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Recombinant VLPs empower RBM peptides showing no immunogenicity in native SARS-COV-2 protein to elicit a robust neutralizing antibody response

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Abstract

New SARS-COV-2 vaccine strategies are still urgently needed, especially for emerging virus mutations and variants. In this study, we focused on analyzing the antigenicity and vaccine potency of linear peptide epitopes located in receptor binding motif (RBM) of spike (S) protein. Nine 12 to 16-mer overlapping peptides (P1-P9) were synthesized chemically and coupled to carrier protein KLH for the immunization in mice. Four of identified peptides were further engineered to present on the surface of recombinant Hepatitis B core antigen (HBcAg) virus-like particles (VLPs) respectively. Antisera obtained from VLPs -immunized mice demonstrated strong reactivity and affinity to S1 protein or inactivated virus and neutralizing activity against virus infection *in vitro*. This study indicates that recombinant VLPs empower peptides which display underprivileged antigenicity in native protein to elicit high levels of neutralizing antibody, providing potential epitope candidates and an effective delivery strategy for the development of a multi-epitope vaccine.

Key words: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2); Vaccine; Virus-like particles (VLPs); Receptor-binding motif (RBM); Epitope

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection has caused a serious global pandemic of a disease called coronavirus disease 2019 (COVID-19). Till now, there are about 267 million confirmed cases and 5 million death cases worldwide.[1] The vaccine is believed to be the most valuable approach to stop the spread of COVID-19 pandemic. At present, several forms of vaccine based on various platforms have been validated by the World Health Organization (WHO) for emergency use, including nanoparticle mRNA vaccine,[2] adenovirus vector vaccine,[3] and inactivated virus vaccine.[4] In

Statement of Significance: Logically, an antibody targeting to key receptor binding sites will produce a direct and crucial effect on blocking the entry of virus into host cells, and thus confer a neutralizing protection. Receptor binding motif (RBM) is the interactive interface between S protein of SARS-COV-2 and its receptor angiotensin converting enzyme 2 (ACE2). We demonstrated that recombinant virus-like particles (VLPs) empowered RBM peptides which are underprivileged for antigenicity presentation in native S protein to elicit high levels of neutralizing antibody. This study provides potential epitope candidates and delivery strategy for the development of a multi-epitope vaccine; in addition, it may also indicate a possible reinforcement strategy for current vaccines to improve protective efficacy especially against virus mutations and variants, through highlighting latent in native protein but potent neutralizing epitopes.

Declaration of Competing Interest: The authors declare no competing financial interests.

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addition, China has also approved a recombinant subunit vaccine.[5] It was reported that CoronaVac, an inactivated virus vaccine, produced a primary efficacy against symptomatic COVID-19 of about 50.7%, and a secondary efficacy against cases requiring assistance and moderate or severe cases of about 83.7% and 100% respectively.[6] It is exciting that new technology-based vaccines such as a lipid nanoparticle-encapsulated mRNA vaccine, even presented to be more effective by showing 94.1% efficacy at preventing COVID-19 illness, including severe disease.[7]

As SARS-CoV-2 infection is new to humankind and the nature of protective immune responses is still not fully understood, it is unclear which vaccine strategies will be more efficient, persistently protective, and safer. The COVID-19 outbreak occurred repeatedly in many places worldwide, and a variety of virus variants such as Alpha (B.1.1.7, first documented in the United Kingdom), Beta (B.1.351, South Africa), Gamma (P.1, Brazil), Delta (B.1.617.2, India), and recent Omicron (B.1.1.529, multiple countries) have appeared and even become dominant.[8] Encouragingly, current vaccines whether mRNA vaccine, adenovirus vector vaccine, or inactivated virus vaccine can still provide effective protection against SARS-COV-2 variants.[9–12] For example, Pfizer or Moderna COVID-19 mRNA vaccines show good protection rate for SARS-CoV-2 variants, showing that the effective rates of preventing B.1.1.7 infection were 86% and 89% and those of preventing B.1.351, P.1 or B.1.617 infection were 82% and 85%, respectively.[9] Antisera collected from the individuals that received inactivated virus vaccine BBIBP-CorV or recombinant dimeric receptor-binding domain (RBD) vaccine ZF2001 preserved some degree of neutralizing titer against mutant 501Y.V2 virus.[10,11] However, recent studies indicated that the protective efficacy of current vaccines was reduced in response to SARS-CoV-2 variants.[13] Omicron variant was reported to have 32 mutations in spike protein, which may produce evasion and significantly limit the neutralization activity of vaccine-induced antibody and increase the risk of reinfections.[14] An *in vitro* finding using authentic SARS-CoV-2 variants indicated that the neutralization efficacy of vaccine-elicited sera against Omicron was severely reduced as compared to the currently circulating Delta variant.[13] Therefore, there is a possibility that the emerging virus mutations and variations may pose a challenge to current vaccines and vaccination strategies.[15,16] New and various vaccine platforms and strategies are still urgently needed to be explored and evaluated. It was listed that more than 300 vaccines based on various platforms, antigen formulations and delivery strategies, including nucleic acid vaccine, attenuated virus vaccine, recombinant subunit vaccine, virus-like particle vaccine, virus or bacterial vector vaccine, and multi-epitope based vaccine, are in clinical trials or preclinical studies.[17]

SARS-CoV-2 invades target cells using human angiotensin-converting enzyme 2 (ACE2) as a receptor.[18,19] It is believed that the receptor-binding domain (RBD) and NTD of SARS-CoV-2 S protein play key roles in the binding of virus to ACE2, and the blockage of the binding is a major mechanism causing SARS-COV-2 neutralization.[20–22] Peptide-based vaccine strategy provides noteworthy benefits in vaccine design and preparation with the development of bioinformatics, synthetic technology and reverse genetics. The distinct virtues of peptide-based vaccine include: easy to screen, simple and convenient antigen source, low antigen complexity, flexible vaccine formulation, economical and rapid, specific immune target, and can be produced without using the pathogen itself.[23,24] It may elicit a more concentrated immune response than a protein, multiple components, or the whole constitution of a pathogen, and thus is capable of focusing on the most conserved epitopes and those that represent the neutralizing and susceptible sites of the virus.[25–27]

Computer-based immunoinformatics and structural biology using resolved 3-dimensional (3D) structures have been widely used to predict the antigenic T cell and B cell epitopes for the SARS-CoV-2 vaccine design, particularly for peptide-based vaccine design.[28] Nine conserved linear B cell epitopes (amino acid residues 15-31, 62-75, 141-152, 208-220, 405-418, 441-448, 657-664, 696-709, and 1154-1169) were predicted to hold high antigenicity, which locate on the surface of the SARS-CoV-2 trimeric of the S protein.[29] Analyzing known antigenic profile of coronaviruses and mapping corresponding regions in the SARS-CoV-2 sequences, five regions on the S glycoprotein (residues 274-306, 510-586, 587-628, 784-803, and 870-893) were predicted to be associated with a high immune response; in addition, in combination with the use of validated bioinformatic tools the prediction may increase the probability of finding promising targets for immune recognition of SARS-CoV-2.[30] Another strategy for identifying linear B cell epitopes is to screen synthetic overlapping peptides with the elicited neutralizing antibodies in convalescent patients. For example, Poh et al reported that two IgG immunodominant regions which are located in close proximity to RBD and at the fusion peptide, respectively, significantly contributed to virus neutralization capacities of convalescent sera.[31] In this study, we focused on identifying neutralizing linear B cell epitopes in receptor binding motif (RBM) of SARS-CoV-2 spike (S) protein, which is directly involved in the interactive interface between SARS-CoV-2 and ACE2 (Figure 1). We demonstrated that recombinant VLPs empowered peptides to elicit high levels of neutralizing antibody. Our study provides potential epitope candidates and delivery strategy for the development of a multi-epitope based SARS-COV-2 vaccine. In addition, it may also indicate a possible reinforcement strategy to current vaccines through highlighting neutralizing epitopes which are underprivileged for antigenicity presentation in native S protein.

