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Short communication

An AAV vaccine targeting the RBD of the SARS-CoV-2 S protein induces effective neutralizing antibody titers in mice and canines

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ABSTRACT

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has resulted in catastrophic damage worldwide. Accordingly, the development of powerful, safe, easily accessible vaccines with long-term effectiveness is understood as an urgently needed countermeasure against this ongoing pandemic. Guided by this strong promise of using AAVs, we here designed, optimized, and developed an AAV-based vaccines (including AAV-RBD(max), AAV-RBD(wt), AAV-2xRBD, and AAV-3xRBD) that elicit strong immune responses against the RBD domain of the SARS-CoV-2 S protein. These immunogenic responses have proven long-lived, with near peak levels for at least six months in mice. Notably, the sera immunized with AAV-3xRBD vaccine contains powerful neutralizing antibodies against the SARS-CoV-2 pseudovirus. Further evidence proven that potent specific antibodies could also be elicited in canines after vaccination with AAV-3xRBD vaccine.

1. Introduction

The coronavirus disease 2019 (COVID-19) pandemic caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) has resulted in catastrophic damage worldwide. Since September 2020, the second and third waves of COVID-19 outbreaks have commenced, and have caused great devastation in many countries, with especially large impacts in Europe and the USA. Accordingly, the development of powerful, safe, easily accessible vaccines with long-term effectiveness is understood as an urgently needed countermeasure against this ongoing pandemic. As of Nov 2020, there are reports of more than 180 COVID-19 vaccines currently under development, 48 of which are undergoing human clinical trials [1]. Fundamentally, there are five major types of vaccines being developed against SARS-CoV-2 infection: whole virus, recombinant protein subunit, DNA/RNA-based technologies, recombinant viral vectors, and virus-like particles (VLP) [1,2]. Beyond these, previous studies have shown that adenovirus associated virus (AAV) can be understood as an attractive vector platform for certain gene therapies [3], and AAV can potentially be deployed as vaccines against infectious diseases. There are demonstrated examples of AAV vaccines including one against influenza [4], one against Simian immunodeficiency virus (SIV) [5]; there is also an AAV vaccine against the causal virus of the 2002–2004 SARS outbreak [6]. Notably, AAV vaccines are particularly useful, because AAV technology enables particularly long-term and potent expression of genetic materials in host cells.

The spike (S) protein is a fusion protein located on the surface of the SARS-CoV-2 virion, and we now know that it has a strong

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binding affinity for human angiotenisin-converting enzyme 2 (ACE2) through its receptor binding domain (RBD) [7]. Thus, the full-length S protein and RBD truncation variants are major targets for the development of neutralizing antibodies against SARS-CoV-2. In the present study, we designed an AAV-based vaccine against the SARS-CoV-2 virus and provide proof-of-concept this AAV vaccine can induce effective immunity in mice. Specifically, we initially tested a variety of secreted fusion antigens comprising the RBD of the S protein and secretory signal peptides (including from S1, oncostatin M (OSM), IgG2, IL-2, Albumin, INS, and tissue plasminogen activator (tPA)). After testing their secretory ability using a mCherry/EGFP indicator system (Supplementary Fig. 1a), we found that the secretory signal peptide from tPA has supports to the best fusion antigen secretion in HEK293T cells (Supplementary Fig. 1b).

Given reports from experiments in rodents that the strength of neutralizing responses is stronger for dimeric and trimeric antigens generally [8], we designed variant AAV vectors with the tPA secretory signal peptide overhang and encoding the wild type RBD (“AAV-RBD(wt)”; residues 318 – 541) or encoding one, (“AAV-RBD(max)”) or two (“AAV-2xRBD”) RBD(max) units, which is a codon-optimized variant of the RBD for human host cell t-RNA preferences designed using online tool, or three (“AAV-3xRBD”) RBDs combined the wild type RBD and two RBD(max) (Fig. 1a and Supplementary Sequences). The expression levels and secretory efficiencies of these four variant AAV vectors were initially monitored in HEK293T cells using western blotting (Supplementary Fig. 1c,d). The AAV-tPA vaccine was set as a negative control (Fig. 1a), and all four variants were expressed and secreted.

