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Amantadine-assembled nanostimulator enhances dimeric RBD antigen-elicited cross-neutralization against SARS-CoV-2 strains

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Introduction

COVID-19 pandemic caused by SARS-CoV-2 infection has spread rapidly worldwide\cite{1}, leading to 4.5 million people deaths in less than two years\(https://covid19.who.int\). The emergence of SARS-CoV-2 variants around the world, such as lineage B.1.1.7 (Alpha) appeared in the southeast of England\cite{2}, lineage B.1.351 (Beta) discovered in India\cite{3} and lineage B.1.617.2 (Delta) identified in the UK\cite{4}, poses a huge challenge for the prevention and control of global COVID-19 pandemic. Till now, vaccination is still the most effective strategy to prevent the spread of COVID-19 pandemic and relieves the clinical symptoms of SARS-CoV-2 infected individuals\cite{5-8}. However, the existing vaccines are suffering the decreased efficacy in protecting the inoculated population from the infection of SARS-CoV-2 variants\cite{9-11}. Therefore, there is an urgent need to develop new vaccination strategies with potent cross-protection against a variety of SARS-CoV-2 strains\cite{12-14}.

To obtain a potent cross-protection, it not only requires a rational design of antigen to induce the critical neutralizing antibody response\cite{15}, but also a potent immunostimulator to increase the magnitude of neutralizing antibody response\cite{16}. For the design of antigen, receptor-binding domain (RBD) derived from spike protein has been widely used as the antigen of anti-SARS-CoV-2 vaccines and can elicit satisfactory neutralizing antibody response in vivo, which is 1.9-4.6-fold stronger than that in COVID-19 convalescent patients\cite{17-20}. In comparison to monomeric RBD, dimeric RBD possesses more stable epitope conformation and anti-biodegradation capability in vivo\cite{21}, both of which are important for inducing the high-level neutralizing antibody response\cite{22-24}. For the selection of immunostimulator, amantadine as a well-known medication has a good biocompatibility and has been approved by FDA in large-scale clinical applications.\cite{25-27}. In the animal model, the adverse reactions of amantadine were also not observed even receiving repeated injections with high dose\cite{28}. Importantly, amantadine...
can enhance antigen-induced immune response and increase the secretion of multiple immunoregulatory cytokines such as interferons and tumor necrosis factors in vivo and in vitro. These results suggest that amantadine plays an immunostimulatory role to activate the immunity [29–32]. However, naked amantadine inevitably suffers an over-rapid elimination from the body, that would discount its immunostimulation capacity [33]. To overcome this weakness, we try to assemble monomeric amantadine molecules together to improve the utilization efficiency and circulation time of amantadine in vivo [34]. Together, the use of amantadine-assembled stimulator may effectively improve dimeric RBD-elicited cross-neutralization against SARS-CoV-2 strains. Fig. 1.

In the current study, we designed a peptide assembly block with the structure of phosphate group-modified tyrosine-phenylalanine-phenylalanine-glycine (YpFFG), and linked amantadine with the C terminal of tetrapeptide by a condensation between an amino group from amantadine and a carboxylic acid group from glycine. After adding alkaline phosphatase (ALP), we observed uniform nanospheres with an amino group from amantadine and a carboxylic acid group from glycine. After adding alkaline phosphatase (ALP) to remove the phosphate group in tyrosine, tetrapeptide-amantadine precursors rapidly assembled into nano-spherical AAS [34–36]. By mixing AAS and dimeric RBD with a mass ratio of 5:1 to vaccinate mice, dimeric RBD induced 4-fold and 1.3-fold stronger IgG and IgM response than aluminum adjuvant-assistant dimeric RBD in mice. Importantly, AAS can significantly improve dimeric RBD-induced neutralizing antibody response against Wuhan-Hu-1 (9-fold), B.1.351 variant (4-fold), B.1.1.7 variant (15-fold) and B.1.617.2 (7-fold). Molecular mechanism analysis revealed that AAS activates RLR signaling pathway to increasing the production of type I interferons, resulting in the maturation and proliferation of DCs and Ths [37,38].

