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ORIGINAL RESEARCH

Adenovirus Nanoparticles Displaying RBD Induce a Protective Immune Response Against BA.5 in Mice

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Introduction: Adenovirus (Ad) vectors demonstrated significant efficacy as vaccine vectors during the COVID-19 pandemic. Hexon is the major capsid protein, and multiple hypervariable regions (HVRs) have been used for displaying exogenous antigens and inducing a strong antibody responses.

Methods: We utilized SpyCatcher/SpyTag technology to incorporate SpyTag into HVR2, 4, and 7 of the hexon of the bivalent vaccine strain rAd3/7, respectively, to construct recombinant Ad, rAd3/7-SpyTag. The receptor-binding domain (RBD) of the SARS-CoV-2 BA5.2 strain fused with SpyCatcher was expressed as SpyCatcher-RBD, Spycatcher-RBD and rAd3/7-SpyTag were incubated in vitro to prepare a novel nanoparticle vaccine candidate, rAd3/7-SpyRBD, against SARS-CoV-2. Characterize rAd3/7-SpyRBD using Western blot, ELISA, transmission electron microscopy (TEM), and particle size measurement, and verify its immunogenicity through mouse immunization.

Results: We have successfully established a universal nanoparticle vaccine platform, rAd3/7-SpyTag, and the RBD protein was successfully displayed on the surface of rAd3/7-SpyTag. Compared with SpyCatcher-RBD, rAd3/7-SpyRBD can rapidly induce the production of antibodies and stronger immune responses. Both Spycatcher-RBD and rAd3/7-SpyRBD provide a protective immune response against BA.5 in mouse model mice and can be used as candidates for SARS-CoV-2 vaccine. We also found that rAd3/7-SpyRBD induced the production of neutralizing antibodies against Ad3 and Ad7, suggested that it could serve as an Ads vaccine candidate.

Conclusion: We developed a universal nanoparticle vaccine platform and obtained a trivalent vaccine candidate rAd3/7-SpyRBD, against SARS-CoV-2, Ad3, and Ad7, and this is the first time to use SpyCatcher/SpyTag technology in a bivalent rAd3/7 vector for trivalent immunity.

Keywords: adenovirus, SARS-CoV-2, nanoparticles, vaccine, SpyTag

Introduction

Adenovirus (Ad) particles are nanoscale viral particles that comprise a non-enveloped icosahedral capsid with a diameter of approximately 65–90 nm, and the capsid is mainly composed of hexon, fiber and penton.[1] Recombinant replication-defective Ad vectors demonstrated significant efficacy as vaccine vectors during the recent COVID-19 pandemic.[2] Exogenous genes are typically designed in the E1 or E3 region and utilize host cell to express transgenic products, while also inducing antigen-specific cellular immune responses.[3] In clinical trials, Ad vector vaccines have been shown to induce effective and safe immune responses

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against various pathogens,[4,5] but Ad vector vaccines typically require heterologous primary booster regimens,[6,7] and associated with high costs and potentially complex vaccine regimens, which restrict the use of Ad vector vaccines.

Ads can also be used in vaccine applications through a novel "capsid chimeric" strategy, in which Ad capsid proteins are highly flexible and can incorporate exogenous antigens into viral capsid proteins, providing the potential for developing protein vaccines based on Ad vectors.[8] Both structural and non-structural proteins of Ads can be used for inserting exogenous genes, mainly including hexon, penton, fiber and pVI etc,[9,10] and these Ads capsid proteins have been used to modify and display exogenous antigens.[11–13] Additionally, optimizing antigens through genetic engineering to enhance antigen-specific cellular immune responses, thereby combining the advantages of Ads-proteins vaccines.[14]

The spontaneous formation of heteropeptide bonds has been development for vaccines, such as SpyCatcher/SpyTag, DogCatcher/DogTag, and SnoopCatcher/SnoopTag. These reactive pairs have previously been used to prepare various virus-like particle (VLP) platforms.[15–17] Furthermore, SpyTag, DogTag and SnoopTag are short peptides containing 16 to 23 aa, and Ad capsid proteins can embed short peptides without affecting the stability of Ads. Therefore, these short peptides could be embedded in the Ad capsid protein in theory, providing the possibility of preparing Ads for nanoparticle vaccines.

Recently, DogTag was designed to be chimeric at HVR5 of the Ad5 hexon protein and to allow the DogCatcher-RBD protein bind to virus surface.[18] Hexon, which has a trimeric structure, is the major capsid protein in Ads, there are 720 hexon monomer proteins per capsid,[19] hexon surface-exposed HVRs (HVR1-HVR7) can be replaced by exogenous sequences,[14,20] and previous research has reported that the short peptides can be inserted into the Ad5 hexon and retained the vector infectivity.[21,22] However, hexon modification often leads to an inability to rescue the virus or poor virus growth.[23] The efficacy of using an Ad5-based hexon as a display vector depends on the insertion site,[24] therefore, it is necessary to develop other types of Ad display vectors.

In previous study, we have constructed a bivalent Ad vaccine strain, rAd3/7, by fusing human Ad type 7 (Ad7) neutralizing epitopes based on the skeleton of human Ad type 3 (Ad3).[25] In this study, based on the rAd3/7 and retaining the neutralizing epitopes of human Ad3 (HVR1) and Ad7 (HVR5), we inserted SpyTag, a short peptide tag (19 aa), into other HVRs (HVR2, 4 and 7) of the hexon of rAd3/7 to establish a universal nanoparticle vaccine platform, rAd3/7-SpyTag. To test this platform, we expressed and purified the RBD protein fused with SpyCatcher, termed SpyCatcher-RBD. After incubating SpyCatcher-RBD and rAd3/7-SpyTag in vitro, we prepared rAd3/7-SpyRBD, a novel trivalent vaccine candidate against SARS-CoV-2, Ad3 and Ad7. The immunogenicity of rAd3/7-SpyRBD and its potential as a candidate trivalent vaccine was then investigated.

Materials and Methods

Plasmids, Cells, and Viruses

The plasmid for the bivalent vaccine strain, rAd3/7, was constructed and preserved in our laboratory. Vero E6 (CRL-1586), 293T (CRL-3216), and A549 (ATCC: CCL-185) cells were purchased from ATCC and kept in our laboratory, which were cultured in DMEM supplemented with 10% FBS and 1% Penicillin/streptomycin. Recombinant Ads, Ad3E, and Ad7E, preserved with enhanced green fluorescent protein (EGFP) and Ad7W (wild-type [WT]), were cultured in our lab. SARS-CoV-2 variants, including the WT, BA5.2, and EG.5, were provided by Prof. Wang Zhongfang's group, and all infectious experiments were conducted in BSL-3 Laboratory (Guangzhou, China).

Protein Expression and Purification

The sequences of SpyCatcher (140aa) fused with SARS-CoV-2 RBD (BA5, residues 316–534; GenBank: OP603965) (name Spycatcher-RBD) were cloned into the expression vector pcDNA3.1. The DNA sequence was optimized and synthesized by Beijing Tsingke Biotech Co., Ltd., and then the SpyCatcher-RBD protein was expressed and purified using CHO cells from GenScript Biotech Corporation (Piscataway, NJ, USA).