Methods

Mice and Ethics statement

Female C57BL/6 mice (6-8 weeks; 16-18 g) were provided by and maintained under specific pathogen-free (SPF) conditions in the Central Animal Care Services of Institute of Medical Biology, Chinese Academy of Medical Sciences (IMBCAMS). All of the animal experiments were approved by the Ethics Committee of Animal Care and Welfare of IMBCAMS (approval number: DWSP201905008) according to the guidelines of CAMS. All efforts were made to minimize animal suffering.

Peptides, oligonucleotides and reagents

A total of 9 overlapping peptides (P1-P9) covering the whole length of RBM, as shown in Figure 1, were chemically

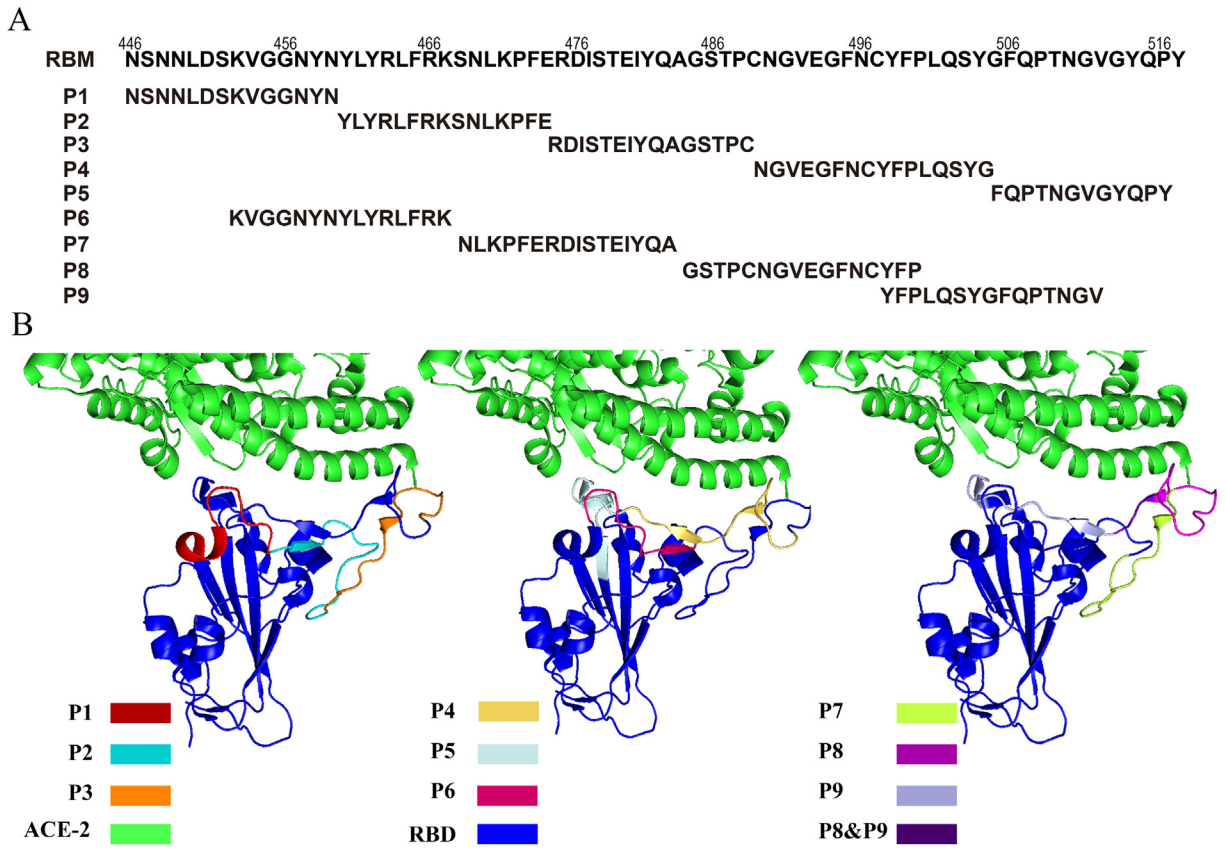

Figure 1. The sequence and distribution of nine overlapping peptides in RBM of SARS-CoV-2. **(A)** The sequences of the peptides. **(B)** An overview of the peptide location on the basis of the resolved complex structure of the RBD binding to the cell receptor ACE2.

synthesized by GL Biochem, Shanghai. The peptides were coupled respectively to carrier protein Keyhole Limpet Hemocyanin (KLH) for immunization in mice. S1 protein was purchased from Sino Biological Co., Ltd. Inactivated SARS-COV-2 virus was prepared and anti-sera derived from rhesus monkey immunized with inactivated virus were provided by Dr. Hongqi Liu and Dr. Longding Liu of biosafety level-3 (BLS-3)/ level-4 (BLS-4) laboratory of the National Kunming High-level Biosafety Primate Research Center, Yunnan, China. Oligonucleotides were synthesized by Sangon Biotech (Shanghai, People's Republic of China) Co., Ltd. Enzymes were purchased from TaKaRa Company (Dalian, People's Republic of China) Co., Ltd.

Preparation of chimeric VLPs

The recombinant plasmids encoding a chimeric protein with antigenic peptides inserted into the immunodominant epitope of hepatitis B core antigen (HBcAg) were constructed as previously reported. Briefly, the two strand fragments of oligonucleotides with BamH I and EcoR I cohesive ends, which encode the antigenic peptides, were prepared by annealing synthetic two complementary single strands of oligonucleotides, and then cloned into a previously constructed recombinant plasmid pThioHis A-NP (pThioHis A was commercially derived from Thermo Fisher Scientific, Waltham, MA, USA), which expresses truncated HBcAg (1-149 amino acid, GenBank accession number: GQ 377581) with BamHI and EcoRI recognition sequences introduced between codes for amino acids 78 and 79. The construction leads to the peptides were presented in the protrusion on the surface of HBcAg virus-like particles (VLPs).

The preparation of VLPs was performed according to a previous report.[32] Briefly, the constructed recombinant plasmids were transformed into *Escherichia coli* DH5α. The engineered bacteria were induced with isopropyl β-d-1-thiogalactopyranoside (IPTG) at 30 °C for 4 h. The expression of chimeric protein HBcAg-peptide was identified by Coomassie brilliant blue staining and western blotting. Briefly, the bacteria samples were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The transferred membrane was blocked with 5% BSA and then incubated with corresponding KLH-peptide immunized serum at a dilution of 1:1000 overnight at 4 °C, and then incubated with HRP-goat anti-mouse antibody (1:8000; GeneTex Inc.) for 1 h. ECL chemiluminiscence detection kit (Thermo Scientific) was used to develop the reaction.