Results

The design and characterization of AAS and dimeric RBD antigen

Amantadine has shown an immunostimulating effect on promoting the secretion of cytokines such as interferons and tumor necrosis factors, which are critical for inducing the activation of immune system [25–27]. In this study, we designed an AAS precursor consisting of an amantadine molecule as immunostimulator and a tetrapeptide (phosphate group-modified tyrosine-phenylalanine-phenylalanine-glycine) as the assembly block. Amantadine was linked with the C terminal of tetrapeptide by a condensation between an amino group from amantadine and a carboxylic acid group from glycine (Fig. 2a). To verify the immunostimulation effect of amantadine, 1 × 10^6 immune cells (DC 2.4) were treated with 10 μg/mL amantadine for 24 h. The expressions of multiple bioactive molecules (CD40, CD80, IL-4 and TNF-α) were significantly enhanced in amantadine-treated cells, in comparison to those in naked cells and tetrapeptide-treated cells (Fig. S1). These results indicated that amantadine, instead of tetrapeptide, possesses the immunostimulation activity against immune cells. Furthermore, we prepared and characterized AAS as following: 1 mg/mL AAS precursor was fully dissolved in 0.9% saline at pH 7.0. By adding 60 U/mL alkaline phosphatase (ALP), we observed uniform nanospheres with around 120 nm diameter by transmission electron microscope (TEM) (Fig. 2b). Dynamic light scattering (DLS) analysis revealed that AAS possesses around 155 nm hydration particle size and ~ 51.4 mV zeta potential (Fig. 2c, d). X-ray photoelectron spectrometry (XPS) showed the ratio of carbon:nitrogen (C:N) is 8:5.1 on the surface of AAS, which is close to that of amantadine (C:N = 10:1). This result suggested that amantadine is very possibly displayed on the surface of AAS (Fig. 2e). We used TEM and DLS to evaluate the stability of AAS either at room temperature or 37 °C for 27 days. The data shows that the morphology, size, zeta potential and hydrate particle size of AAS are stable (Fig. S2b, S2c). We also detected the enrichment degree of AAS in multiple organs (heart, liver, spleen, lung, kidney and lymph node) at different times (0, 3 and 6 h). The enrichment of AAS is obvious in all these organs after 3 h. AAS can still be observed in liver after 6 h (Fig. S3b). After 3 h, naked amantadine had only a limited enrichment in liver, lung and kidney, but disappeared in heart, spleen and lymph node (Fig S3b). After 6 h, amantadine is
undetectable in all these organs (Fig S3b). These results indicated that AAS has a higher accumulation and a longer residence time in vivo than naked amantadine (Fig. S3).

For the design of dimeric RBD antigen, two monomeric RBDs (35 kDa, Fig. S2a) were contacted together via a four-glycine flexible peptide linker (Gly-Gly-Gly-Gly). Western blot analysis verified the expected molecular mass of dimeric RBD which was around 70 kDa (Fig. 2f).

We also detected the interactions between AAS and dimer RBD by quantifying the equilibrium dissociation constant (K\textsubscript{D}) between AAS and dimer RBD using surface plasmon resonance (SPR) assay. As shown in Fig S4, AAS solutions with a series of concentrations (from 1.25 to 60 μM) flowed over the surface of chip immobilized with dimer RBD. The K\textsubscript{D} value is 0.15 μM. The data demonstrated that AAS can bind with dimer RBD. We prepared AAS-RBD vaccine by mixing AAS and dimeric RBD antigen with a mass ratio of 5:1 for the further animal vaccination.

