MHC I Immunogenicity tool from IEDB.

Selection and synthesis of linear B cell and $CD8^+$ T cell epitopes

The linear B cell and CTL cell epitopes of SARS-CoV-1 S protein obtained from IEDB were analyzed as above and dominant epitopes which contain CTL epitopes or have high conservation respectively within coronaviruses were selected for homologous comparison to SARS-CoV-2. Seven linear B cell epitopes (designated as B) including four potential B cell epitopes (designated as BS1 including B1, B2, B3, B4) from S1 subunit and three B cell epitopes (denoted as BS2 including B5, B6, B7) from S2 subunit and three potential CTL epitopes (designated as T

including T1, T2, T3) were screened out. The position of all the selected epitopes was visualized by using Swiss-PdbViewer4.1 with a 3D-rendering structure of SARS-CoV-2 spike glycoprotein (PDB: 6VSB). The selected 10 epitope peptides (3 T cell and 7 B cell) with purity of >95% were synthesized and purified *in vitro* by Jill biochemical (Shanghai) Co., Ltd.

Preparation and Characterization of BMSNs

BMSNs were synthesized according to our previous work[6]. Briefly, 0.2g Cetyltrimethylammonium bromide (CTAB, Thermo Fisher Scientific) and 175.7mg Na_2HPO_4 were dissolved in 70mL deionized water, followed by 0.27mL ammonia and 20mL ethanol added. After magnetic stirring at 40℃ for 30 minutes, 1mL Tetraethyl orthosilicate (TEOS, Sinopharm) was added. Then, 82.5mg $CaCl_2$ was immediately added and reacted for 24h at 40℃. Finally, BMSNs-CTAB particles were obtained by washing with ethanol and deionized water at 8000rpm for three times. The surfactant CTAB was removed by calcination with the heating rate of 1℃/min, and the temperature was kept at 550℃ for 6h.

To track the distribution of nanoparticles, BMSNs were functionalized with green fluorescein isothiocyanate (FITC, Alfa Aesar) according to the previous reported methods[7]. The FITC-labeled nanoparticles were denoted as BMSNs-FITC.

Transmission electron microscopy (TEM) images of BMSNs were taken on Tecnai G2 F20 (FEI, American) at 200kV. The small angle X-ray diffraction (XRD) pattern was recorded on D8 Advance (Bruker, Germany) by continuous scanning mode from 0.6° to 6° at a scanning interval of 0.02°. The nitrogen (N_2) adsorption desorption isotherm and pore size distribution was operated on ASAP 2020 instrument (Micromeritics, USA) by static adsorption at 77K.

Loading and Release of epitope antigens into BMSNs

BMSNs (10mg) were fully mixed with 0.2mg/mL individual antigen peptide solution separately and adsorbed for 24h at room temperature. Centrifugation was performed at 8000rpm for 8min, washed twice with deionized water to remove the non-adsorbed peptide. Each epitope peptide loaded particles, marked as B@BMSNs or T@BMSNs (B stands for B1, B2, B3, B4, B5, B6 or B7. T stands for T1, T2 or T3), was collected by vacuum freeze-drying. BS1/T@BMSNs means mixture of individual B1-B4 loaded BMSNs and individual T1-T3 loaded BMSNs. Meaning of

BS2/T@BMSNs and BS1/BS2/T@BMSNs similar to BS1/T@BMSNs. Loading amount (mg/g) of individual peptide was calculated separately by the absorption value of the supernatant measured by UV-vis spectrometer.

The peptide-loaded nanoparticles (B2@BMSNs, B6@BMSNs and T3@BMSNs) were released in PBS solution at 37℃ with pH value of 5.0 and 7.4, respectively. The supernatant was collected at 1h, 3h, 5h, 7h, 9h, 12h, 24h and 48h. The cumulative release rate (%) was calculated by the absorbance value of the supernatant detected by UV-vis spectrometer.

***In Vitro* Cytotoxicity**

The toxicity of BMSNs, B/T and B/T@BMSNs on RAW264.7 was analyzed by CCK-8 assay. BMSNs with various concentrations (1, 5, 10, 25, 50, 100μg/mL) and 100μg/mL B/T@BMSNs with the corresponding concentration antigens of B1, B2, B3, B4, B5, B6, B7, T1, T2, T3 were cultured with RAW264.7 for 24h. After treated with cell counting kit-8 (CCK-8, DOJINDO), the absorbance value was measured by microplate spectrophotometer at 450nm.