Rescue and Purification of Recombinant rAd3/7-SpyTag

Based on the backbone of rAd3/7, we retained the neutralizing epitopes, HVR1 and HVR5 of Ad3 and Ad7, and used reverse genetic technology to insert SpyTag tags into HVR2, 4, and 7 of the rAd3/7 hexon. Other studies have reported that

Table 1 Primers Used to Incorporate SpyTag into the HVRs of the rAd3/7 Hexon

Primers	Sequence (5′ to 3′)
Pac18303F	GCGCCGTCGCTGCTATTA
Avr23600R	TCTGTTGTTTCTCTGCCTAGGAGAAC
HVR2-R	ACGATATGGGCACCAGAGCCGGTAATGTCTTTCCCAATTTGCAA
HVR2-F	GGCTCTGGTGCCCATATCGTGATGGTGGACGCCTACAAGCCTACCAAAAAGCCCATTTATGCCGATAAAAC
HVR4-R	ACGATATGGGCACCAGAGCCTGTTGGTTTTACTTTTCTGTTTTTAGCT
HVR4-F	GGCTCTGGTGCCCATATCGTGATGGTGGACGCCTACAAGCCTACCAAAGTTGAAACTGAGGAACCAGATATTGAT
HVR7-R	ACGATATGGGCACCAGAGCCTTTAACTTTAATGCCTTGATACCTGTG
HVR7-F	GGCTCTGGTGCCCATATCGTGATGGTGGACGCCTACAAGCCTACCAAATGGGAAAAAGATGCTAATGTTGATAC
HVR-identF	CGCTACCAGAAAGCCCACC
SpyTag-identR	CACCATCACGATATGGGCAC
HVR-seq-F	CGCCCAATACATCTCAGTGGAT
HVR-seq-R	GGCGTGTACTTGTAAACATCTGGA

these HVRs can accommodate exogenous epitopes and successfully rescue recombinant Ads.[20,26,27] The primers used for recombinant molecular cloning are listed in Table 1. The plasmid with the SpyTag tag was by *Asi*SI and then rescue the recombinant rAd3/7-SpyTag in 293T cells. Recombinant Ads that were successfully rescued were identified using PCR and genome sequencing. The recombinant Ad rAd3/7-SpyTag was purified via CsCl gradient ultracentrifugation, and the virus particle (VP) were determined by $Nanodrop_{2000}$ at 260 nm, and then conversion factor of 1.1×10^{12} VPs per absorbance unit. Purified recombinant rAd3/7-SpyTag was inactivated β-propiolactone before validation in vitro and animal experiments.

Growth Characteristics of rAd3/7-SpyTag

The sample culture infective doses ($TCID_{50}$) of the virus were determined following previously reported procedures,[28] and was performed to evaluate growth characteristics of rAd3/7-SpyTag. Briefly, the A549 cells were seeded into 24-well plates (3599, Corning) and cultured for 24 h, then infected with 1000 $TCID_{50}$ rAd3/7-SpyTag and Ad3E. After incubation for 2 h, the cells were washed with PBS and then added serum-free DMEM. Finally, cells are collected every 12 h after infection until 72 h of infection, after three freeze-thaw cycles, the supernatant were taken for detecting $TCID_{50}$.

Western Blot

Each Ads particle has 720 copies of hexon and based on the same number of molecules, 2×10^{10} VPs (the number of molecules is: $2\times10^{10}\times720$) of rAd3/7-SpyTag is equivalent to 1μg of SpyCatcher-RBD (the molecular weight is 40.48 kDa) molecules. Then equal molar ratios of rAd3/7-SpyTag and SpyCatcher-RBD were incubated overnight at 4°C. Then mixed with 4×Protein SDS PAGE Loading Buffer (TAKARA, 9173), and boiled for 5 min. After separated by using 10% SDS polyacrylamide gel electrophoresis, then the protein transferred onto PVDF membranes (IPVH00010, Merck millipore), and blocked with blocking buffer (5% non-fat dry milk (A600669-0250)) in PBST (0.05% Tween-20 in PBS) overnight at 4°C. Subsequently, the antibodies: SARS-CoV-2 spike antibodies (40592-MM117; Sino Biological, Beijing, China) were incubated for 1 h at room temperature. Then, the membranes were washed four times with PBST and incubated with a secondary antibody: goat anti-mouse HRP-IgG (H+L) (ab6789-1 mg, Abcam, Cambridge, UK). Finally, using an Electro-chemiluminescence assay kit (Beyotime, P0018M), imaging was performed on an iBright™ CL1500 Imaging System (iBright FL1500, Thermo Fisher Scientific).

Enzyme-Linked Immunosorbent Assay (ELISA)

For the ELISA experiment, 96-well ELISA plates (40302; Beaver Bio, USA) were coated with the recombinant Ads rAd3/7-SpyTag, Ad7W (10^{10} VPs/mL) or SARS-CoV-2 Spike RBD protein (1 μg/mL; 40592-V08H130; Sino Biological) and stored overnight at 4°C. For the coated with rAd3/7-SpyTag and Ad7W plates, were washed once with PBST, and blocked in blocking buffer (3% BSA in PBST) for 2 h. Then, 2 μg/mL of SpyCatcher-RBD or 3% BSA was added to the plates, and incubated at room temperature for 4 h. After washed three times with PBST, SARS-CoV-2 Spike Antibody (40592-MM117, Sino Biological) was added and incubated at 37°C for 1 h. For the coated with RBD

protein plates, mouse serum for detecting antibody titer was added at dilutions of 10^{-3} to 10^{-7}, for detecting antibody kinetics titer was added at dilutions of 10^{-4}, and incubated at 37°C for 1 h. All the plates were washed four times with PBST, a secondary antibody: goat anti-mouse IgG (H+L)-HRP (ab6789-1mg; Abcam) was added to the plates and then incubated at 37°C for 1 h. Finally, all plates were washed four times with PBST and the reactions in all plates were using TMB substrate (N0160, biohao biotechnology) at room temperature for 5–10 min and stopping by 2 M H_2SO_4 (A11J1AAQ, BOLINDA), then the OD450 was measured by microplate reader.

Transmission Electron Microscope (TEM)

Equal molar ratios of rAd3/7-SpyTag and SpyCatcher-RBD were incubated overnight at 4°C, then add three times the volume of stationary liquid. TEM analysis was performed by Wuhan Baiqiandu Biotechnology Co., Ltd.

Particle Size Analysis

After incubating rAd3/7-SpyTag and SpyCatcher-RBD in equimolar ratios at 4°C overnight, Zetasizer instruments (Malvern Instruments Ltd., Malvern, UK) were used to measure the particle size. Ad nanoparticles were diluted in PBS according to the instrument requirements for the volume of the test sample. Among all the measured data, the Z-mean was the average particle size obtained from the intensity distribution; therefore, we used the Z-mean (nm) to represent the particle size.

Mice Immunization and Infection

The 6-8-week-old female BALB/C and K18-ACE2 mice used in this experiment were purchased from GemPharmatech Co., Ltd. All mice were fed under specific requirements for food, microorganisms, temperature, humidity, airflow, noise, etc. All procedures were conducted in accordance with the "Guiding Principles in the Care and Use of Animals (China)" and approved by the Laboratory Animal Ethics Committee of the Guangzhou National Laboratory (Approval No. GZLAB-AUCP-2023-05-A4).

The rAd3/7-SpyTag quality of 2×10^{10} VPs is approximately 12μg, and we select a dose with lower SpyCatcher-RBD for immunizing, so the immunization dose for rAd3/7-SpyRBD was rAd3/7-SpyTag: 2×10^{10} VPs and SpyCatcher-RBD: 1 μg. The controls received SpyCatcher-RBD (1 μg) or rAd3/7-SpyTag (2×10^{10} VPs). The mice were immunized with three 50-μL doses (25-μL immunogen and 25-μL Addavax (InvivoGen, vac-adx-10)) by injecting into the lateral thigh muscle (IM) or intranasal immunization (IN), and the vaccination for mice at weeks 0 (prime), 2 (boost), and 4 (boost) in all groups. Tail vein blood was collected at weeks 0, 2, 4, and 6 after immunization, and then isolated the serum from the tail vein blood to analyze the differences in the rate of antibody production using ELISA. All the sera were kept at −20°C until use.

After immunization, K18-hACE2 mice were anesthetized with isoflurane and then infected with the SARS-CoV-2 BA5.2 strain (10^5 FFU) by intranasally. The body weight and survival rates of K18-hACE2 mice were continuously monitored until 10 d post-infection. Three days post-infection, three mice in each group were intraperitoneally injected by avertin with 200–300 mg/kg, then the mice were euthanized by cervical dislocating and lung samples were collected during necropsy to detect lung viral titers and pathological changes. The weight loss of mice exceeds 20% or ten days post-infection, the mice were euthanized by cervical dislocating after intraperitoneally injected by avertin.

ELISPOT Assay

Mouse IFN-γ and IL-4 precoated ELISPOT kit (2210002 and 2210402, respectively) were used to detect the T cell immune response induced by rAd3/7-SpyRBD and SpyCatcher-RBD. Firstly, prepared the suspension of splenic cells from individual mice, and seeded into the plates with a density of 5×10^5. Then, 10 μg/mL of SARS-CoV-2 Spike RBD protein (40592-V08H130; Sino Biological) was added to the wells, an unrelated protein, RSV-F protein was added to the negative control, and PFA of the kits serves as a positive control. Subsequently, all experimental methods were conducted according to the instructions, and the spots on the plates were counted by ELISPOT counter (CTL-ImmunoSpot® S6, USA) after stopping the reaction.