The biocompatibility of AAS

We evaluated the biocompatibility of AAS in vivo. According to the published protocol[29], we injected 100 μg AAS per each mouse at day 0, and assessed the acute toxicity caused by AAS at day 14. In comparison to blank mice, AAS-injected mice showed the almost consistent weight growth (Fig. 3b). It indicated that the injection of AAS has not influence the growth of mouse body weight. Moreover, we harvested the heart, liver, spleen, lung, kidney and brain, and evaluated the organ coefficient (the ratio of organ weight/body weight). The organ coefficient based on these samples was not significantly different between AAS-injected mice and blank mice (Fig. 3c). Several critical biochemical indicators from mouse serum samples are quantified. The expression of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (CRE) and urea nitrogen (UREA) in AAS-injected mice were very close to that in blank mice (Fig. 3d). Immunohistochemical analysis also demonstrated that neither the infiltration of inflammatory cells nor organ necrosis was observed in the heart, liver, spleen, lung and kidney from AAS-injected mice (Fig. 3e). Together, all these results demonstrated that AAS has a satisfactory biocompatibility.

The molecular mechanism of AAS in regulating the host immune system

By the transcriptomes analysis, we investigated the molecular mechanism of AAS in activating the host immune system. Mice were intramuscularly injected with 50 μg AAS (Fig. 4a). Blank mice were used as control. 3 × 10^6 immune cells isolated from lymph nodes were lysed to harvest fresh RNAs for the transcriptomes analysis. According to the standard of gene expression (fold change > 2, p-adjusted < 0.05), we obtained 250 differentially expressed genes (DEGs). Principal components analysis (PCA) indicated that AAS treatment can cause a significant change of total gene expression in mice (Fig. 4b). Heat map of DEGs visually presented a different profile of gene expression between AAS-treated mice and blank mice (Fig. 4c). By the analysis of kyoto encyclopedia of genes and genomes (KEGG) pathway, we enriched multiple AAS regulated signaling pathways (Fig S5) and found that RIG-I like receptor (RLR) signaling pathway showed the highest correlation (Fig. 4d). We also observed a significantly increased profile of RLR signaling pathway-related gene expression in AAS-treated mice (Fig. 4e). By the gene ontology (GO) enrichment analysis, we obtained multiple critical AAS regulated biological function modules (Fig. 4f). Most of these modules closely correlated with the production of type I interferons that can potently activate the innate immune system.
system. We further performed weighted gene co-expression network analysis (WGCNA) to visualize an AAS regulated protein-protein interaction (PPI) network (Fig. 4g). Three key genes (Oasl2, RIG-I and IRF3) located in the central position of this PPI network. Oasl2, RIG-I and IRF3 are also three checkpoint molecules in the activated RIG-I like receptor signaling pathway. These results highlighted the importance of RIG-I like receptor signaling pathway in mediating AAS-regulated immune responses.

Retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) are key intracellular sensors of virus infection, mediating the expression of type I interferons to establish an antiviral immune response. To activate the RLR signaling pathway, AAS needs be internalized into DCs. To verify the mechanism of the internalization of AAS, we performed the inhibition experiment of phagocytosis and endocytosis. The treatment of cytochalasin B, an inhibitor of endocytosis can inhibit AAS uptake by DCs. The inhibition rate of cytochalasin B is 11.72% (Fig S9a). The treatment of cytochalasin D, an inhibitor of phagocytosis, can stronger inhibit AAS uptake by DCs. The inhibition rate of cytochalasin D is 46.03% (Fig S9a). Together, DCs can more effectively internalize AAS via phagocytosis, in comparison to endocytosis. In order to verify AAS can induce the activation of innate immune response, we measured the expression level of IFN-β in DCs by qRT-PCR. The results show that the expression level of IFN-β in DCs stimulated by AAS was significantly higher than that in control and Alu group (Fig. S9b). Together, we revealed a potential mechanism that AAS activates RLR signaling pathway to increase the expression of type I interferons that would result in the activation of innate immune system in vivo.
The effect of AAS on regulating dimeric RBD-elicited humoral response and cross-neutralization