Next, to assess the immunogenicity of these SARS-CoV-2 AAV vaccines, groups of BALB/cJ mice were vaccinated via intramuscular injection various doses of AAV viral genomes (10^9, 10^{10}, and 10^{11} viral genomes) at day 0, and sera were collected on post-vaccination days 9, 16, and 30 (Fig. 1b), and we used enzyme-linked immunosorbent assay (ELISA) to measure humoral immune responses. Compared to AAV-tPA or PBS vaccinated control animals, we detected significantly elevated IgG responses at day 9 in the animals given the AAV-RBD(max), AAV-2xRBD, and AAV-3xRBD vaccines at the 10^{11} genomes dose (Fig. 1c); no elevation was detected in animals given the AAV-RBD(wt) vaccine (Fig. 1c). At 10^{10} genomes dose, only the animals given AAV-RBD(max) vaccine shows the obvious IgG responses (Supplementary Fig. 1f). At 10^9 genomes dose, no elevation of IgG responses was detected in animals given all variants of AAV vaccine (Supplementary Fig. 1e) For day 16, we observed high levels of specific IgG responses in AAV-RBD (max), AAV-2xRBD, and AAV-3xRBD vaccinated mice given the 10^{11} viral genome dose (Fig. 1d). Notably, an elevated IgG response was also detected in the sera of the animals given the AAV-RBD(wt) vaccines relative to control at the day 16 (data not shown).

Further elevated IgG responses were detected in the day 30 sera, and specific antibodies in the AAV-2xRBD and AAV-3xRBD vaccinated samples were detected even when using a titration dilution of 1: 512,000 (Fig. 1e-g). Overall, the 10^{11} AAV viral genome dose of the AAV-3xRBD vaccine elicited the highest level of IgG response (Fig. 1e). Notably, high level of specific antibodies remained at day 180 sera from AAV-2xRBD and AAV-3xRBD vaccinated samples (Fig. 1h). Comparing the immune responses induced by different vaccine doses showed that the serum anti-RBD antibodies were induced in a dose-dependent manner, and the 10^{10} viral genomes could elicit considerable IgG response, but 10^9 viral genomes were incapable, for both the AAV-2xRBD and AAV-3xRBD vaccines (Fig. 1i,j). $10^{10} \sim 10^{11}$ genome particles is a reasonable dose employed in intramuscular injection in rodents to elicit immunity with AAV-based vaccines [9]. Taken together, we found that our fusion RBD-tPA AAV vaccine could elicit specific IgG responses to the S protein in dose-dependent manner in mice, and found that the AAV-3xRBD vaccine variant elicited the strongest response among others.

To better model the immunogenicity of AAV vaccine, we tested AAV-3xRBD vaccine in canines (beagle). Two dogs were vaccinated with a 5×10^{10} and 5×10^{11} viral genomes, respectively, and one dog was injected with the PBS as a control by day 0. Our results showed that AAV-3xRBD vaccine could elicit high level of specific antibodies at day 30 with 5×10^{11} viral genomes (Fig. 1k). In addition, the antibodies were also detectable in sera vaccinated with 5×10^{10} viral genomes (Fig. 1k,l). Given the limitation of funding, no more animals were performed in the present study, however our findings still indicate the qualitative potential immunogenicity of AAV-3xRBD vaccine.