Live SARS-CoV-2 Neutralization Assay

Mouse serum collected two weeks after three immunizations and was used for the neutralization test, and the procedures were described as follows.[29] Briefly, sera were inactivated at 56°C for 30 min, and then serially diluted from 1:32 to 1:4096 at a 1:2 ratio, mixed with equal volume of live SARS-CoV-2 variants BA5.2, EG.5, and WT (100 FFU/well), the mixture were in cubated at 37°C for 1 h. Then the 1.6% CMC were added to the plates and incubated 37°C for 24 hours, and the plates were added 4% paraformaldehyde for fixed with Triton X-100 for permeabilized. Then, the cells were subsequently incubated with anti-SARS-CoV-2 N protein polyclonal antibody (40143-T62; Sino Biological), and Goat Anti-rabbit IgG (H+L) secondary antibody (109–035-088; Jackson). Finally, the foci were visualized using TrueBlue peroxidase substrate (KPL, Gaithersburg, MD, USA) and counted using an ELISPOT reader (Cellular Technology Ltd., Cleveland, OH, USA).

Adenovirus Microneutralization Test

Before the neutralization test, the $TCID_{50}$ of Ad3E and Ad7E was determined using routine procedures.[28] Briefly, all mouse sera were inactivated at 56°C, A549 cells were seeded in 96-well plates (3599, Corning) with a density of 2×10^4 and cultured at 37°C for 24 h, then 100 $TCID_{50}$ of the virus and equal volume of the two-fold serially diluted serum were added to the cells. The mixtures were incubated at 37°C for 1 h and then added to the cells. After the mixtures incubated at 37°C for 2 h, the cells were washed with PBS and added serum-free DMEM. Finally, the cells were cultured at 37°C for 2–3 d, and the neutralization titers were determined based on the number of virus fluorescence or significant visible cytopathic effects (CPE).

IgG Subtype Detection

To further validate the T cell immune response induced by the two vaccines in mice, we examined the IgG1/IgG2a ratio using ELISA. Firstly, the 1 μg/mL SARS-CoV-2 Spike RBD protein (40592-V08H130; Sino Biological) were coated on ELISA plates (40302; Beaver) overnight at 4°C. Plates were washed with PBST and then blocked with blocking buffer (3% BSA in PBST) at 37°C for 2 h. The week 6-collected serum was then diluted at 1:5000 in blocking buffer and incubated at 37°C for 1 h. After washing four times with PBST, goat anti-mouse IgG1 (115–005-205, Jackson), IgG2a (115–005-206, Jackson), IgG2b (115–005-207, Jackson), IgG2c (115–005-208, Jackson), and IgG3 (115–005-209, Jackson) were diluted in blocking buffer and added to the ELISA plate. After incubated at 37°C for 1 h, the plates were washed four times with PBST and the reactions in all plates were using TMB substrate (N0160, biohao biotechnology) at room temperature for 5–10 min and stopping by 2 M H_2SO_4 (A11J1AAQ, BOLINDA), then the OD450 was measured by microplate reader.

Detected Viral Load in Mouse Lung Tissue

Three days after infection, lung samples were homogenized after weighed, and then supernatant of the sample was collected for detecting the virus titer. The supernatant was ten-fold serially diluted and incubated with Vero E6 cells at 37°C for 1 h, then added the 1.6% CMC, 4% paraformaldehyde, Triton X-100 and staining, were determined as the live SARS-CoV-2 neutralization assay according to the previously described.

Pathological Analysis

Three days post-infection, the lung samples were fixed with 4% formalin, and make paraffin sections. Pathological sections are subjected to histological analysis using HE staining. All procedures were performed at Wuhan Servicebio Technology.

Statistics

Paired or unpaired two-sided Student's and Multiple *t*-tests were used to analyze differences intergroup comparisons. Statistical analyses were performed using Prism 8.0 software, and the statistical results are expressed as mean ± SEM. Statistical significance was categorized as follows: $*P<0.05$; $**P<0.01$; $***P<0.001$; NS: not significant.

Results

Construction of Recombinant Ad rAd3/7-SpyTag

Hexon is the most abundant component on the surface of Ad. Therefore, it is an attractive choice for generating Ad antigen-display vectors. Inserting an exogenous antigen epitope into the hexon protein allows each Ad particle to display 720 copies. We used reverse genetic technology to insert the 19aa SpyTag peptide into the hexon HVR2, 4, and 7 of rAd3/7 and constructed three recombinant Ad plasmids (Figure 1A). Then we utilized the recombinant Ad-SpyTag to achieve covalent coupling of the fusion protein with SpyCatcher on the surface (Figure 1B).

Three types of recombinant Ads were continuously passaged, and only rAd3/7-HVR4- SpyTag (here after named rAd3/7-SpyTag) was successfully rescued, showing fluorescence and CPE (Figure 2A). We then extracted the viral genome for PCR identification and genome sequencing (data not shown). Our results showed that rAd3/7-SpyTag, with the SpyTag inserted into HVR4, was successfully rescued. Finally, to test the stability of rAd3/7-SpyTag, we compared the growth characteristics of rAd3/7-SpyTag and Ad3E. As shown in Figure 2B, rAd3/7-SpyTag and Ad3E showed similar replication kinetics, indicating that embedding SpyTag into HVR4 of rAd3/7 did not affect its stability.

Characterization of rAd3/7-SpyRBD

To test the purified SpyCatcher-RBD protein (Figure 3A) can bind to rAd3/7-SpyTag in vitro, Western blot, ELISA, TEM and particle size measurements were performed. Western blot performed using a spike monoclonal antibody identified a 210-kDa band (a monomeric form of hexon-RBD complex) for rAd3/7-SpyRBD and a 50-kDa band for SpyCatcher-RBD, indicating that SpyCatcher-RBD can bind to rAd3/7-SpyTag (Figure 3B). The hexon monomer is approximately 106 kDa, and the size of hexon-RBD is 146 kDa in theory, this may be the binding of SpyCatcher-RBD to rAd3/7-SpyTag in vitro, which affects the migration of electrophoresis. The ELISA results showed that only proteins coated with rAd3/7-SpyTag and incubated with SpyCatcher-RBD were recognized by the spike monoclonal antibody. Ad7W was not recognized by the spike monoclonal antibody after incubating SpyCatcher-RBD or 3% BSA (Figure 3C). In addition, ELISA experiments also showed that the SpyTag tag embedded in HVR4 was exposed on the surface of rAd3/7