We evaluated the effect of AAS on regulating dimeric RBD-elicited humoral response that is an important indicator for evaluating the performance of anti-SARS-CoV-2 prophylactic vaccines. 6–8 weeks Balb/c mice were immunized twice at day 0 and 10, using 50 µg AAS and 10 µg dimeric RBD (AAS+RBD group), or 35 µg aluminum adjuvant and 10 µg dimeric RBD (Alu+RBD group), or 10 µg dimeric RBD (RBD group). Serum samples were collected at day 21. The magnitude of RBD-specific IgG response in AAS+RBD group is significantly higher than that in Alu+RBD group (4-fold) and RBD group (3.5-fold) (Fig. 5b). IgG subclasses profile revealed that, in comparison to commercial aluminum adjuvant, AAS promoted dimeric RBD antigen to induce 3.5-fold, 3-fold and 3.8-fold stronger IgG2a, IgG2b and IgG3 response, instead of IgG1 response (Fig. 5b). Moreover, RBD-specific IgG subclasses (IgG2a, IgG2b and IgG3) response in AAS+RBD group increased around 2.6-fold, 3-fold and 1.35-fold, in comparison to that in RBD group (Fig. 5b). Both IgG2a and IgG2b are two crucial IgG subclasses to neutralize viral infection [40], suggesting that AAS may upregulate the titer of RBD-induced neutralizing antibody. IgG3 dominantly mediates antibody-dependent cellular cytotoxicity (ADCC) to kill infected cells [41], suggesting that AAS possibly enhance RBD-triggered cytotoxicity against infected cells. Moreover, the magnitude of IgM response in AAS+RBD group were 1.2-fold higher than that in Alu+RBD group, and 1.2-fold higher than that in RBD group (Fig. 5b). Enhanced RBD-specific IgM response would benefit from the prevention of viral infection in early stage [42].

We next investigated whether AAS can improve dimeric RBD-elicited neutralizing antibody (NAB) response in mice. The magnitude of NAB response is defined as the serum dilution of 50% neutralization (IC50) against SARS-CoV-2. In AAS+RBD group, two vaccinations induced 9-fold and 5-fold stronger NAB response against Wuhan-Hu-1 strain than that in Alu+RBD group and RBD group (Fig. 5c). The emergence of SARS-CoV-2 variants raises a serious challenge for controlling virus spread. We therefore evaluated the capacity of AAS in improving dimeric RBD-induced NAB against SARS-CoV-2 variants. NAB response against B.1.1.7 strain in AAS+RBD group is 15-fold stronger than that in Alu+RBD group and 18-fold stronger than that in RBD group (Fig. 5c). NAB response against B.1.617.2 strain in AAS+RBD group is around 6-fold stronger than that in Alu+RBD group and RBD group (Fig. 5c). Similarly, 4-fold and 6-fold enhanced neutralization against B.1.351 strain is induced in AAS+RBD group, in comparison to Alu+RBD group and RBD group (Fig. 5c). Together, these results demonstrated that AAS can significantly enhance the cross-neutralization against different SARS-CoV-2 strains in mice.

We further carried out a series of functional inhibition experiments to verify whether AAS enhances RBD-induced humoral response and cross-neutralization via activating RLR signaling pathway. IRF3 is a checkpoint molecule within RLR signaling pathway. We firstly select malachite green oxalate as IRF3 inhibitor. Malachite green oxalate can inhibit the IKBKE kinase-dependent activity of IRF3 to disturb the function of RLR signaling pathway [43]. 100 µg malachite green oxalate was injected into the mice receiving 50 µg AAS and 10 µg dimeric RBD (INHIBITOR I group). The magnitude of RBD-specific IgG, IgG2a, IgG2b, IgG3 and IgM response in INHIBITOR I group decreased 3.4-fold, 3-fold, 3.3-fold, 2-fold and 1.5-fold, in comparison to that in AAS+RBD group (Fig. 5b). Similarly, the magnitude of neutralizing antibody against Wuhan-Hu-1, B.1.1.7, B.1.351 and B.1.617.2 in INHIBITOR I group is 7-fold, 14-fold, 4-fold and 7-fold lower than that in AAS+RBD group (Fig. 5c). Moreover, we also used GSK690693 which can not only inhibit the IKKβ kinase-dependent activity of IRF3, but also inhibit the activation of STING-dependent IRF3 [44], to inject the mice receiving 50 µg AAS and 10 µg dimeric RBD (INHIBITOR II group). The magnitude of RBD-specific IgG, IgG2a, IgG2b, IgG3 and IgM response in INHIBITOR II group is 3-fold, 2.8-fold, 3.3-fold, 2-fold and 1.5-fold lower than that in AAS+RBD group (Fig. 5b). In INHIBITOR II group, the magnitude of neutralizing antibody against Wuhan-Hu-1, B.1.1.7, B.1.351 and B.1.617.2 also decreased 17-fold, 9-fold, 7-fold and 6-fold (Fig. 5c). Overall, these results demonstrated that AAS failed to enhance dimeric RBD-induced humoral response and cross-neutralization efficacy once the activity of RLR signaling pathway is suppressed.