Having detected strong IgG responses, we next explored whether the antibodies induced in mice upon vaccination with the AAV-3xRBD have neutralization activity against a SARS-CoV-2 pseudovirus. Briefly, this pseudovirus harbors capsid of the SARS-CoV-2 S protein and harbors a luciferase gene in the viral genome. We incubated the pseudovirus with diluted sera collected from the mice immunized with AAV-3xRBD or AAV-tPA vaccine (10^{11} viral genome dose, day 30 sera). The specific IgGs could competitively bind to the S protein to block the entry of the pseudovirus into HEK293T cells stably expressing known receptor ACE2, revealed as a significant decrease in luciferase activity. Specifically, we observed that immune sera showed half maximal neutralization concentration (EC50) at a calculated dilution of 1: 2750 (Fig. 1m).

We also performed histological staining as an initial assessment of the potential safety of this AAV-3xRBD vaccine. No pathologic changes were detected in liver, lung, kidney, spleen, heart, muscle, or brain of the vaccinated mice, which we sacrifice on post vaccination day 60 (Supplementary Fig. 2a-g). Further, no obvious adverse events (*e.g.*, behavioral changes or altered appetite) were observed. Notably, only weak capsid-specific IgG responses was observed in the sera of mice, resulted from the exposure to the AAV (Fig. 1n).

Vaccines are understood as likely the most efficient countermeasure to protect individuals from the threat of SARS-CoV-2 infection. In the present study, we designed an AAV vaccine that elicited powerful immunity against SARS-CoV-2 with a single intramuscular injection, and with the RBD of SARS-CoV-2 as the antigene delivered by AAV vector. Heparan aulfates (HS) are carbohydrate moieties of HS proteoglycans (HSPGs), which are ubiquitously distributed in the cytomembrane [10]. It has been demonstrated that the HS could adhere to the S protein of SARS-CoV-2, thereby contributing the binding of S protein with the ACE2 expressed on host cells [11]. Interestingly, the bindings of ACE2 and HS with SARS-CoV-2 are both mediated by RBD domain [11]. Previous studies indicated that antigens that capable of binding the HS undergo an increased Antigen-Presenting Cells (APCs) uptake and hence contribute to T-helper cell stimulation [12]. Antigens endowed with the ability to target the HS could be of great value to enhance adaptive immune response and to promote the immunogenicity in the vaccine development [13]. Given that RBD has the ability of binding to HS, RBD of SARS-CoV-2 was selected as antigen in the present study.

Notably, the vaccine is based on AAV vectors, which have an established safety record because of their FDA-approved use in human gene therapies. Also, AAV-based vaccines can be scaled up readily, since the existing manufacturing platforms for making AAV gene therapies are well understood and could be adapted to produce AAV vaccines at scale. Further, given that SARS-CoV-2 is a single strand RNA virus with multiple known mutations, it bears emphasis that AAV vaccines are adaptable vaccine technologies that can support easy exchange of the genetic code in very short time windows, likely enabling rapid production of updated vaccine types to protect against novel variations that arise. Notably, the intrinsic hardy nature of AAVs endows the vaccines remaining