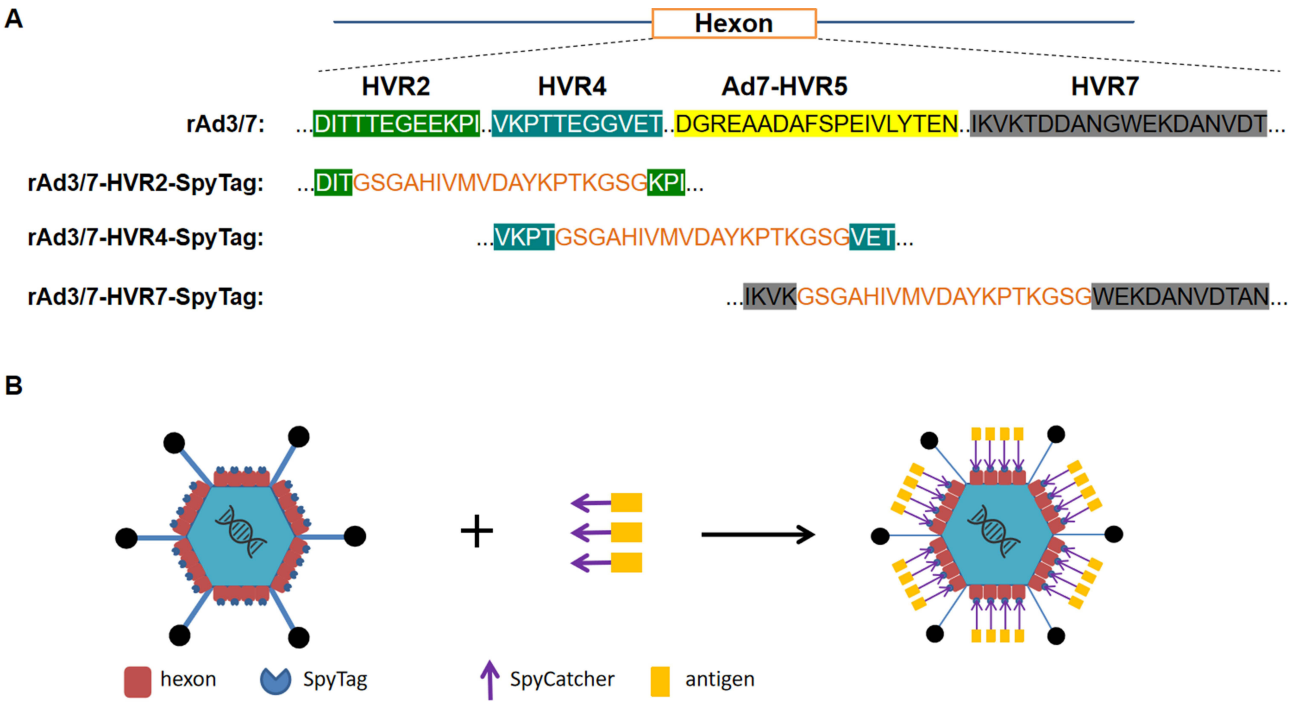

Figure 1 Schematic diagram of rAd3/7-SpyTag construction. (**A**) Design-modified Ad hexon sequences and SpyTag are inserted into HVR2, 4 and 7. (**B**) SpyTag is inserted into the hexon HVRs and the SpyCatcher is displayed with the fused antigen on the surface of the Ads capsid through covalent coupling. Antigens were observed on the capsid via the co-incubation of Ad and antigen components in rapid and spontaneous reactions.

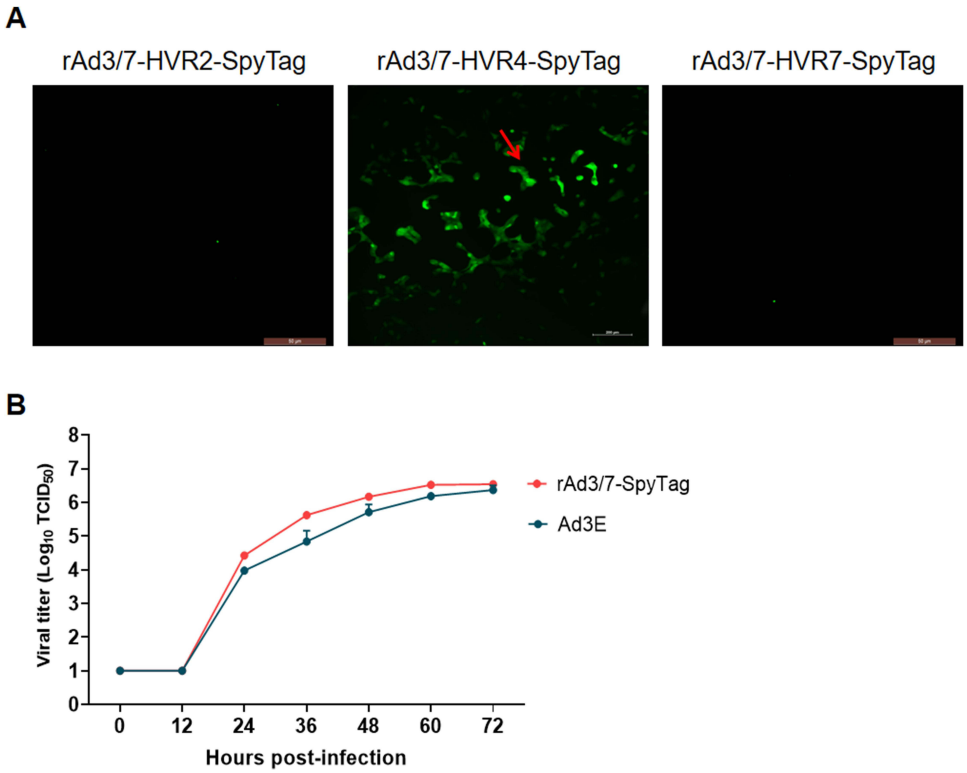

Figure 2 Rescue and characterization of rAd3/7-SpyTag. (**A**) After transfecting 293T cells with three types of Ad plasmids and continuous passaging, only rAd3/7-HVR4-SpyTag could observe fluorescence, and fluorescence represents the rescued live virus (red arrow). (**B**) Growth kinetics of rAd3/7-SpyTag. A549 cells are infected with 1000 $TCID_{50}$ and harvested at different time points. The number of infectious particles at each time point was determined by $TCID_{50}$ assay.

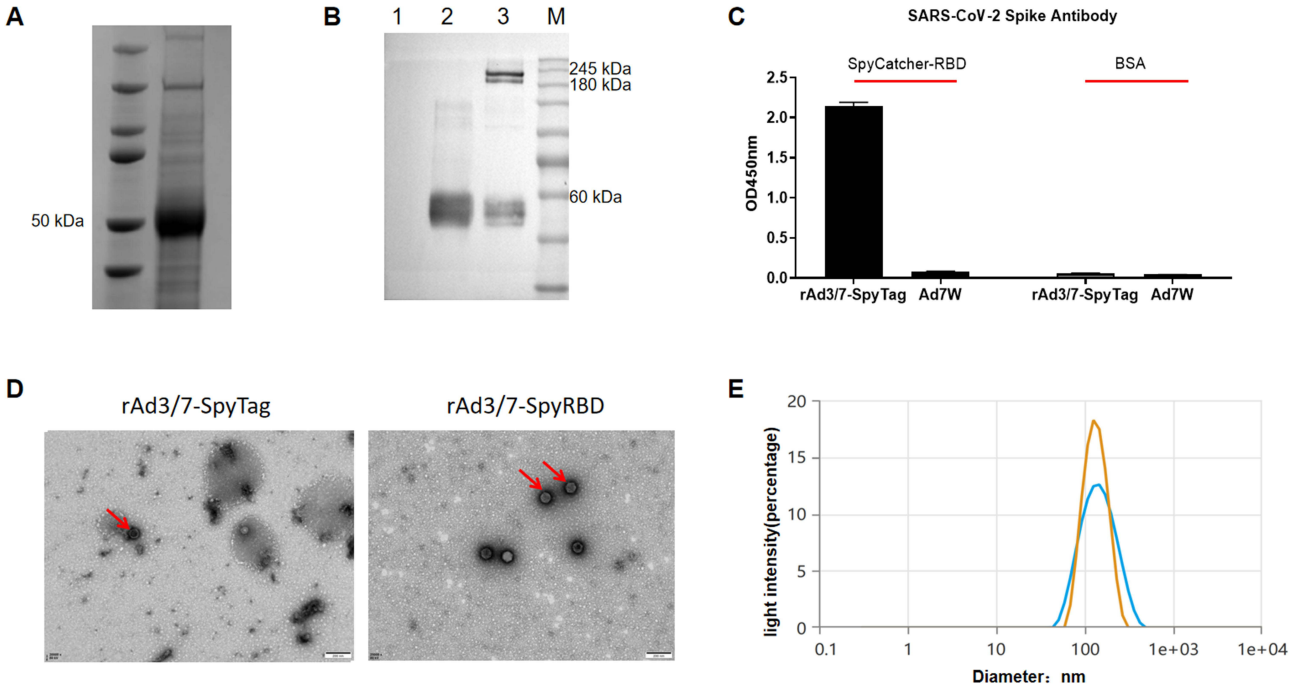

Figure 3 Analysis of rAd3/7-SpyTag and SpyCatcher-RBD binding in vitro. (**A**) Purity detection of Spycatcher-RBD protein using Coomassie solution. (**B**) Western blot: rAd3/7-SpyTag, rAd3/7-SpyRBD and SpyCatcher-RBD protein were transferred onto PVDF membranes, and analysis with SARS-CoV-2 spike antibodies. (M) protein marker, Lane 1: rAd3/7-SpyTag, Lane 2: rAd3/7-SpyRBD, Lane 3: SpyCatcher-RBD. (**C**) ELISA, coated with rAd3/7-SpyTag or Ad7W, incubated with SpyCatcher-RBD protein or BSA, followed by the addition of a spike monoclonal antibody and secondary antibody. (**D**) TEM analysis of Ads particles displaying RBD. Particles are indicated by red arrows, and diameters calculated from vertex to vertex. (**E**) Nanoparticle size and zeta potential analysis. The rAd3/7-SpyRBD is labeled with Orange lines, rAd3/7-SpyTag is labeled with blue lines, and particle size is represented by the Z-mean (nm).