***In Vitro* Endocytosis**

The endocytosis of T3 and T3@BMSNs in RAW264.7 cells was observed by confocal laser scanning microscopy (CLSM, ZEISSLSM880). T3@BMSNs with a concentration of 50μg/mL in DMEM was added into the cultured RAW264.7 cells. After two hours incubation, the supernatant was discarded and the RAW264.7 cells were washed for three times by PBS and continuously cultured with fresh DMEM for another 6h, 12h and 24h, respectively. Equivalent concentration of T3 were cultured with RAW264.7 cells for 6h. Then the treated cells were fixed with paraformaldehyde and nucleus was stained by DAPI (blue). The samples were observed under CLSM after sealed with a ProLong Antifade Kit (Life Technologies).

***In vivo* biosafety assessment of B/T@BMSNs**

Specific pathogen-free (SPF) female BALB/c mice (6 to 8 weeks) were used for biosafety assessment of B/T@BMSNs *in vivo*. PBS group and BMSNs group were intramuscular immunized with PBS (50μL) and B/T@BMSNs nanoparticles (50μL, 1mg/mL), respectively. The living state of mice was observed and their body weight change was recorded for 14 days after immunization. Three mice from PBS or BMSNs groups were euthanized, and mice tissues

including heart, liver, spleen, lung, kidney, stomach, brain and muscle, were separated and fixed overnight at 4℃. After embedded in paraffin, the tissues were processed by routine histopathological techniques for hematoxylin and eosin (H&E) staining. The sections were photographed under optical microscope and analyzed for tissue damage.

Immune response induced by B/T@BMSNs nanoparticles

The mice of three experimental groups including BS1/T@BMSNs, BS2/T@BMSNs and BS1/BS2/T@BMSNs were intramuscular immunized twice with corresponding peptide-loaded nanoparticles (1.5μg each antigen/ mouse/ 200μL). The mice of control groups were injected with PBS or BMSNs respectively with the same method as experimental groups. As shown in Fig. S1, on the 14th day after the second immunization, the sample of serum and spleens were collected for humoral and cellular immunity analysis.

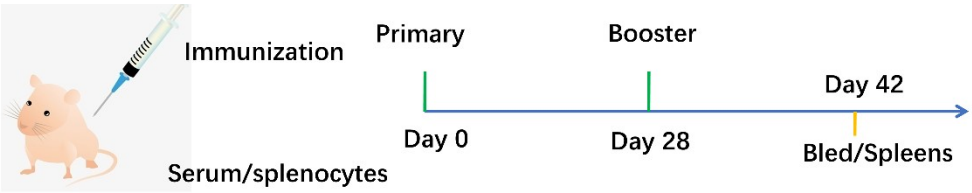


Fig. S1 Schematic diagram of mouse inoculation procedure

Evaluation of humoral immunity of B/ T@BMSNs nanoparticles

Mice serum was centrifugated at 3000rpm for 10min and peptide-specific and recombinant S protein-specific antibodies were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, 96-well microtiter plates (Corning, NY, USA) were coated with each peptide (B1, B2, B3, B4, B5, B6, B7), mixed peptides (BS1 and BS2) or S1+S2 recombinant protein (40589-V08B1, SinoBiological) in PBS with 200μL per well at 4℃ overnight. Plates were washed three times with PBS-T (PBS solution containing 0.05% Tween-20) and blocked (PBS-T containing 3% BSA) at 37℃ for 2h. Plates were washed again followed by adding serial diluted sera and incubated at 37℃ for 2h. After washing, HRP-conjugated goat anti-mouse secondary antibodies (IgG, SounthernBiotech) with 100μL/well at a dilution of 1:10000 were added into the plates and incubated at 37℃ for 1h. After final washing, plates were developed using TMB (3, 30, 5, 50-tetramethylbenzidene) (Sigma) substrate in a 100μL volume for each well and incubated for 15-20min at room temperature. The reaction was stopped with TMB stop solution and plates were read on ELISA reader (synergy H1, Bio-Tek) within 30min at 450nm.