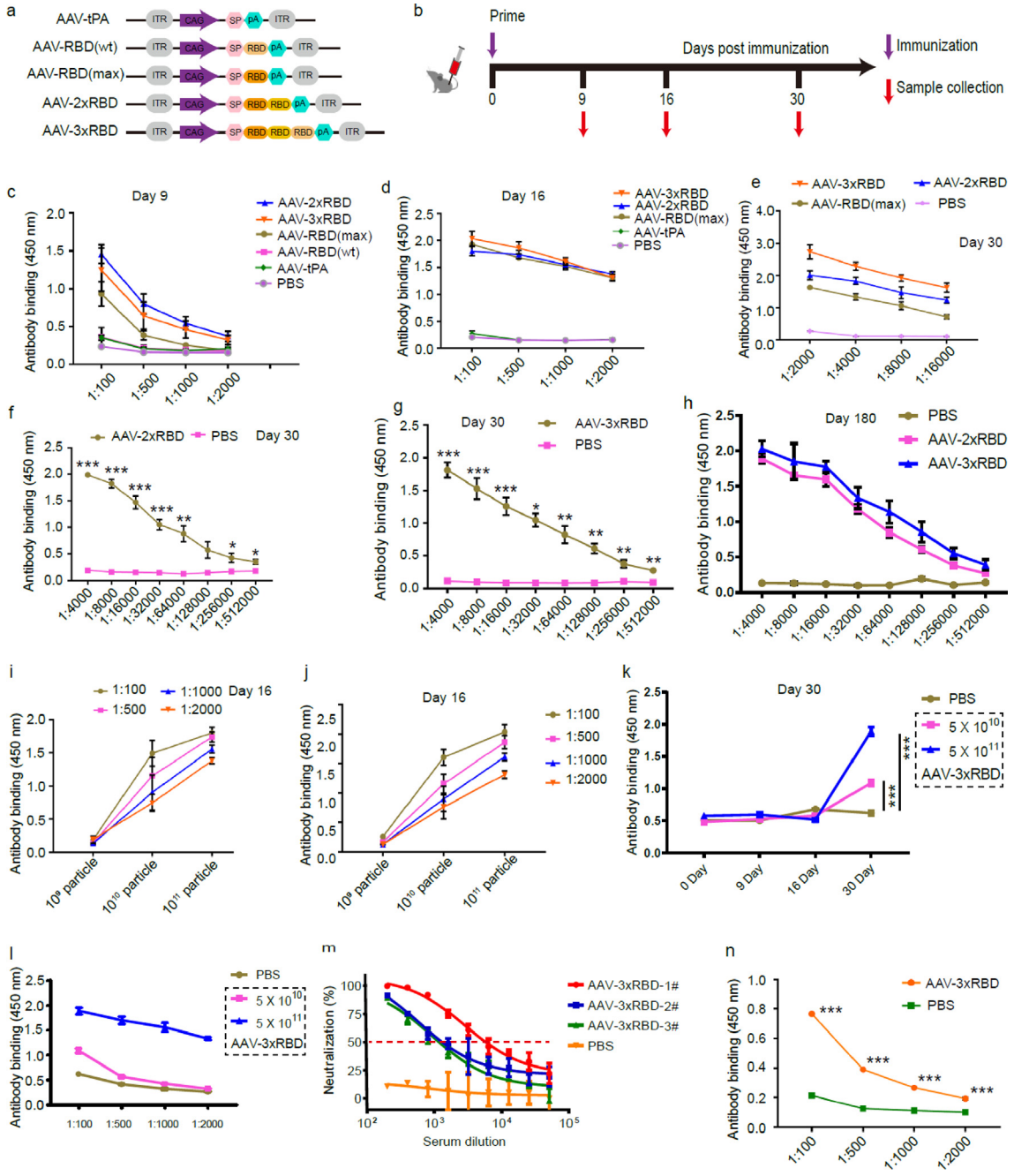

Fig. 1. Serum antibody response against RBD of the SARS-CoV-2 S protein in ELISA assay. (a) The experimental design of each AAV-vaccine vector. Vectors contains a CAG promoter (purple), secretory signal peptide (sp, light pink), various antigen (gradual yellow), and ploy A (pA, blue) that flanked with two ITR elements. The RBDs were linked with linker sequence in AAV-2xRBD and trimeric AAV-3xRBD (Supplementary Sequence). (b) The scheme of mice immunization. Mice were either mock immunized via intramuscular in caudal thigh muscle with AAV-tPA or PBS, or were vaccinated with AAV-RBD(wt), AAV-RBD(max), AAV-2xRBD, and AAV-3xRBD. A total of 54 mice were randomly divided into 6 groups that received PBS, AAV-tPA, AAV-RBD(wt), AAV-RBD(max), AAV-2xRBD, and AAV-3xRBD, respectively. The 9 mice in each AAV group were further divided into 3 groups that received 10^9, 10^{10}, and 10^{11} genomes, respectively. Time points of prime vaccination and serum sample collection are indicated by purple and red arrows, respectively. (c) Sera were collected at day 9 and were tested at indicated dilution for IgG against RBD by ELISA. The group of mice received 10^{11} genomes was showed in the figure. (d) Sera were collected at day 16 and were tested at different dilution for IgG against RBD by ELISA. The group of mice received 10^{11} genomes was showed in the figure. (e) Sera were collected at day 30 and were tested at indicated dilution for IgG against RBD by ELISA. The group of mice received 10^{11} genomes was showed in the figure. (f,g) Gradient dilution of the sera collected on 30 days and corresponding specific IgG level were curved using ELISA. The groups of mice immunized with AAV-2xRBD (f) and AAV-3xRBD (g) upon 10^{11} genomes were analyzed in the figures. (h) Gradient dilution of the sera collected at 180 days and corresponding specific IgG level were curved using ELISA. The groups of mice immunized with AAV-3xRBD upon 10^{11} genomes were analyzed, $n = 2$ in each groups at day 180 sera. (i-j) The dosage effects on IgG titer were measured using ELISA, sera were collected on 16 days. (j) The SARS-CoV-2 pseudovirus was employed to assess the neutralization ability of infection by the sera at day 30 immunized with AAV-3xRBD vaccine or AAV-tPA upon 10^{11} genomes. (k) The canine sera were collected at day 0, 9, 16, and 30, and then tested at dilution of 1 : 100 for IgG against RBD. Note that two dogs were received with $5x10^{10}$ (pink) and $5x10^{11}$ (blue) viral