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particles. Finally, we observed two particles of different sizes under TEM, rAd3/7-SpyRBD with a size of 90.4 nm and rAd3/7-SpyTag with a size of 84.5 nm (Figure 3D). Then nanoparticle size analyzer analysis the particle sizes of rAd3/7-SpyRBD and rAd3/7-SpyTag were 143.3 nm and 128.7 nm, respectively (Figure 3E), indicating that SpyCatcher-RBD protein can bind to rAd3/7-SpyTag particles. Based on these results, indicate the SpyCatcher-RBD protein can bind to and be displayed on the surface of rAd3/7-SpyTag particles.

Immunogenicity Analysis of rAd3/7-SpyRBD

To evaluate the efficacy of rAd3/7-SpyRBD immunization, we performed the following vaccination plan: the vaccines were injected intramuscularly into four female BALB/c mice with three times, an initial dose followed by two booster immunizations administered at weeks 2 and 4 (Figure 4A). The results of anti-RBD antibody kinetics showed that almost no anti-RBD antibodies can be detected in the mice immunized without Addavax adjuvants (Figure 4B), which maybe the dilution factor exceeding the limit of detection (dilute 10^4 times). When Addavax adjuvant was used, after one booster immunization, mice in the rAd3/7-SpyRBD and SpyCatcher-RBD groups began to produce antibodies against RBD, and rAd3/7-SpyRBD mice produced anti-RBD antibodies significantly faster than mice immunized with SpyCatcher-RBD alone ($P<0.05$, Figure 4B). Our results show that RBD displayed on Ad can rapidly induce antibody production under the action of the Addavax adjuvant. After three immunizations, there were no difference in anti-RBD

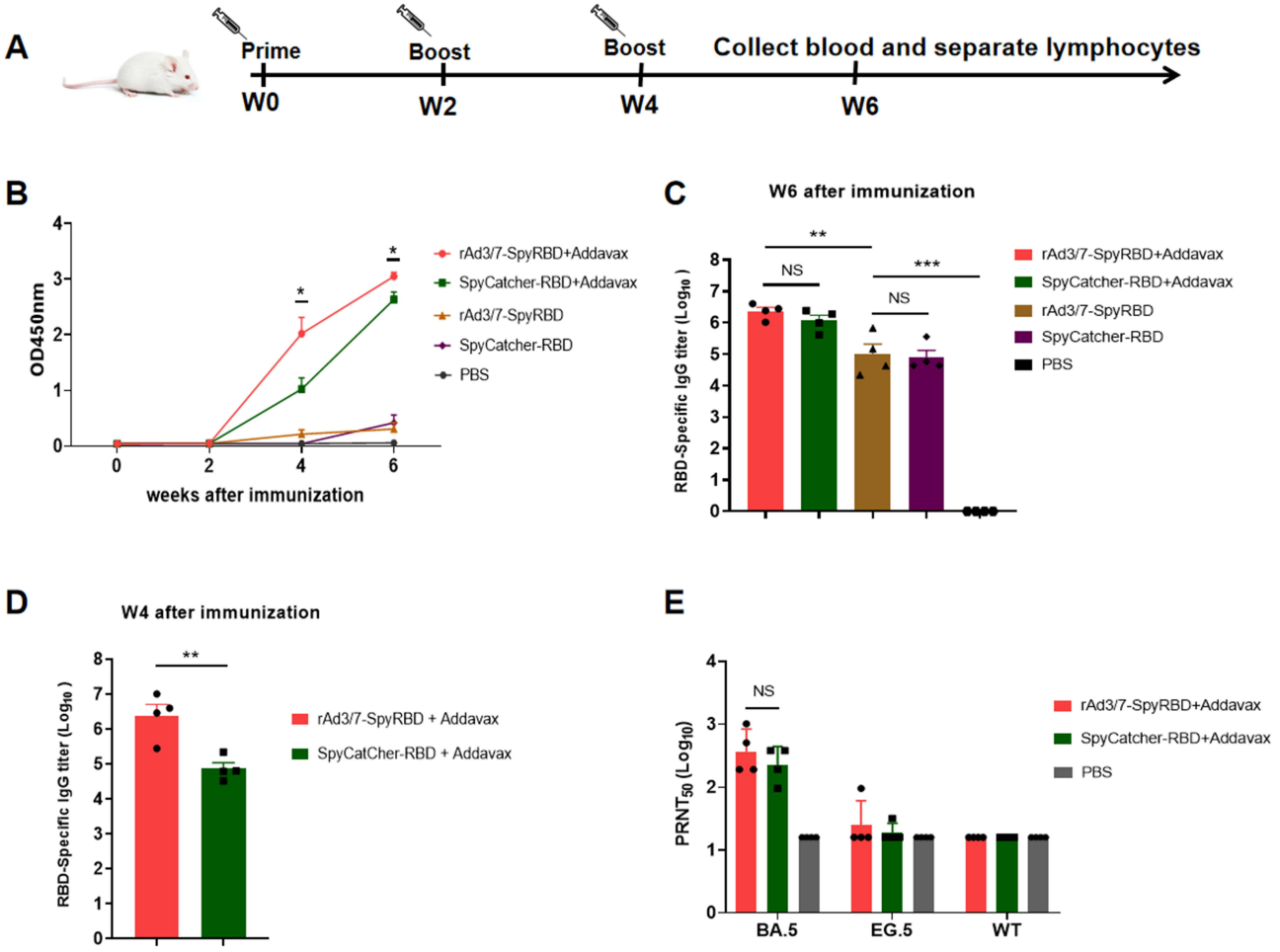

Figure 4 Antibody responses induced against SARS-CoV-2 RBD in mice. (**A**) Schematic diagram of immunity and sampling. (**B**) Dynamics of vaccines targeting SARS-CoV-2 RBD antibody production, and all serum was diluted 10^4 times. (**C**) Analyze the RBD-specific IgG titers in mouse serum after three immunizations. (**D**) Analyze the RBD-specific IgG titers in mouse serum after two immunizations. (**E**) Neutralizing antibody titers in the mouse serum against live viruses, SARS-CoV-2 BA5.2, EG.5 and the WT. The RBD-specific IgG titers was calculated using GraphPad Prism 8.0 software and analyzed using non-linear regression (curve fit). The cutoff value was defined as 2.1 times the OD value of PBS immune serum (diluted 10^3 times), and the corresponding serum dilution factor calculated based on the cutoff value was the antibody titers. Statistical analyses were performed using *t*-tests. All results are expressed as mean ± SEM. *$P<0.05$; **$P<0.01$; ***$P<0.001$; NS: not significant.

antibody titers between the rAd3/7-SpyRBD and SpyCatcher-RBD, which maybe due to multiple immunizations and adjuvant effects ($P>0.05$, Figure 4C). Furthermore, compared to the PBS group, rAd3/7-SpyRBD can also induce an immune responses without adjuvants ($P<0.001$, Figure 4C), but rAd3/7-SpyRBD could not induce a strong antibody responses through intranasal immunization (data not shown).

Next, to test whether rAd3/7-SpyRBD has VLP effect, we analyzed the tail vein blood collected twice during immunization showed that the anti-RBD antibody titer in SpyCatcher-RBD group was $10^{4.8}$, whereas that in the rAd3/7-SpyRBD group was $10^{6.3}$, the antibody titer increased exceed 10-fold ($P<0.01$, Figure 4D), indicating that immunization with rAd3/7-SpyRBD twice can observe VLP effects. Overall, our results indicate that rAd3/7-SpyRBD immunization can rapidly induce antibody production and a higher antibody titers.