Mice serum IgG antibody competition against ACE-2 was also performed by ELISA with a similar process as above. ELISA plates were coated with full length (S1+S2) spike protein containing a C-terminal His tag (cat. 40589-V08B1, Sino Biologics) at 2μg/mL with 100μL/well. After sealing with blocking solution, the experiments were divided into three groups. For the first group, ACE-2 with a human Fc tag at various concentrations was added and incubated at 37℃ for 2h. For the second group, mice serum (1:40 dilution) was firstly added to ELISA plate to bind to S1+S2 recombinant protein, and then ACE-2 with different concentrations was added. For the last group, after plates were incubated with serial diluted serum (20, 40, 80, 160, 320), 100ng/mL ACE-2 was added. And then, Goat Anti-Human IgG Fc-HRP (SouthernBiotech) secondary antibodies at a dilution of 1:10000 were added and incubated at room temperature for 1 h. After final wash plates were developed using TMB Substrate and the reaction stopped with TMB stop solution. Plates were read at 450nm using ELISA reader (synergy H1, Bio-Tek) within 30min and competition curves were plotted using originpro 8.0 software.

Determination of immune response type by cytokines and antibody isotypes assay

The Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-5) type cytokines in the cultured splenocytes supernatants were quantitatively measured by ELISA as previously described[8]. Briefly, splenocyte suspensions were prepared from the spleens of individual vaccinated mice using Mouse 1×Lymphocyte Separation Medium (DKW33-R0100, Dakewe Biotech). Splenocytes (1×10^6) were cultured in a 24-well culture plate (Corning, NY, USA) in the presence or absence of the selected CTL epitope peptides (T1, T2, T3) and the PMA+Ionomycin (cat. 2030421, Dakewe Biotech) were used as the positive stimulant. Cultures were maintained in a 5% CO_2 incubator at 37℃ for 72h and the supernatants were collected and stored at -80℃ for subsequent assays. The cytokine levels (IFN-γ, IL-2, IL-10 and IL-4) in the splenocytes supernatants were detected by the respective ELISA kits (Thermo Fisher Scientific), as recommended by the manufacturer.

The different IgG antibody isotypes (IgG1, IgG2a and IgG2b) were also determined by ELISA to identify the differential Th2 and Th1 immune response induced by B/T@BMSNs nanoparticles. Briefly, serial dilutions of mouse sera on Day 42 were incubated in the plates coated with BS1 and BS2 respectively (200ng/well). Following washes, goat anti-mouse IgG1, IgG2a and IgG2b-HRP conjugated (1071-05, 1081-05 and 1091-05, SouthernBiotech) were added to the plates and incubated at 37℃ for 1h. The TMB (Sigma) was used as the substrate and the optical density at

450nm was measured. Antibody titers were defined as the dilution at which the mean absorbance of the sample was 2.1-fold greater than that of the control serum and the ratio of IgG(2a+2b)/IgG1 was calculated.

Statistical analysis

The experimental results were analyzed by originpro 8.0 software and expressed as mean ± standard deviation (SD). Two-tailed Student's t test and one-way ANOVA were used and p-values less than 0.05(*p<0.05, **p <0.01, and ***p<0.001) were considered to be statistically significant.