The collected bacteria were lysed with ultrasonic, and then the recombinant proteins in supernatant were purified by a procedure that consists of ammonium sulfate precipitation, iodixanol density gradient centrifugation and gel filtration chromatography. The VLPs were observed and imaged by transmission electron microscopy (TEM)(Japan) at 80 KV. The size distribution and polydispersity index (PDI) of the VLPs

were measured in triplicates using a particle size analyzer (Nano ZS, Malvern Instruments, UK).

Immunization

C57BL/6 mice were immunized with either KLH-peptide (P1-P9) with Alum (Thermo Scientific, USA) or KLH-peptide (P1-P9) with complete/incomplete Freund's adjuvant (CFA/IFA) (Gibco, USA) or HBcAg-peptide (P2, P3, P4, and P6), respectively, in three separate experiments. The mice were subcutaneously inoculated with 200 μl antigens at three-point injection every other week for a total of three times. For immunization with KLH-peptide/Alum, 160 μg protein with 2 mg Alum per mouse was given for primary immunization and a dose of 80 μg protein was used for the following two booster immunizations. For an enhanced immunization procedure, complete and incomplete Freund's adjuvants were used to replace Alum, and 320 μg, 160 μg and 160 μg of antigen were given, respectively, for three immunizations. For immunization with HBcAg-peptide VLPs, 50 μg of protein with 2 mg Alum was inoculated into mice. The serum samples were collected at one week after each immunization, and splenocytes were isolated for analyzing peptide-specific T cell proliferation at the endpoint of the experiment using KLH-peptide/Alum to immunize mice.

Enzyme-linked immunosorbent assay (ELISA)

For detecting the levels of specific IgM and IgG antibody with ELISA, peptides, S1 protein, or inactivated virus particles were coated onto microplates at 4 °C overnight, respectively. And then, 50 μl of serum sample was added and incubated at 37 °C for 1 h, followed by incubation with HRP-labeled IgG at 37 °C for 45 min. Finally, TMB single-component substrate solution (Solarbio, Beijing, China) was used to develop color at 37 °C, and 2 M H_2SO_4 was added to stop the reaction. The OD_{450} value was read with an ELISA reader. For detecting the titer of specific IgG induced by recombinant VLPs, the antisera from the HBcAg peptides VLP and the control HBcAg VLP groups were diluted as 1:2000, 1:4000, 1:8000, 1:16,000, 1:32,000, 1:64,000, 1:128,000, 1:256,000. When the OD_{450} value of a tested serum is 2.1 times of the control serum at the same dilution, the reciprocal of the dilution is defined as the titer.

To analyze the affinity of the antisera, a similar experiment was performed with a modification that the serum samples were added with a mix with different concentrations of NaSCN.

To detect if the peptide was exposed on the surface of VLPs, capture antibody was coated onto the plates, and then VLPs samples were added, followed by using KLH-peptide immunized serum to provide detection antibodies.

Isolated splenocytes were stimulated with corresponding peptide and then analyzed for T cell proliferation. Briefly, a cell proliferation assay was performed following the manufacturer's instructions for the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega). Briefly, the isolated splenic lymphocytes were adjusted to 5×10^4 cells/ml and maintained in DMEM supplemented with 10% FBS, 100 U penicillin/ml, and 100 μg streptomycin/ml at 37 °C with 5% CO_2. Corresponding peptide was added to wells at concentrations of 5 μg/ml. After incubating cells at 37 °C for 48 h, 20 μl of MTS reagent was added to each well. After an additional incubation for 2 h at 37 °C, the optical absorbance at 490 nm was determined for each well.

Neutralization experiment

To evaluate the neutralizing activity of peptides-elicited antibodies against SARS-COV-2 infection, Vero E6 cells (4×10^4 /100 μl / well) were seeded in 96-well plates and cultured overnight. Pre-incubate the 50 μl of immunized mouse serum (diluted in different dilutions with medium) with 50 μl of 100 tissue-culture infectious dose (TCID50) of SARS-COV-2 in medium at 37 °C for 1 h, and then add the mixture to Vero E6 cell. Plates were incubated for 3-5 days at 37 °C in a 5% CO_2 incubator. Cytopathic effect (CPE) of each well was recorded under microscopes, and the neutralizing titer was calculated by the dilution number of 50% protective condition.[33]

Results

Immunogenicity of nine overlapping peptides in RBM

The synthetic peptides were coupled to KLH respectively and emulsified with aluminum adjuvant. The mice were immunized subcutaneously three times at two-week intervals (Figure 2, *A*). One week after the primary immunization, peptide-specific IgM responses were checked with ELISA. As shown in Figure 2, *B*, P1, P7, P3, P4, P5 and P6 elicited IgM responses in a descending order, while P2, P8, and P9 didn't show clear responses. The sera were collected at one week after each immunization and analyzed for the kinetics of the anti-peptide IgG responses (Figure 2, *C*). In a descending order, P1, P5, P3, P7, P2, P4 and P6 induced clearly IgG responses, while P8 and P9 failed to induce IgG production (Figure 2, *D*).

The analyses on the immunogenic characteristics of the peptides in native S protein

In a parallel experiment, the mice were immunized with KLH-peptides conjugates with complete CFA for primary immunization and IFA for the two boosters, and the immunization procedure was the same as that shown in Figure 2, *A*. As shown in Figure 3, *A*, as compared to using alum as adjuvant, the antibody levels were generally increased, and the responsive variation among mice was reduced. To reflect the potentials of peptide-induced antibodies to recognize native S protein, S1 protein was coated on microplates to replace peptides in ELISA. Antisera against P7, P6, P1, P4, and P3 presented distinct reactivity to S1 protein (Figure 3, *B*). Using inactivated virus-immunized rhesus monkey antisera to recognize peptides, there was no specific reaction found in the test (Figure 3, *C*). As controls, coated S1 protein and inactivated virus presented clear reactivity to monkey sera. Isolated splenocytes from immunized mice were incubated and stimulated with 9 peptides separately, and cell proliferation was checked. Stimulation with P7 and P6 showed clear cell proliferation as compared to the other peptides, indicating that the two peptides include important T cell epitopes (Figure 3, *D*).