Finally, in the live SARS-CoV-2 neutralization assay, both SpyCatcher-RBD and rAd3/7-SpyRBD had similar neutralizing activities, with no significant difference in their neutralizing antibody titers ($P>0.05$, Figure 4E). In the cross-experiments on SARS-CoV-2 WT and variant EG.5, only the antiserum induced by rAd3/7-SpyRBD had lower neutralizing activity against variant EG.5; none of the antisera demonstrated neutralizing activity against the original wild-type (WT) strain.

rAd3/7-SpyRBD Induced T Cell Immune Response in Mice

To detect the T cell immune types induced by SpyCatcher-RBD and rAd3/7-SpyRBD vaccines, we measured the levels of the cytokines IFN-γ and IL-4 by via ELISPOT. Our results showed that both rAd3/7-SpyRBD and SpyCatcher-RBD vaccination induced the production of IFN-γ and IL-4 (Figure 5A and B), indicating that both vaccines induced T cell

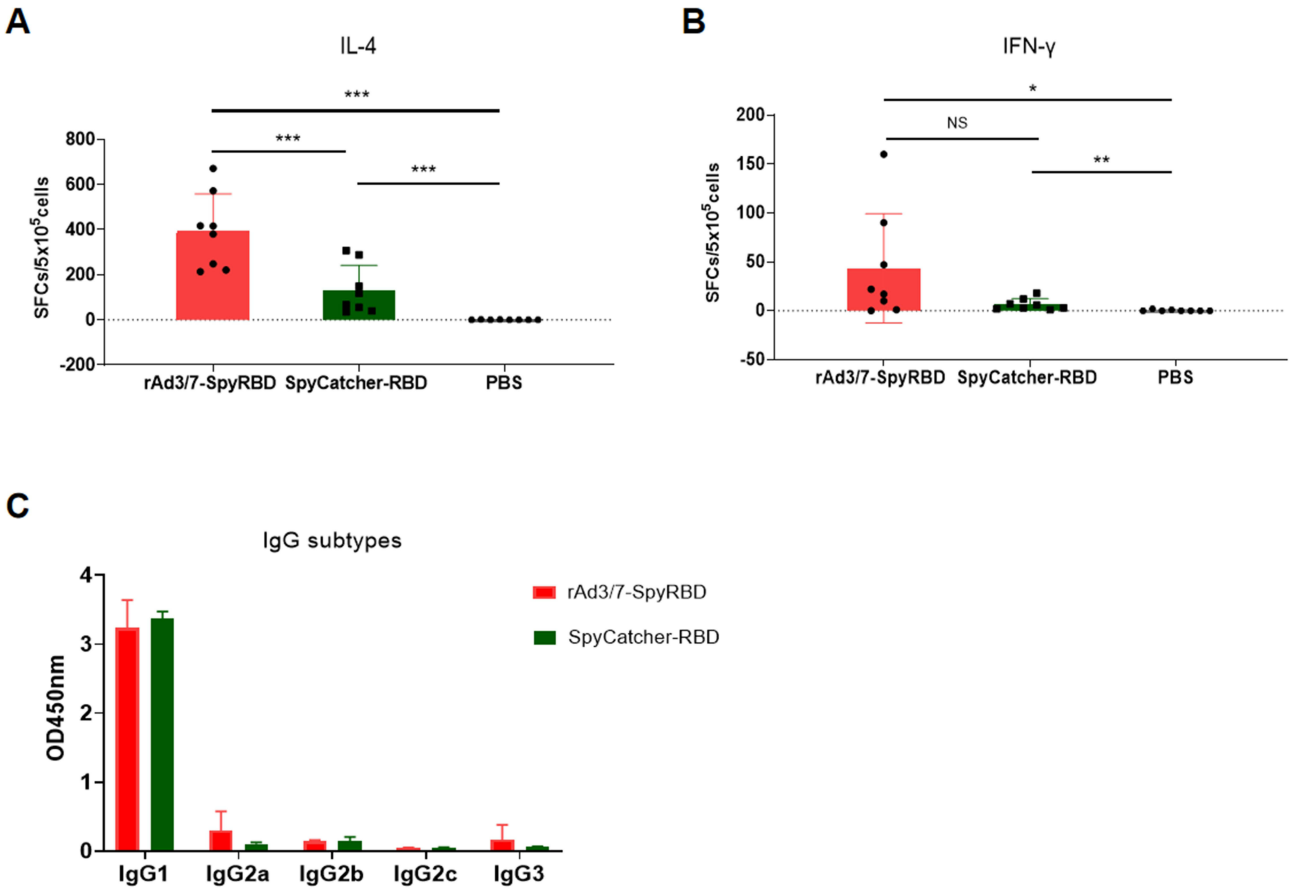

Figure 5 Analysis of T cell immune responses in mice. (**A** and **B**) ELISPOT detection of the cytokines IFN-γ and IL-4. (**C**) ELISA analysis of mouse IgG antibody subtypes. Statistical analyses were performed using *t*-tests. All results are expressed as mean ± SEM. *$P<0.05$; **$P<0.01$; ***$P<0.001$; NS: not significant.

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immune responses. Notably, higher IL-4 levels were detected following rAd3/7-SpyRBD and SpyCatcher-RBD immunization, indicating that both vaccines induced a Th2-skewing immune response.

To further validate the immune response induced by the two vaccines in mice, we examined the IgG1/IgG2a ratio by using ELISA. The ELISA results showed that the main antibody subtype produced by the two vaccines was IgG1 (Figure 5C), IgG1 production is a marker of the Th2 response, and high IgG1 level indicates induced a Th2-biased immune response, further confirming that both vaccines induced a Th2-biased immune response.

rAd3/7-SpyRBD Induced a Neutralizing Antibody Response Against Ad3 and Ad7

To evaluate the potential of rAd3/7-SpyRBD as a trivalent vaccine, we tested the hexon antibodies response in mice after vaccination with rAd3/7-SpyTag and rAd3/7-SpyRBD. Firstly, anti-hexon antibodies were produced after a single vaccination dose, and compared with rAd3/7-SpyTag, the RBD protein binds to the hexon decreased the rate of hexon antibody production ($P<0.001$, Figure 6A). Then we analyzed the anti-hexon antibody titers, both rAd3/7-SpyRBD and rAd3/7-SpyTag can induce the production of hexon antibodies with or without adjuvant (Figure 6B). However, after immunization with adjuvant, the anti-hexon antibody titers in the rAd3/7-SpyRBD group were lower than rAd3/7-SpyTag group ($P<0.01$, Figure 6B).

Finally, we detected the neutralizing titers of Ad3E and Ad7E in mouse serum. Compared with PBS group, all the vaccination group can induce the neutralizing antibody to Ad3E and Ad7E. After immunization with or without adjuvants, compared with rAd3/7-SpyTag, rAd3/7-SpyRBD did not affect the neutralizing activity against Ad3E, but