genomes of AAV-3xRBD vaccine, respectively, and one dog was injected with PBS (brown) as a control (l) The canine sera were collected at day 30, and then tested at indicated dilution for IgG against RBD. $n = 1$ dog in (k) and (l). (m) The SARS-CoV-2 pseudovirus was employed to assess the neutralization ability of infection by the mice sera at day 30 immunized with AAV-3xRBD vaccine or AAV-tPA upon 10^{11} genomes. (n) Assessment of immune responses to AAV vectors were tested against the Cap protein of AAV8 using ELISA. Sera on 30 days from mice vaccinated with AAV-3xRBD (10^{11}genomes) and PBS were shown. $n = 3$ mice per calculated point for all panels. $^*p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$, for statistics, see Supplementary Table 1-11.

stable and active at room temperature for storage for one month [14], which potentially facilitates the low- and middle-income countries lacking the accessibility and storage to mRNA vaccine to control the COVID-19 pandemic.

Comparing the various readouts of immunogenicity between vaccines, laboratories, and assays seems difficult. It has been reported that the macaques immunized with AAV vaccine showed the minimal to no virus detection in both upper and lower respiratory tract when confronting SARS-CoV-2 challenging, indicating the powerful immunity response elicited by AAV vaccine [14]. The ChAdOx1 nCoV-19 and BNT162b, which represent two popular types of approved vaccines in USA and Europe, are not easy to completely eliminate viral particles in the airway of Non-human primate models when confronting SARS-CoV-2 challenging [15,16]. In addition, both AAV and adenovirus vaccines can provide protection for full efficacy from a single dose, while mRNA vaccines require twice or thrice injection to achieve the comparable level of immunological response [17]. Moreover, although AAV vaccines showed reduced protection against variants of SARS-CoV-2 virus, it still providing at least comparable cross-protection against variants given by Pfizer vaccine [14,18,19]. Collectively, the current studies and our findings reveal that the AAV-3xRBD vaccine can provide the long-term potent expression of antigen of RBD to elicit powerful immunity against SARS-CoV-2 infection. A great challenge is that no approved AAV vaccines have yet been deployed in clinical application, so substantial preclinical and clinical evaluations in the vaccination context would be required for successful deployment of our RBD-tPA fusion AAV vaccine against SARS-CoV-2 infection.