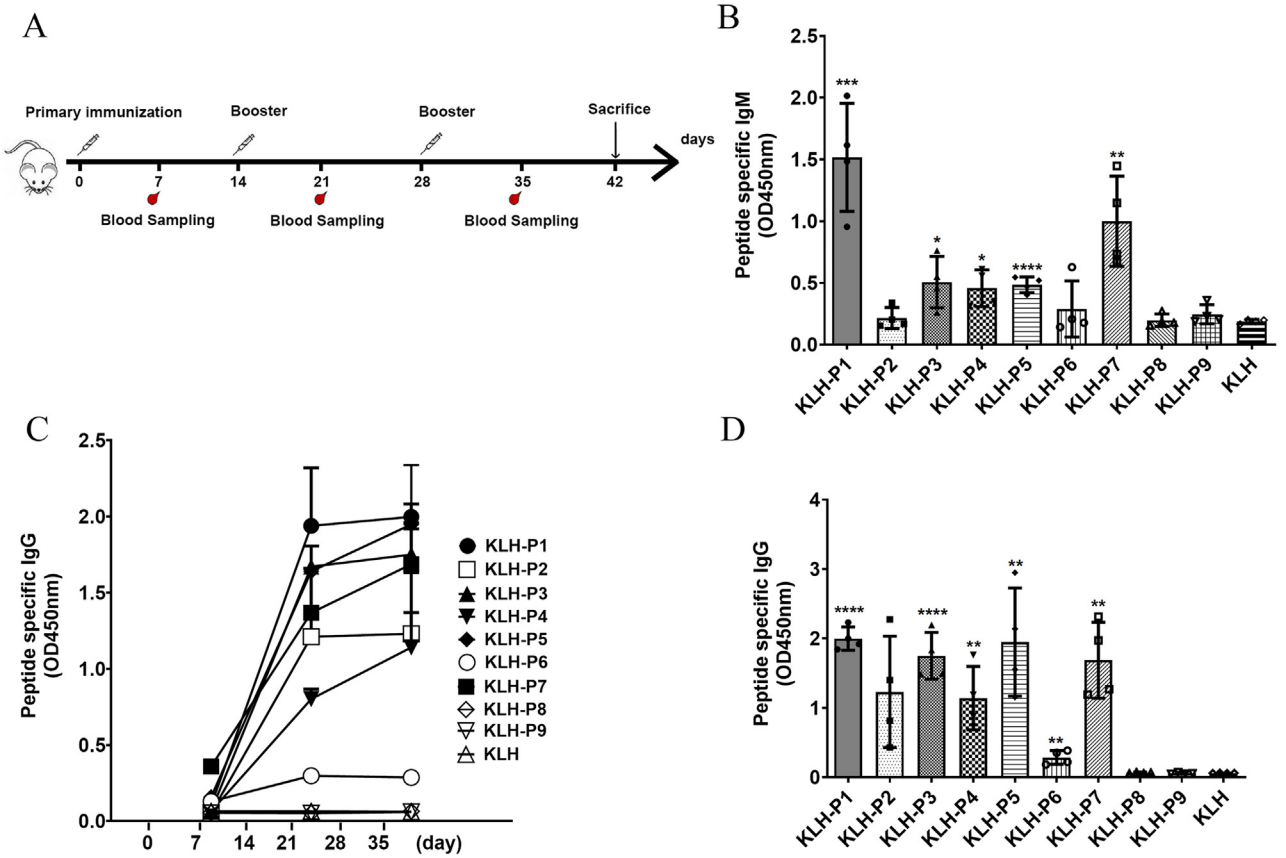

Figure 2. The peptides coupled with KLH induce specific antibody responses. **(A)** A schematic diagram for the experiment of mouse immunization. The conjugates were emulsified with aluminum adjuvant to immunize mice. **(B)** Anti-peptide IgM antibody response detected with ELISA at one week after the first immunization ($n = 4$ mice per group). **(C)** Kinetics of anti-peptide IgG responses. The samples were collected at one week after each immunization ($n = 4$ mice per group). **(D)** The peptide-specific IgG responses detected one week after the third immunization were shown as a histogram ($n = 4$ mice per group). Each group was statistically compared with KLH group. Data were analyzed by unpaired *t* test; $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

The peptides-elicited antibodies present neutralizing activity against virus infection in vitro

SARS-COV-2 virus is able to infect Vero E6 cells *in vitro* and causes cellular pathogenic effects, which provides a useful platform to test the neutralizing capability of antisera. SARS-CoV-2 caused a severe cytopathic effect (CPE) in Vero E6-infected cells, including cell rounding, detachment and death (Figure 4, *A*). Preincubation with antisera prevented the entry of virus into Vero E6 cells and thus the occurrence of cellular pathogenic effects. The test for antisera derived from immunized mice using Alum as an adjuvant showed that P3 antiserum presented the highest neutralizing potential with a neutralizing titer of 128, followed by P2, P4, and P6 which showed a titer of 32 (Figure 4, *B*). Using antisera produced by using Freund's adjuvants, the neutralizing capability of P6 was significantly elevated to a titer of 256 (Figure 4, *C*), most likely due to its distinctly enhanced antibody response.

Recombinant Protein Expression, Purification and Identification

Further, P2, P3, P4, and P6 were presented respectively on the surface of HBcAg VLPs through gene recombinant, which are named as HBcAg-P2, HBcAg-P3, HBcAg-P4, and HBcAg-P6, respectively. As shown in Figure 5, *A*, the peptide is inserted between amino acids 78 and 79 in the immunodominant domain of HBcAg. The theoretical molecular weights of HBcAg-P2, HBcAg-P3, HBcAg-P4 and HBcAg-P6 are 19.4 kDa, 19.1 kDa, 19.2 kDa and 19.3 kDa, respectively. SDS-PAGE analysis showed that there were obvious protein bands that appeared with desired molecular sizes after inducing with IPTG, indicating that recombinant HBcAg-peptide proteins are expressed successfully (Figure 5, *B*). The expressed proteins were specifically recognized by corresponding KLH-peptide induced antiserum in Western blot (Figure 5, *C*). The lysate supernatant of recombinant bacteria was separated by size-exclusion chromatograph, and the HBcAg-peptides were purified (Figure 5, *D*, lane 1). Further, the proteins were ultracentrifuged with iodixanol density gradient and separated according to sedimentation coefficient. The recombinant HBcAg-peptide proteins were enriched in fractions 7, 8 and 9 (Figure 5, *D*), which are similar to the behavior of native HBcAg VLPs, indicating the HBcAg-peptides form into VLPs. The presence of VLPs was identified by electron microscope (Figure 5, *F*) showing a diameter of 20-30 nm. The purity of VLPs was analyzed with HPLC-SEC (Figure 5, *G*), and the results showed that the sample peaks of