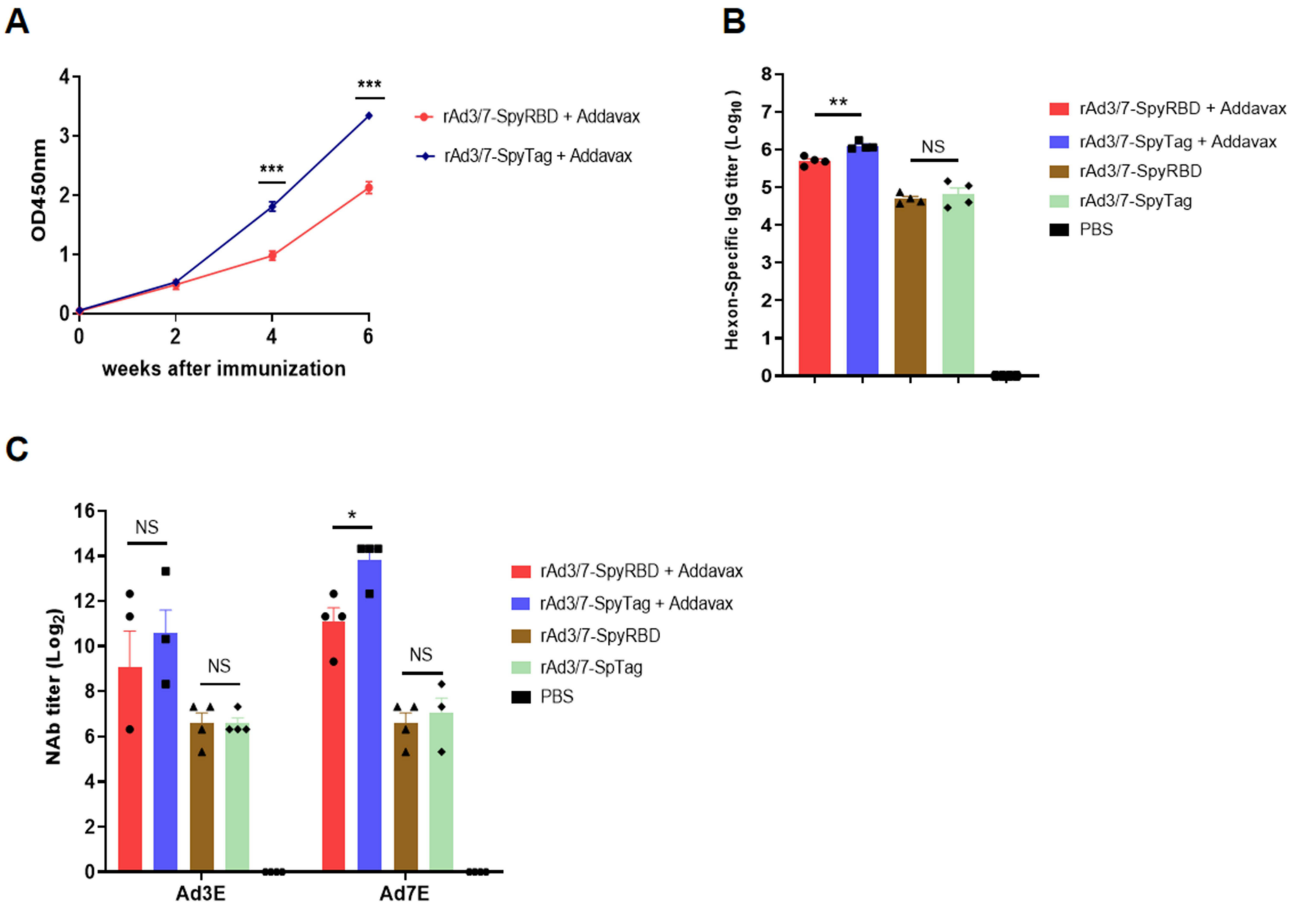

Figure 6 Analysis of anti-hexon antibody production by rAd3/7-SpyRBD. (**A**) Dynamics of hexon antibody production in rAd3/7-SpyRBD and rAd3/7-SpyTag, and all serum was diluted 10^4 times. (**B**) Analyze the Hexon-specific IgG titers in mouse serum after two immunizations. (**C**) Neutralizing antibody titers of rAd3/7-SpyRBD and rAd3/7-SpyTag against Ad3E and Ad7E. The antibody titers was calculated using GraphPad Prism software and analyzed using non-linear regression (curve fit). The cutoff value was defined as 2.1 times the OD value of PBS immune serum (diluted 10^3 times), and the corresponding serum dilution factor calculated based on the cutoff value was the antibody titers. Statistical analyses were performed using *t*-tests. All results are expressed as mean ± SEM. **P*<0.05; ***P*<0.01; ****P*<0.001; NS: not significant.

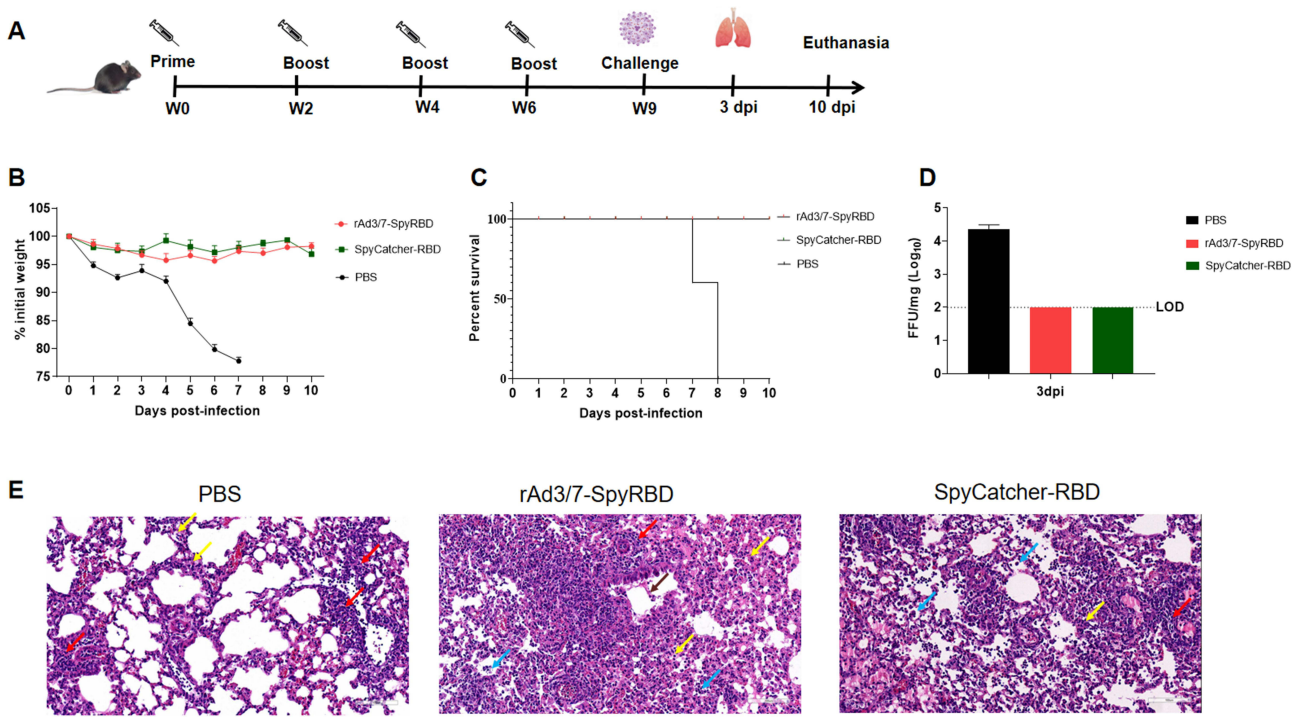

Figure 7 rAd3/7-SpyRBD and SpyCatcher-RBD immunization provides protection against SARS-CoV-2 BA5.2 challenge in a mouse model. (**A**) Scheme of the immunization process in K18-hACE2 mice. Three weeks after immunization, the mice were infected with 10^5 FFU of SARS-CoV-2 BA.5. The mice are monitored daily until 10 d post-infection. Pathological changes and viral loads in the lungs are detected 3 d post-infection. (**B** and **C**) Mice were intranasally infected with 10^5 PFU of the SARS-CoV-2 BA5.2 virus. Mortality and body weight are monitored daily until 10 d post-infection (n=5 mice per group). (**D**) To obtain virus titers, the lungs are homogenized at the indicated time points and titrated on Vero E6 cells. Titers are expressed as PFU/g or FFU/g of samples (n=3 mice per group per time point). (**E**) Paraffin-embedded lung sections from infected mice were stained with hematoxylin and eosin on day 3 post-infection. Scale bar =100 μm. Pulmonary pathology showing granulocyte infiltration (yellow arrow), focal lymphocyte infiltration (red arrow), endothelial cell proliferation (black arrow), and macrophages and granulocytes (blue arrow).

the neutralizing activity against Ad7E was lower than rAd3/7-SpyTag when using adjuvants for immunization ($P<0.05$; Figure 6C). This indicated that the neutralizing epitope of Ad7 was chimeric in the HVR5, whereas the RBD was displayed in HVR4, where the two epitopes were in close proximity, affecting the production of neutralizing antibodies against Ad7. Overall, our results indicated that rAd3/7-SpyRBD may be serve as a trivalent vaccine candidate.

rAd3/7-SpyRBD Induced Protective Immunity Against SARS-CoV-2 BA5 Challenge

To evaluate the protective effect of rAd3/7-SpyRBD and SpyCatcher-RBD in vivo, we performed the following immunization and challenged plan (Figure 7A). After challenged with the BA5.2 strain, the PBS group showed significant weight loss, with a decrease of over 20%; they died within 7–8 d post-challenge. In contrast, mice vaccinated with rAd3/7-SpyRBD and SpyCatcher-RBD showed a slight reduction in weight loss within 3–4 d, and no deaths were observed during the 10 d of monitoring (Figure 7B and C), indicating that vaccination with rAd3/7-SpyRBD and SpyCatcher-RBD can protect mice against BA5.2 infection.