The effect of AAS on regulating the function and behavior of immune cells

Although we have verified that AAS can activate RLR signaling pathway to enhance dimeric RBD-elicited humoral response and cross-neutralization against SARS-CoV-2 strains, it is still unknown how AAS regulates the functions and behaviors of critical immune cells to enhance RBD-specific humoral response and cross-neutralization. To answer this question, we assessed the maturation and proliferation of three types of immune cells (B cells, DCs and T helper cells) from draining lymph nodes in AAS+RBD group, INHIBITOR I group and INHIBITOR II group (Fig. 6a). Flow cytometry analysis showed that the expression of CD40 and CD86 on the surface of B cells, that are two critical indicators to evaluate B cell maturation, was significantly higher in AAS+RBD group, in comparison to that INHIBITOR I group and INHIBITOR II group (Fig. 6b). This result indicated that AAS-mediated activation of RLR signaling pathway would promote the maturation of B cells in mice. Previous studies have solidly demonstrated that both DCs-mediated antigen presentation (the first signal) and T helper cells-assistant B cell activation (the second signal) are required for the maturation of B cells [46]. We therefore evaluated the effect of AAS on regulating the functions and behaviors of DCs and T helper cells. Higher CD40 and CD86 expression on the surface of DCs were detected in AAS+RBD group, in comparison to INHIBITOR I group and INHIBITOR II group (Fig. 6c). Meanwhile, the abundance of T helper cells (CD3+CD4+ T cells) in AAS+RBD group (73%) significantly increased, in comparison to that in INHIBITOR I group (66%) and INHIBITOR II group (64%) (Fig. 6d). We also analyzed the ratio of Th1 cells and Th2 cells in AAS+RBD group. The percentage of Th1 cells was 2.7-fold higher than Th2 (Fig. 6c). Together, AAS activate RLR signaling pathway to promote the maturation and proliferation of DCs and T helper cells, finally resulting in the activation of B cells to produce potent neutralizing antibodies in vivo.

Discussion

Amantadine is a well-known medicine for the indications in infectious and neurology diseases such as influenza, Parkinson’s disease and drug-induced extrapyramidal symptoms [27,47]. Recently, Y. Zhang, R. Wang, C. He et al. Nano Today 43 (2022) 101393
several observational clinical studies have also reported that amantadine can prevent SARS-CoV-2 infection and symptomatology [48]. However, the underlying mechanism of amantadine in preventing SARS-CoV-2 is still unexplored [49]. Multiple published works reported that amantadine or its derivatives can stimulate immune cells such as DCs and macrophages to produce cytokines [29–31]. DCs and Ths can provide the first signal (DCs-mediated antigen presentation) and the second signal (Ths-assistant activation) to activate B cells to produce potent cross-neutralizing antibodies against SARS-CoV-2 strains [50,51].

Of note, our transcriptomes analysis suggested that amantadine molecules displayed on the outermost layer of AAS may be in charge of activating RLR signaling pathway. By comparing with the transcriptomes data between naked amantadine-treated and AAS-treated mouse group, we observed the similar kyoto encyclopedia of genes and genomes (KEGG) pathways and protein-protein interactions (PPI). KEGG pathways showed that the activation of RLR signaling pathway is obvious in naked amantadine-treated mice [Fig S6], which is consistent with that in AAS-treated mice (Fig. 4d). Similarly, PPI analysis also showed that, in naked amantadine-treated mice, three RLR signaling pathway-related genes (Oasl2, RIG-I and IRF3) located in the central position (Fig S7), which is consistent with that in AAS-treated mice (Fig. 4g). Furthermore, we analyze the correlation coefficients between AAS-treated samples and naked amantadine-treated samples. The data showed a high correlation between AAS and amantadine (Fig S8). Together, these results supported that amantadine is a major factor to activate RLR signaling pathway in mice.