2. Materials and methods

2.1. Gene cloning and AAV packaging

The sequence of wild type RBD (residues 318–541, wt) was derived from original Wuhan SARS-CoV-2 virus (NC_045512). Meanwhile, to further enhance the immunity of RBD, humanized sequence (online humanized tool:https://www.genscript.com.cn/gensmart-free-gene-codon-optimization.html), dimerized sequence, and trimerized sequence of RBD were also employed as antigens and were cloned into an ITR-flanked AAV-backbone to construct the AAV-RBD(max), AAV-2xRBD, and AAV-3xRBD vector, respectively (Supplementary sequences). The native RBD and the secretory signal peptide were clone into the ITR-flanked AAV-backbone to construct the AAV-RBD(wt) and AAV-tPA vector, respectively. The plasmid was co-transfected with pHelper and pAAV-RC into HEK293T cells to produce recombinant AAV2/8 virus genomes. Optiprep density gradient centrifugation was employed for further purification. All purified virus genomes were tittered by quantitative PCR and were qualified by transmission electron microscope.

2.2. Animals and immunization

All animal procedures were carried out in compliance with the Animal Care Committee at the Institute of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Reference NA-100426). Immunogenicity was assessed at 9, 16, 30, and 180 days after the initial vaccination of six-week old female BALB/cJ given a single intramuscular injection (IM) to the caudal thigh muscle with 10^9, 10^{10}, or 10^{11} viral genomes of each AAV vaccine. Control mice received PBS or AAV-tPA vaccine with 10^9, 10^{10}, and 10^{11} viral genomes. The titer test and neutralization test of the specific IgG as well as assessment of immune responses to AAV vectors were performed on the same batch of mice, n = 3 in each group. The H&E staining were performed on another batch of mice, n = 2 in each group. The adult beagles weighting 20 ± 2 kg were employed and vaccinated with AAV-3xRBD vaccine by intramuscular injection in hind leg. Three dogs were received with 50 μl of PBS, AAV-3xRBD containing 5×10^{10} viral genomes and 5×10^{11} viral genomes, respectively. Sera were collected at day 0, day 9, day 16, and day 30, and then analyzed by ELISA.

2.3. Enzyme-linked immunosorbent assay (ELISA)

For sera SARS-CoV-2-IgG detection, stripwell ELISA plates (Costar, 42592) were coated with 75 ng/ well of S protein (Yeasen biotech, 40592) in coating buffer (NCM Biotech, E30500) at 4 °C for 16 hr. The plates were blocked with 5% Bovine albumin at 37 °C for 2 hr and subsequently incubated with serial dilutions (1 : 100, 1 : 500, 1 : 1000, 1 : 2000, 1 : 4000, 1 : 8000, 1 : 16000, 1 : 32000, 1 : 64000, 1 : 128000, 1 : 256000, and 1 : 512000) of sera for overnight at 4 °C. Following washes, anti-muse IgG (Beyotime, A0216, 1 : 500) and anti-human IgG (Proteintech, SA00001-17, 1 : 4000) - horseradishperoxidase conjuages were used as secondary Abs and 3,5,3′5′-tetramethylbenzidine (TMB; Beyotime, P0209) was used as the substrate to examine the Ab responses, which developed for 5–10 min at RT. The reaction was stopped using stop solution (Beyotime biotech, P0215), and then the titers were read at 450 nm. Threshold for positivity was set at 3xOD value of negative control (serum obtained from the mice prior to start of the experiment). For sera IgG against capsid of AAV2/8 detection, the plates were coated with 10^9 viral genomes/well. After blocking, serial dilutions of sera from mice vaccinated with PBS and AAV-3xRBD were incubated overnight at 4 °C.