In pathological analysis, PBS group revealed high viral loads in the lungs after 3 d post-infection. In contrast, the viral loads in the lungs were below the limit of detection (LOD) in the rAd3/7-SpyRBD and SpyCatcher-RBD vaccine groups (Figure 7D), indicating that both vaccines could clear the virus in mice. HE staining of the lung sample of mice 3 d post-infection showed that the PBS group had significant lung lesions and abundant lymphocyte infiltration around the pulmonary vessels after SARS-CoV-2 BA5.2 challenge. However, the same pathological changes were observed in the lungs of the rAd3/7-SpyRBD and SpyCatcher-RBD groups (Figure 7E).

Discussion

In clinical vaccine development, there is an increasing exploration of multivalent antigen presentation on self-assembled protein scaffolds. The self-assembled nanoparticle frameworks have been used to develop for the SARS-CoV-2 vaccine,

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include Ferritin,[29,30] two-component protein nanoparticle, I53-50[31] Lumazine Synthase,[32,33] SpyCatcher003-mi3 (60 potential conjugation sites).[34,35] The RBD protein is directly linked to the nanoparticle or coupled to the nanoparticle by a heteropeptide bond, which can significantly enhance the neutralizing antibody reaction. Recently, there have been studies using the hexon of human Ads type 5 to display RBD protein, which has increased the neutralizing antibody titer of SARS-CoV-2 and achieved equivalent or superior T cell immunogenicity.[18] In this study, we employed a bivalent vaccine strain, rAd3/7, to display the RBD protein. In order to prepare the trivalent vaccine of Ad3, Ad7 and SARS-CoV -2, we retained the neutralizing epitopes HVR1 and HVR5 of Ad3 and Ad7. Using SpyTag/SpyCatcher technology, and finally successfully rescued the recombinant Ads with SpyTag in HVR4 (Figure 2A). Hexon modification may lead to virus rescue failure, our previous study constructed the EV71 vaccine by incorporating a neutralizing epitope SP70 (15aa) into HVR1, 2, 4, 5 and 7 of the hexon of Ad3, respectively, and successfully rescued recombinant Ads in HVR1, 2, and 7.[36] The length of the embedded SpyTag is 19aa (both sides of the sequence contain a GSG linker), which may lead to the failure of HVR2 and 7 rescue in rAd3/7.

However, using rAd3/7-SpyTag as a VLP display platform still requires adjuvants to enhance the immune response (Figure 4B and C). Although HVR4 was exposed on the hexon surface in the absence of an adjuvant, the RBD displayed on the hexon surface did not necessarily have a dominant antigenic effect. Another possibility is that Ads have strong immunogenicity. The dominant epitope of rAd3/7 is located in HVR1, resulting in a concentrated immune response against Ads. Immunized with adjuvants, rAd3/7-SpyRBD induced the production of anti-RBD antibodies more quickly and increased the antibody titer by exceeding 10-fold compared with SpyCatcher-RBD (Figure 4B and D). However, there were no difference in neutralizing antibody titers induced by rAd3/7-SpyRBD and SpyCatcher-RBD (Figure 4E). We speculated that multiple immunizations and the use of adjuvants might have obscured the intrinsic adjuvant effects of Ads. Previous studies have shown that Addavax among the most potent adjuvants for SARS-CoV-2 subunit vaccines.[37,38] A study by van Oosten et al showed that immunization of mice with S1-VLP and only showed the effect of VLP in the first immunization, the S1 subunit and S1-VLP induced similarly immune responses after the second immunization.[39] In another study, without the addition of an alum adjuvant, SpyCatcher-M2e and noro-VLP + SpyCatcher-M2e induced low titers of anti-M2e antibodies in mice.[40] Some studies have showed that the importance of adjuvants in prepared vaccines. For example, Iwata-Yoshikawa et al, without appropriate adjuvants, immunization of mice can lead to histopathology and insufficient neutralizing antibodies after infection,[41] highlighting the need for future investigations on the mechanisms behind how the affect of adjuvants in vaccines.

Surprisingly, in the SARS-CoV-2BA5 challenge experiment, K18-hACE2 mice vaccinated with rAd3/7-SpyRBD and SpyCatcher-RBD can provide effective protection. Compared with the PBS group, the viral load in the lungs was significantly reduced in immunized groups (Figure 7D), indicating SpyCatcher-RBD can also be candidates for SARS-CoV-2 vaccines. Previous studies have shown that SpyCatcher may enhance immune responses, for example, Lampinen et al found that SpyCatcher-M2e as a immunogen, inducing strong M2e-response in mice.[42] In addition, anti-SpyTag /SpyCatcher antibodies were detected in almost all human serum samples,[43] indicating that SpyCatcher is immunogenic. Our results also suggest that SpyCatcher enhances antigen immunogenicity.

Plasma IgG is the dominant antibody that protects the lungs, and only IgA can prevent upper respiratory tract infections;[44] However, most vaccines are administered via intramuscular or subcutaneous injection and stimulate the production of serum antibodies.[45] Puhach et al demonstrated that intramuscularly administered vaccines have a high IgG responses in serum, but IgA responses were weakly,[46] and Ad5-nCoV delivered via inhalation triggers mucosal immune responses against SARS-CoV-2 and other variants.[47] In this study, rAd3/7-SpyRBD could not induce a strong antibody responses through intranasal immunization (data not shown). So, inhaled immune preparations can be further developed in the future, as the intranasal administration of rAd3/7-SpyRBD and SpyCatcher-RBD may induce better mucosal immune responses. Owing to the rapid spread and mutation rate of SARS-CoV-2, it may have higher binding affinity, enhanced transmissibility, and higher antibody escape rates. Our system may be used for updates and iterations of SARS-CoV-2 vaccines.

The versatility and flexibility of the SpyCatcher/SpyTag system make it widely applicable in vaccine development. This system can quickly develop new vaccines through adjustments and combinations. Compared to other protein VLP platforms, rAd3/7-SpyTag combines the advantages of SpyCatcher/SpyTag and Ads, making it easier to cultivate and prepare. Using rAd3/7-SpyTag as a display platform, we can prepare a trivalent vaccines. However, this research had some limitations.

Firstly, although it can induce neutralizing antibodies against Ad3 and Ad7 (Figure 6), we did not perform protection experiments for Ad challenge due to a lack of suitable Ad infection animal models. Secondly, with the continuous evolution of SARS-CoV-2, possibly not exclusively the BA.5 and EG.5 variants, new variants may be more transmissive and more antibody-evasive. Many mutations were observed in the RBD of SARS-CoV-2 variants, so the vaccine based on RBD may be ineffective against the new variants.[48] Therefore, we should attempt to use rAd3/7-SpyTag to update the vaccine composition or display other conservative antigens to prepare multivalent COVID-19 vaccines.

Conclusion

In summary, we developed a nanoparticle vaccine platform, rAd3/7-SpyTag, which allowed the attachment of a SpyCatcher-fused antigen to HVR4 of hexon surface, which can induce antibody production quickly. Our platform can also be applied to research and development of vaccines for other newly emerging and reemerging major infectious pathogens. Using this platform, we prepared a nanoparticle vaccine candidate, rAd3/7-SpyRBD, which induced a protective immune response against SARS-CoV-2 in mice. Moreover, rAd3/7-SpyRBD immunization simultaneously induced neutralizing antibodies against SARS-CoV-2 BA.5 variants, Ad3 and Ad7, indicating that it may be a trivalent vaccine candidate.

Data Sharing Statement

All data generated or analyzed during this study are included in this published article and its supplemental files. The primary data is available from the corresponding authors upon reasonable request.

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Disclosure

The authors declare no conflicts of interest in this work.

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