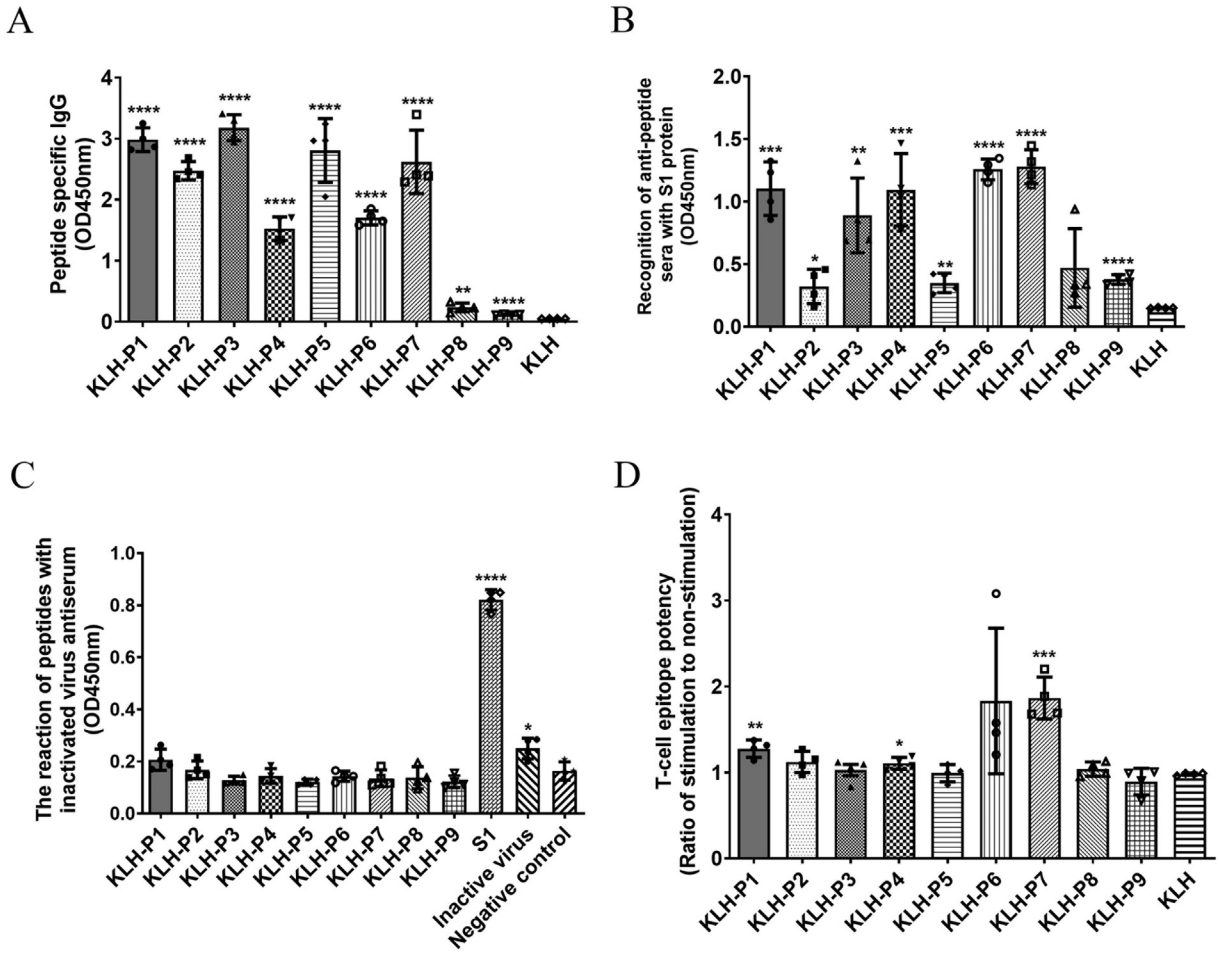

Figure 3. The analyses on the immunogenic characteristics of the peptides in native S protein. The peptides coupled with KLH was emulsified with Freund's adjuvant to immunize mice. **(A)** The peptide-specific IgG responses detected at one week after the third immunization (n = 4 mice per group). **(B)** The recognition of anti-peptide sera with S1 protein (n = 4 mice per group). **(C)** The reactivity of the antiserum derived from a rhesus monkey immunized with inactivated virus with coated peptides (n = 4 mice per group). **(D)** The analysis on potential T cell epitope in RBM. The proliferation of splenic lymphocyte was detected by MTS, and the potency was reflected by calculating the ratio of proliferation of stimulation with peptides *in vitro* to nonstimulation (n = 4 mice per group). Each group was statistically compared with KLH group. Data were analyzed by unpaired t test; *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.

both purified HBcAg and HBcAg-P3 VLPs appeared at the same time. And, the size distribution of VLPs was detected by dynamic light scatterometer (DLS), showing both the particles are homogeneous with a similar mean size (Figure 5, *H*). The results displayed that the purified recombinant proteins existed mainly in the form of VLPs; in addition, HBcAg-peptide VLPs have similar behavior to HBcAg VLPs.

Recombinant VLPs boost peptide-induced antibody responses

The recombinant VLPs were coated on microplate for ELISA. As shown in Figure 6, *A*, HBcAg-P2, P3, P4, and P6 VLPs were clearly recognized by antisera derived from the mice immunized with corresponding KLH-peptide conjugates as compared to HBcAg VLPs, indicating that the peptides were successfully presented on the surface of recombinant VLPs. The sera were collected at one week after the second and third immunizations and specific IgG responses were detected. All the VLPs induced specific IgG responses, and among them, HBcAg-P3 and HBcAg-P6 induced quite high levels of IgG (Figure 6, *B*). Especially, HBcAg-P6 induced antibody level is significantly higher than that induced by KLH-P6.

Further, the affinity of these antibodies to peptides or S protein was examined using ELISA with the addition of increased concentration of NaSCN, which was used to interfere with the binding of antibodies to peptides or S1 protein. As shown in Figure 7, *A*, the antibodies induced by HBcAg-P3 and HBcAg-P6 presented higher affinity to the peptides in comparison with those of HBcAg-P2 and HBcAg-P4. Using S1 protein to replace peptides, HBcAg-P6 induced antiserum showed far better affinity to S1 than the other three antisera (Figure 7, *B*). The reactivity of VLPs -elicited antibodies to inactivated virus was further detected in ELISA. Although all the four antisera could react with inactivated virus, HBcAg-P6 serum exhibited better recognition than the other three sera (Figure 7, *C*). The neutralizing ability of the antibodies induced by the HBcAg-P6 VLPs was checked with cell neutralization assay, the results showed that HBcAg-P6 serum had stronger ability than the antiserum from Alum-adjuvanted KLH-P6 immunization to suppress SARS-COV-2 infection of Vero-E6 cells (Figure 7, *D*).