In this study, as far as we know, we for the first time revealed that amantadine can upregulate the production of type I interferons to activate the innate immune system in a RLR signaling pathway-dependent manner. Moreover, using different inhibitors to block RLR signaling pathway can not only suppress AAS-induced maturation and proliferation of immune cells such as B cells, DCs and T helper cells, but also reduce AAS-enhanced neutralizing antibody response in vivo. In line with our findings in the current study, other
researchers also reported the effect of Oasl2 in activating the function of RIG-I (a checkpoint gene in RLR signaling pathway) and enhancing the expression of type I interferons\[37\], both of which can activate the host innate immune system. The activation of innate immune system is a critical step for the enhancement of antigen-induced acquired immune responses\[52,53\]. Therefore, our findings inspired us to develop amantadine as a potent immunostimulator to enhance antigen-elicited immune response.

However, multiple shortcomings such as unsatisfactory water solubility, low utilization efficiency, excessive dosage and short half-life period \textit{in vivo} limited the use of naked amantadine as an immunostimulatory\[33,54,55\]. In this work, we employed the assembly strategy to solve these problems. In many cases, assembly strategy has been successfully applied to amplify the function of monomeric elements such as protein, peptide, chemical small molecule \textit{etc.} in the field of biomedicine\[56–60\]. We here linked amantadine with a tetrapeptide that can not only provide assembly drive force, but also increase the water solubility of amantadine-based assembly. By constructing a new amantadine-tetrapeptide molecule, we managed to assemble monomeric amantadine molecules together and improved \textit{in vivo} utilization efficiency of amantadine, finally amplifying the immunoreactivity of amantadine.

The design of antigen is another critical factor to determine vaccine efficacy\[61\]. RBD of spike protein is an attractive antigen candidate in SARS-CoV-2 vaccines and can trigger high-quality humoral responses to neutralize SARS-CoV-2 infection \textit{in vivo}. However, monomeric RBD requires a high vaccination dosage and only induces modest immune response without potent immunostimulatory in the human body\[22\]. In comparison to monomeric RBD, dimeric RBD have stronger anti-degradation ability, more stable epitope conformation and more sufficient immunogenicity\[22,24\]. We therefore selected dimeric RBD as the antigen in this study. We only need to simply mix RBD and AAS.
Moreover, the preparation process of AAS-RBD vaccine is convenient and robust. Together, by rationally designing and selecting the combination of immunostimulatory and antigen, our vaccination strategy based on AAS and dimeric RBD can elicit the potent RBD-specific humoral responses. Importantly, vaccine-elicited cross-neutralization against different SARS-CoV-2 strains was significantly enhanced as well. Recent data exposed some hidden troubles due to the continuous mutation of SARS-CoV-2. The enhanced cross-neutralization induced by our AAS-dimeric RBD vaccination strategy provides hope in overcoming the continuous mutation of SARS-CoV-2. In the further, the extended application of AAS-dimeric RBD vaccination strategy would also benefit from the prevention of COVID-19 worldwide.

Materials and methods

Preparation and characterization of AAS

AAS percursor was synthesized in GL biochem (Shanghai) Ltd. 0.1 mg/mL AAS percursor was dissolved in 0.9% saline and adjusted to pH 7.0 with Na2CO3 solution. AAS was prepared by adding 60 U/mL alkaline phosphatase (ALP, TAKARA) and keeping at the room temperature for 10 min. The morphology and size of AAS were observed by the transmission electron microscope (TEM, FEI Tecnai T20). The zeta potential of AAS was detected by Malvern Zetasizer (Zetasizer Nano ZS). Both C and N on the surface of AAS were determined by X-ray photoelectron spectrometry (XPS, Thermo Fisher Scientific).