2.4. Pseudovirus packaging and neutralization assay

The murine leukemia virus (MLV) Gal-Pol packaging plasmid, transfer plasmid containing a luciferase reporter gene, and a plasmid encoding the wild-type S protein were co-transfected into HEK 293 T cells to produce MLV-based SARS-CoV-2 pseudovirus according to a previously described protocol [20]. The cells were incubated with the plasmids for 4 h, followed by exchanging of the transfection mixture for fresh DMEM medium supplemented with 10% FBS and subsequent incubation for another 48 h at 37 °C. The culture supernatant was harvested and further filtered through 0.45 μm pore-size filters.

A neutralization assay based on the MLV-pseudovirus was performed by measuring infection of hACE2 stably over-expressed HEK 293 T cells. 50 μl of the pseudovirus was preincubated with the sera from the immunized mice at various dilutions and control sera for 1 h at 37 °C, then the mixture was added to the attached ACE2-stable 293 T cells. After incubated for 2 h at 37 °C, sera-pseudovirus containing media was replaced with fresh DMEM with 10% FBS. At 48 h after viral infection, the luciferase assay system (Promega, E1500) was employed to measure the luciferase activity. Then, calculated the EC50 to measure the neutralization ability of the vaccine.

2.5. Secretory peptide screening

The plasmids for secretory peptide screening were described in supplementary data (Supplementary Fig. 1a), these plasmids were separately transfected into HEK293T cells cultured in 24 well plate with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. After 24 h incubation, the attached cells were digested and subsequently measured according to the fluorescence intensity of mCherry and EGFP with a flow cytometry. Then the fluorescent intensity ratio of mCherry and EGFP for various secretory peptide were calculated. Each sample was performed in triplicates.

2.6. Western blot

The HEK293T cells were cultured in 24 well plate with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, then transfected each well with 2 μg of AAV-RBD(wt), AAV-RBD(max), AAV-2xRBD, and AAV-3xRBD plasmid. Each sample were performed in triplicates. After 48 h incubation at 37 °C in 5% CO2 atmosphere, the supernatant were harvested and denatured by heat shocking. A total of 7 μl protein were loaded into each well and then further separated by SDS-PAGE. Following electrophoresis, proteins in the gel were transferred to polyvinylidene difluoride membranes. After blocking for 1 h with 5% non fat milk, the membranes were incubated overnight at 4 °C with antibody against RBD (SinoBiological, 40592-R001, 1 : 2000). followed by incubation with a horseradish peroxidase labeled goat anti rabbit IgG secondary antibody (abcam, ab205718, 1 : 500) at room temperature for 1 h. Signals were detected by application of ECL Western Blotting Detection Reagent.

2.7. The hematoxylin and eosin stain (H&E)

The mice immunized with AAV-3xRBD (10^{11} genomes) and PBS were sacrificed at 60 days post injection (n = 2 mice per group), The tissues consisting liver, lung, heart, muscle, kidney, spleen, and brain were harvested after perfusion with PFA. Then, tissues were further dehydrated through a series of graded ethanol baths and embedded into paraffin wax. Finally, H&E staining was conducted.

2.8. Data analysis

Data are presented as mean ± SEM. All the conclusions are based on the double blind experiments. One-way ANOVA followed by a Tukey's Multiple Comparison test was applied for the comparison among multiple treated groups (≥3) (Fig. 1c,d,e,h,i,j,k,l,m, and Fig. S1b,e,f). The comparison between two treated groups were analyzed by One-way ANOVA (Fig. 1f,g, and n).P value<0.05 was considered statistically significant. All the p values were presented in Supplementary tables.

Author contributions

H.Z., X.H., and X.W. jointly conceived the project, designed experiments, and supervised the whole project. X.H., F.L, and C.F. designed vectors and performed experiments. F.L. and C.F. prepared AAVs. Q.W., R.X., J.T. and S.G. assisted with mice immunization. Y.C. assisted with statistical analysis. Q.W., Y.L. and M.L. assisted with animal keeping. FL.C assisted with human sera collection and H&E staining. F.L., C.F. and X.H. wrote the paper. H.Z., X.H., and X.W revised the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2022.01.030.

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