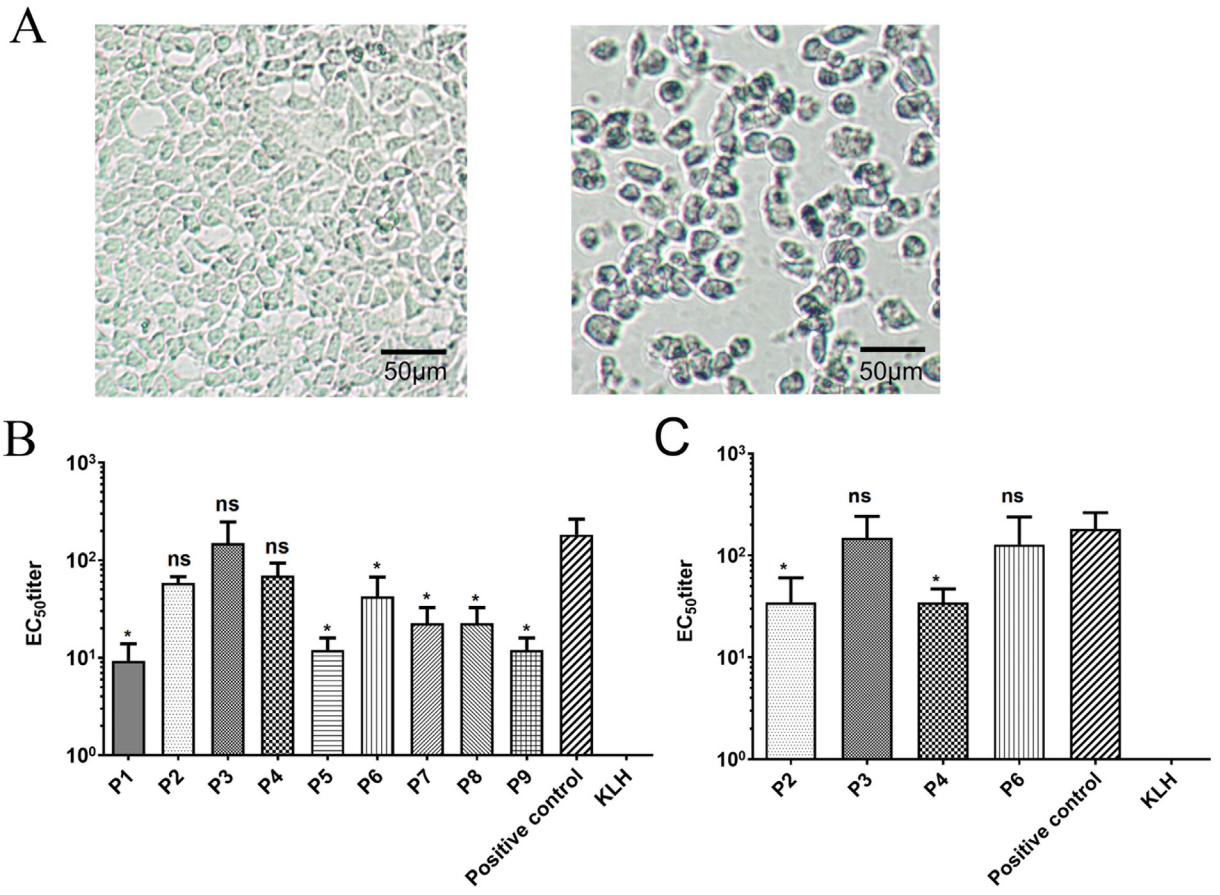

Figure 4. The KLH-peptide conjugates-elicited antibodies present neutralizing activity against virus infection *in vitro*. The antisera were preincubated with SARS-COV-2 for preventing virus infection of Vero E6 cells. **(A)** The observation of cell pathological effects. Left: control cells; right: virus -infected cells with pathological effects. **(B)** Neutralization assay for the antisera derived from the mice immunized with KLH-peptides adjuvanted with Alum (n = 3 mice per group). **(C)** Neutralization assay for the antisera derived from the mice immunized with KLH-peptides adjuvanted with Freund's adjuvants (n = 3 mice per group). Each group was statistically compared with inactivated virus-immunized rhesus monkey serum (positive control). Data were analyzed by unpaired t test; $^*P < .05$. ns means no statistically significant differences.

Discussion

Peptide-based vaccine strategy holds the following advantages over some other vaccine approaches. Firstly, it can be developed fast without the need for scale culture of a virus strain, and convenient to timely adjust and cope with the challenge of emerging virus mutations by including specific peptides[34]; secondly, through rational selection and combination of antigenic peptides, specific desired immune responses can be induced while ineffective and even detrimental responses will be deleted, thus favoring avoidance of possible vaccine-associated enhanced disease (VAED) or antibody-dependent enhancement (ADE) of infection and diseases.[35,36] In addition, peptide vaccine may represent an ideal alternative when pathogens cannot use whole-cell methods to produce vaccines due to several issues such as causing immunopathogenesis or propagation *in vitro* not being available. However, peptide vaccines also face great challenges. Peptides are unstable *in vivo* and tend to be less immunogenic than protein or virus and thus may fail to induce a strong immune response; however, it can be effectively improved through the application of delicately designed adjuvant and delivery system. Polymer nanoparticles, self-assembling nanofibers, or Fe^{++} mediated nanoparticles have been widely used as nanoscale vaccine platforms to boost peptide-specific responses.[37–39] In addition, recombinant virus-like particles (VLPs) are also an ideal vaccine delivery system.[32,40,41] However, the linear B cell epitopes generally induce the antibodies showing weaker binding affinity and correspondingly weaker neutralizing activity as compared to conformational epitopes, which limit the use of linear epitopes as a vaccine antigen.[42] Logically, an antibody targeting to key receptor binding sites will produce a direct and crucial effect on blocking the binding of virus to its receptor and its entry into targeting cells, and thus confer a neutralizing protection. Therefore, an important consideration for peptide-based vaccine is to elicit strong responses of high affinity antibody targeting to key sites. Based on this basic concept, in this study, we focused on identifying antigenic epitopes in RBM of spike protein of SARS-COV-2, which is the interactive interface between S protein and its receptor ACE2.[20] Predicting antigenic epitopes through a bioinformatics strategy is frequently used. The antigenic peptides are mainly predicted by hydrophilia/hydrophilicity, secondary structure, surface exposure, and statistical data of identified epitopes.[43] However, the predictive accuracy is unpredictable