Mice

Balb/c mice (female, 6–8 weeks) were from the Institute of Medical Biology, Chinese Academy of Medical Sciences (CAMS), and were cultured in specific pathogen-free (SPF) condition at the Central Animal Care Services of Institute of Medical Biology, CAMS. All mice experiments were approved by the animal ethics committee of Institute of Medical Biology, CAMS. Animal ethics review number is DWSPJ202008 021.

The culture and evaluation of dendritic cells (DCs)

DC 2.4 cell line was from American Type Tissue Collection (ATCC). DCs were cultured at 37 °C and 5% CO2 with the complete dulbecco’s modified eagle medium (DEME, Gibco) that was supplemented by 10% fetal bovine serum (FBS, Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco). We employ flow cytometry analysis to verify the effect of amantadine on regulating the maturation and function of DCs. DCs were cultured on a 6-well plate with 1 × 106 cells per well. After culturing for 12 h, DCs were stimulated with either 10 μg tetratrapeptide (YpFFG) or 10 μg amantadine. 24 h later, DCs were stained with DAPI-live/dead reactive dye (Invitrogen) to determine the cell viability. Both CD40 and CD86 on DCs were stained with anti-CD40-FITC and anti-CD86-PE/Cyanine 7 monoclonal antibodies (Biolegend) to determine cell maturation. Both IL-4 and TNF-α secreted by DCs were stained with anti-IL-4-Brilliant Violet 421 and anti-TNF-α-PerCP/Cyanine 5.5 monoclonal antibodies (Biolegend) to determine the function of DCs. At least 50,000 DCs were collected by BD LSR Fortessa (BD Biosciences). The data was analyzed with Flowjo software (Tree Star, Ashland, OR).

Cellular uptake inhibition

DC 2.4 cells were plated at 5 × 105 cells per well in 24-well plates at 37 °C with 5% CO2. After 24 h, DC 2.4 cells were incubated with 5 μM cytoplasmal B (Meilunbio) or 5 μM cytoplasmal D (Meilunbio) for 1 h, and then incubated with 5 μg Cy5-labeled AAS for 2 h. DC 2.4 cells were harvested, washed twice with PBS containing 2% fetal bovine serum (FBS), fixed with 2% paraformaldehyde for 10 min, resuspended in PBS containing 2% FBS, and analysed by flow cytometry (Beckman Coulter).

The stability of AAS

The AAS dispersed in PBS were separately stored in a glass vial at room temperature and 37 °C for 27 days. Transmission electron microscopy (TEM) was performed to study storage stability of AAS. Aliquots were taken at day 0, 9, 18, 27 and absorbed onto the copper-coated carbon grid for 2 min. The excess solution was manually removed with the filter paper. Subsequently, AAS were negatively stained with 2% phosphotungstic acid for 10 s and air-dried at room temperature overnight. The morphology was observed using FEI Tecnai T20 at an accelerating voltage of 80 kV. Moreover, the storage stability of the AAS was evaluated in terms of the particle size and zeta potential monitored by dynamic light scattering analysis (DLS). The nanoparticle suspensions were separately stored obtained at room temperature and 37 °C.

Transcriptome sequencing analysis

Nine mice were used for the transcriptome analysis in this study. We used the Balb/c mice for AAS injection. The samples were collected at 24 h hour after AAS injection. Transcriptome sequencing was completed by Amooroad Gene Technology. Transcriptomic analysis was performed by CASJC Supercomputing Center. Differentially expressed genes were obtained by DESeq2 package using Rstudio. The visualization of principal component analysis (PCA) was performed with stats and scatter plot 3d packages using Rstudio. Heat map was generated using ggplot2 package in Rstudio. Violin plot was draw with ggpubr package by Rstudio. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis was carried out using Metascape. Based on the database for Annotation Visualization & Integrated Discovery (DAVID); Gene Ontology (GO) enrichment analysis was applied. With the utilization of weighted gene co-expression network analysis (WGCNA), the protein-protein interaction (PPI) network was visualized by Cytoscape software. Each three replicate samples of AAS and amantadine were applied for relevance analysis. Correlation heatmap was performed with stats package using Rstudio.