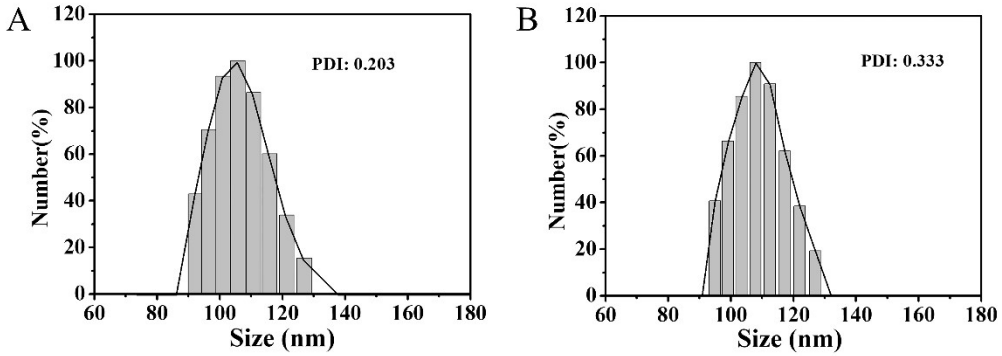
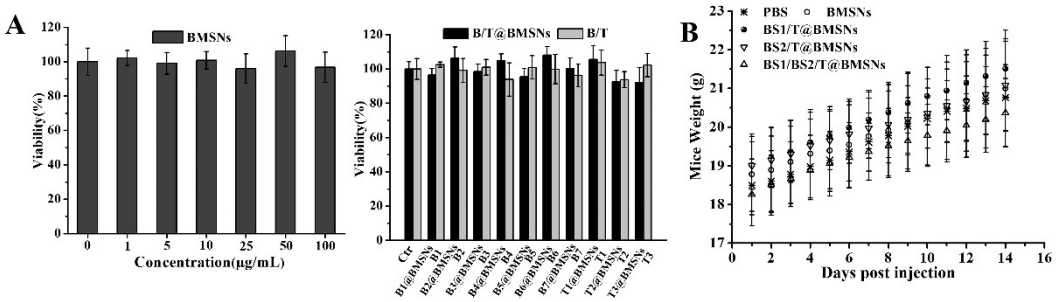


Fig. S2 Hydrodynamic particle size distribution of (A) BMSNs and (B) peptide loaded BMSNs (T3@BMSNs as representative example).



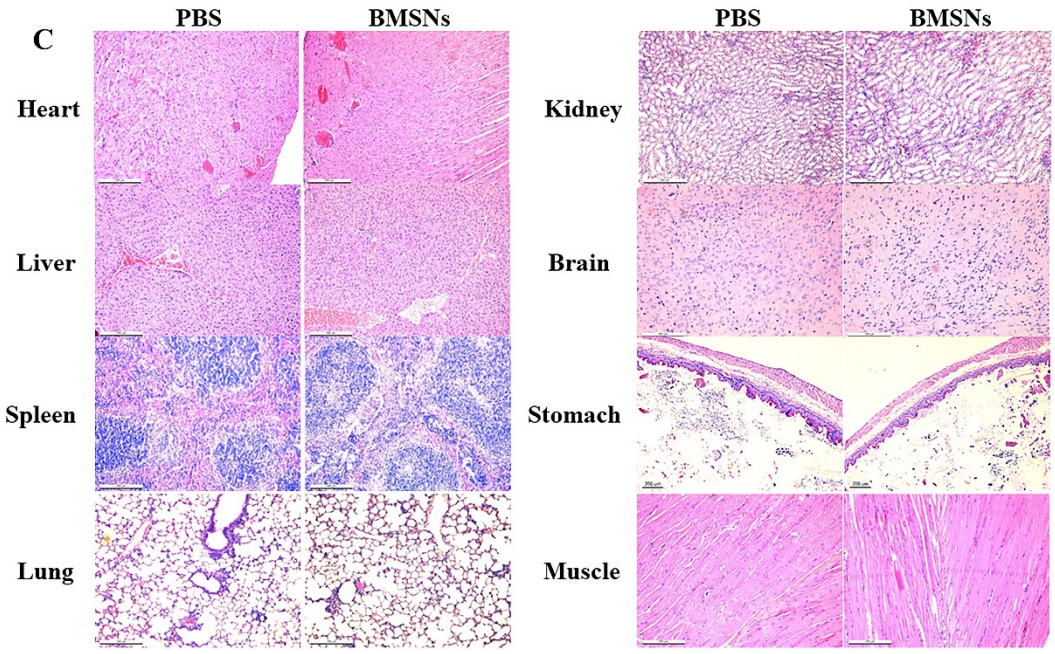


Fig. S3 (A) Cytotoxicity of BMSNs with different concentrations and B/T@BMSNs, B/T with concentration of 100ug/mL on Raw264.7. (B) Body weight diversity curve with time for mice. (C) Pathological section of heart, liver, spleen, lung, kidney, brain, stomach and muscle from mice of PBS and BMSNs groups. Scale bar=200μm (n=5).

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