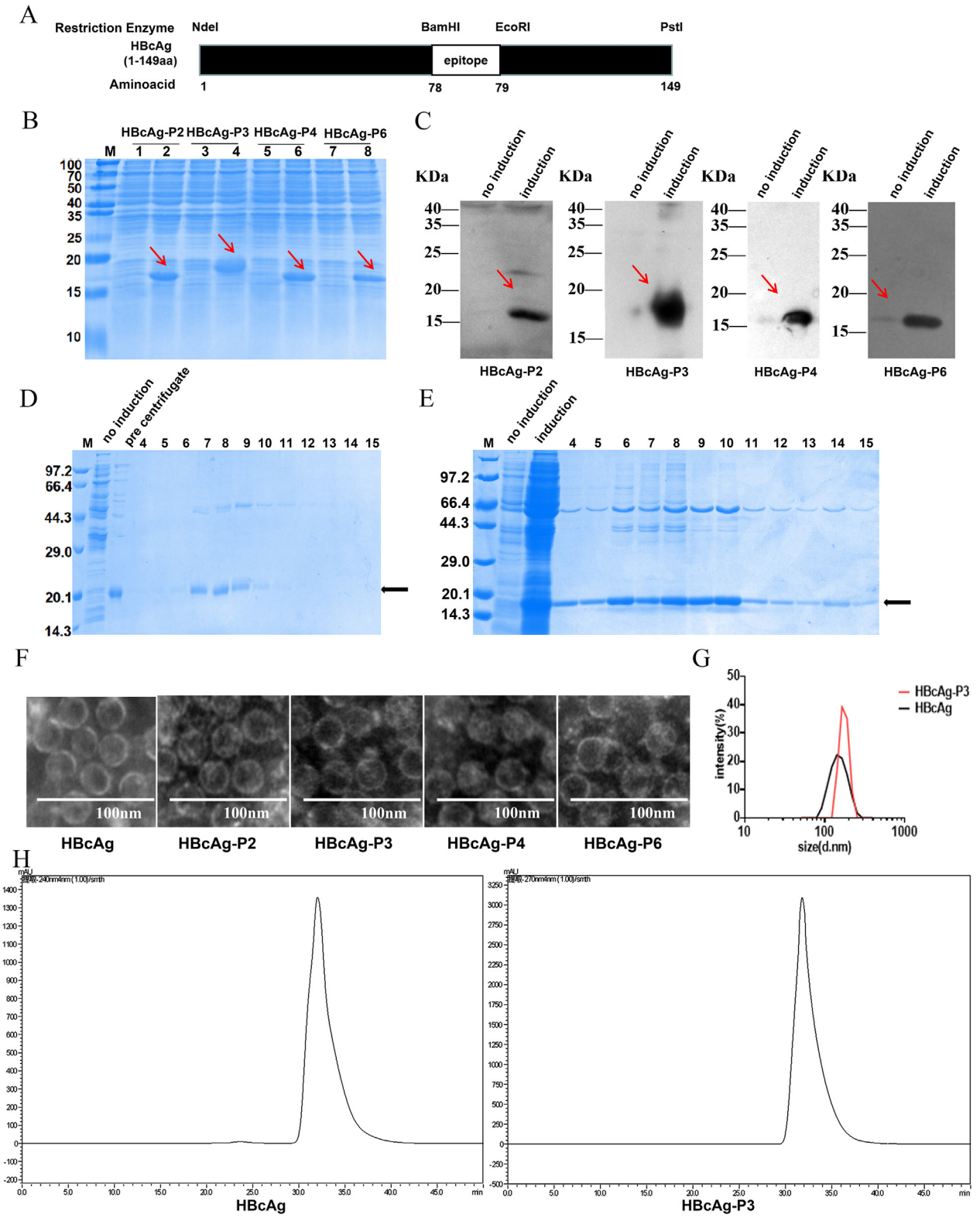

Figure 5. The epitope candidates were presented on HBcAg virus-like particles through gene recombination. **(A)** A schematic diagram for the recombinant construction of chimeric HBcAg-peptides. **(B)** The expression of recombinant HBcAg-peptide proteins. 1, 3, 5, 7: without induction of IPTG; 2, 4, 6, 8: after induction. **(C)** Western blot identification using KLH-peptides-elicited antisera. **(D)** SDS-PAGE analysis on density gradient centrifugation of the proteins purified with size exclusion chromatography (SEC). M: molecular markers; 1: bacteria without induction; 2: the recombinant protein purified with SEC; 4-15: the fractions collected from iodixanol density gradient from top to bottom. **(E)** SDS-PAGE analysis on density gradient centrifugation of HBcAg purified with ammonium sulfate precipitation; 4-15: the fractions collected from iodixanol density gradient from top to bottom. **(F)** Electron microscope observation of the assembly of recombinant proteins into VLPs. **(G)** The morphology analysis of VLPs using a particle size analyzer. **(H)** The purity of VLPs was analyzed with HPLC-SEC.

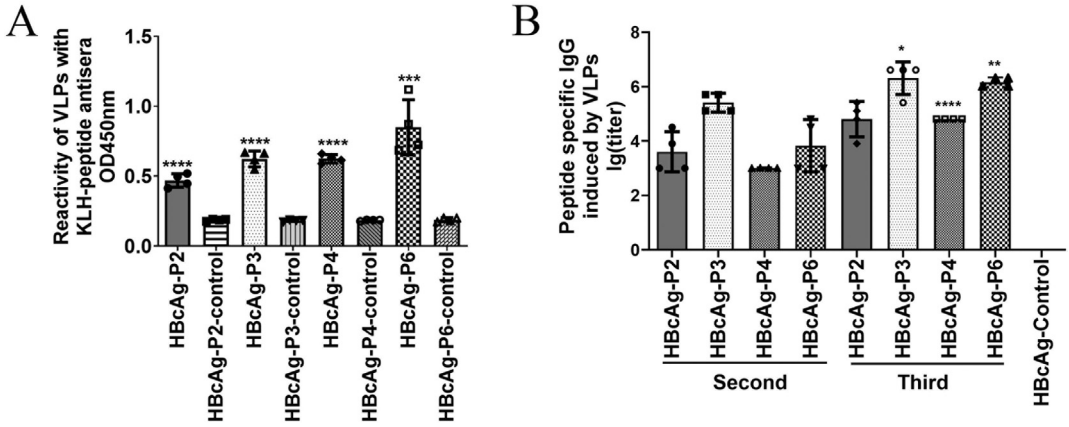

Figure 6. Recombinant VLPs booster peptide-induced antibody responses. **(A)** The recognition of VLPs by the antisera derived from KLH-peptides immunized mice with ELISA. The microplate was coated with HBcAg VLP-peptides or HBcAg VLPs as control. The corresponding KLH-peptide antiserum was added separately. Each HBcAg VLP-peptide group was compared with its corresponding HBcAg control ($n = 4$ mice per group). **(B)** The VLPs elicited peptide-specific IgG responses detected with ELISA. The microplate was coated with each peptide ($n = 4$ mice per group). A mixture of P2, P3, P4, and P6 was coated and reacted with HBcAg VLP antiserum to serve as a control. Each group was compared the second and third time; data were analyzed by unpaired *t* test; $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$, $^{****}P < .0001$.

and possible effective epitopes may be missed. As for SARS-COV-2, some groups have reported their epitope prediction for guiding peptide-based vaccine design,[29] but mostly lack functional verification for using as a vaccine antigen. Another method to analyze B cell epitope of SARS-COV-2 is to screen sets of synthetic peptides using convalescence sera of COVID-19 patients.[44] This method has apparent advantages since the reactivity clearly indicates that the peptide induced specific antibody and thus is definitely an antigenic epitope in virus protein. However, whether it holds the potential as a vaccine antigen still needs to be further identified; further, the strategy may neglect some peptides which show poor or even no antigenicity in native proteins and thus induce no antibody production upon virus infection, but they locate at key neutralizing sites and hold antigenic potentials if properly presented. In this study, we synthesized a set of overlapping peptides and conjugated them to KLH. Our concept is actually quite simple, that is, using appropriate adjuvant and delivery system to empower the peptides to have more antigenicity to boost the responses of immune system; in combination with careful assessment of the production and neutralizing activity of induced specific antibodies, we have chances to find useful neutralizing epitope candidates including those underprivileged in native proteins.

As an early antigenic screening, we chose the commonly used vaccine carrier protein KLH for peptide coupling. The approach provides a powerful carrier effect through stimulating innate immunity and T cell response to ensure that the synthetic peptide effectively induced antibody responses if peptide itself possessed antigenicity. By conjugating to carrier KLH and adjuvanting with alum, 7 of 9 RBM peptides in this study successfully induced peptide-specific antibody response.

After testing the neutralizing activity of these antisera with a live virus-based cell neutralizing assay, we found P2, P3, P4, and P6 presented clearly neutralizing activity with P3 showing the strongest effect. Analyzing the responsive titers and neutralizing titers of the antibodies, P6 drew our attention successfully since its antibody titer is significantly lower than the others. Using an antiserum derived from a parallel experiment with the stronger adjuvant CFA/IFAs instead of alum for immunization, we did find that the antibody responses to the peptides were enhanced, especially for P6. Not surprisingly, P6 antiserum from FAs presented a higher neutralizing activity than that from alum adjuvant, and became even higher than the other peptide sera. Interestingly, inactivated virus-induced antiserum in rhesus monkey didn't recognize these synthetic linear peptides. The above results indicated that peptides which show low or even no antigenicity could be empowered to induce a robust specific antibody responses if using appropriate carrier and adjuvant; in addition, these peptides might provide crucial neutralizing activity. Comparing chemically conjugating the peptides to a carrier protein, recombinant virus-like particles have dominant advantages to deliver peptides since the peptides are highly ordered, are organized and presented with high copy onto the VLPs, which provide strong stimulation signals to immune systems; besides, 20-30 nm size of VLP facilitates uptake, processing and presentation of antigen by antigen presenting cells (APCs) and migration of antigen into lymph nodes where there are more APCs. By using VLPs, we elicited high titers of specific antibodies against the peptides. Obviously, using VLPs as an antigen delivery platform did help elicit a more robust antibody response than KLH. The antibody titers are not certainly correlated with protective effects especially in the case of peptide-based vaccine. In addition to the location of peptides in native protein and virus, the affinity of antibody is also a key factor. After showing that P6 antiserum could effectively bind to virus particles, we further demonstrated that it had strongest affinity to peptides and S1 protein among all the tested peptide-induced sera using a brief binding assay with the addition of NaSCN during the incubation of antisera.

In our results, KLH-P2 and KLH-P5 antisera had high peptide-specific antibody titers (Figure 3, *A*) but weak recognition to S1 protein (Figure 3, *B*). We speculated that the difference of antibody binding activity to peptide and S1 protein

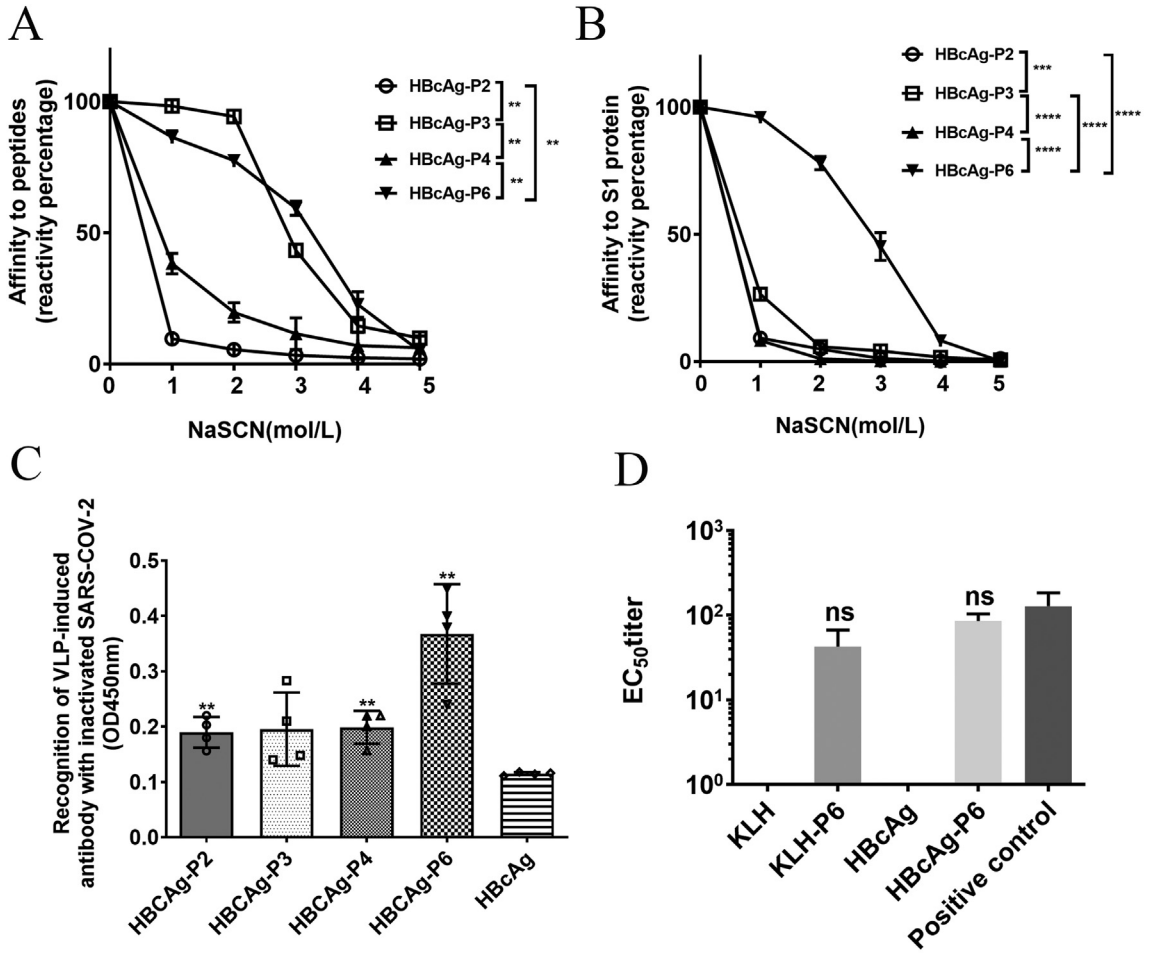

Figure 7. Analyses on the affinity and neutralization activity of VLPs-induced antisera. **(A)** The affinity analysis on the reaction of VLPs-induced antisera to peptides in ELISA. The peptides were coated and the serum samples were added with different concentrations of NaSCN. The affinity is reflected by calculating the reactivity percentage ($n = 4$ mice per group). **(B)** The affinity analysis on the reactivity of VLPs-induced antisera to S1 protein ($n = 4$ mice per group). The upper panel shows the comparisons between all groups, analyzed by two-way ANOVA. **(C)** The recognition of inactivated SARS-COV-2 by VLPs-induced antisera. Each group was statistically compared with HBcAg group ($n = 4$ mice per group). **(D)** The analysis on neutralization ability of VLPs-induced antisera ($n = 3$ mice per group). Each group was statistically compared with inactivated virus-immunized rhesus monkey serum (positive control), Data were analyzed by unpaired *t* test.*$P < .05$, *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.

may attributed to (1) peptides in S1 protein may present some differences on secondary structure comparing with coated free peptides in ELISA due to the restriction of flanking structure elasticity and influences of spatially adjacent amino acid residues; (2) the exposure of the peptides is poor or key antigenic determinants of the peptides may be covered in S1 protein. Similarly, KLH-P1 antiserum was highly reactive towards S1-protein but showed low neutralizing activity. We speculated that it might be because ELISA results reflect the number of binding antibodies, while neutralization activity much more depends on the binding strength of antibody to antigen and the location of recognized site.

Taken together, in this study we identified antigenic peptides in RBM of SARS-COV-2 S protein, which hold a potential of eliciting specific antibodies and providing neutralizing protection, and we also showed that using recombinant VLPs as a delivery platform to highlight the antigenicity of the peptide epitopes was able to dramatically empower peptides showing no immunogenicity in native S protein to elicit a robust antibody response. This study provides an important supplement to the profile of the linear neutralizing B cell epitopes discovered by screening with COVID-19 patient sera, and surmount the lack of experimental verification of informatics predicted epitopes. This study provides a promising candidate peptide for the development of a multiple epitope-based vaccine; in addition, boosting the antibody responses against peptide P6 may be considered to be included in the design of a new vaccine of SARS-COV-2 or as a reinforcement for current vaccines to improve protective efficacy and the capability of coping with emerging virus mutations and variants.

Credit Author Statement

Qiong Long: Validation; Formal analysis; Investigation; Data Curation; Writing – original draft; Writing – review & editing; Project administration; Funding acquisition.

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Mengli Yang: Investigation; Data Curation.
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