RNA isolation and quantitative real-time PCR (qRT-PCR)

DC 2.4 cells were plated at 1 × 106 cells per well in 6-well plates overnight. DC 2.4 cells were incubated with 50 μg AAS, 35 μg Alu, 20 μg LPS and complete dulbecco’s modified eagle medium (DEME) for 12 h. Total RNA was extracted from DC 2.4 cells using RNAeasyTM Isolation Reagent (Vazyme), following manufacturer’s instructions. 500 ng of total RNA was reverse transcribed into cDNA using HiScript® III RT SuperMix for qPCR (Vazyme), following manufacturer’s instructions. qRT-PCR was performed with ChamQ Universal SYBR qPCR Master Mix (Vazyme). The housekeeping gene β-actin was used for normalization. The mouse primer sequences were: IFN-β forward, 5’-ATGACTGTGTTGGTCCAGC-3’; IFN-β reverse, 5’-TACACTCTTAACTGACTAGATT-3’; β-actin forward, 5’-AACAGTCCGCT AGAAAGAC-3’; β-actin reverse, 5’-CGTGTACATCGTAAAGAC-3’. Data were normalized to β-actin expression.

Gene expression values were normalized to β-actin expression and are quantified by the 2−ΔΔCt method.
Surface plasmon resonance (SPR) assay

The interaction of AAS and dimer RBD was studied by their binding ability using a Biacore S200 (Washington, DC, USA). The phosphate-buffered saline solution (PBS) (137 mM NaCl, 10 mM phosphate, 2.7 mM KCl, pH 7.4) was used as a running buffer. Dimer RBD was captured on the chip. AAS, as the analyte, was diluted in running buffer, with a concentration series (1.25–60 μM) in running buffer. The buffer was set to flow over the captured dimer RBD, and the obtained response units (RU) were recorded. Then, the sensor chip surface was regenerated with sodium acetate solution (pH 5.0) for 60 s. For the Binding Level Screen, AAS were screened at 200 mM on a surface of > 10,000 RU of RBD, with a 120 s contact time and a 180 s dissociation time. The contact time was 400 s and dissociation time was 400 s. Dose-response data was collected in the traditional multicycle format. The data was automatically fitted to the 1:1 binding model for both kinetics and steady-state affinity.

The organ enrichment test in vivo

Balb/c mice were utilized to study the enrichment degree of each organ of AAS. The organ enrichment test, the mice were randomly divided into three groups. The mice of the treatment group were injected by tail vein with Cyamine 7 NHS ester is labeled on AAS by condensation reaction (50 μg AAS group) and Cyamine 7 NHS ester is labeled on amantadine by condensation reaction (10 μg Amantadine group). The control group received the PBS solution with the same amount of Cyamine 7 NHS ester. Before fluorescence imaging, the heart, liver, spleen, lung, kidney and lymph node in mice were harvested. The imaging of all the organs were performed at the at 0, 3 and 6 h post administration.

Mice vaccination

6–8 weeks Balb/c mice were from the Institute of Medical Biology, Chinese Academy of Medical Sciences and Peking Union Medical College. The animal experiments were approved by the Yunnan Provincial Experimental Animal Management Association. Mice were randomly divided into six groups (n = 5) as following: (1) 50 μg AAS and 10 μg dimeric RBD (AAS+RBD group); (2) 35 μg aluminum adjuvant and 10 μg dimeric RBD (Alu+RBD group); (3) 50 μg AAS, 10 μg dimeric RBD and 100 μg Malachite green oxalate (INHIBITOR I group); (4) 50 μg AAS, 10 μg dimeric RBD and 100 μg GSK690693 (INHIBITOR II group); (5) 10 μg dimeric RBD (RBD group); (6) 0.9% saline (Control group). Mice received AAS, dimeric RBD, Alu adjuvant and saline by intramuscular injection at day 0 and 10. Both malachite green oxalate and GSK-690693 were injected into mice in an intraperitoneal manner. After 10 days of the final immunization, mice were sacrificed to harvest spleens and serum samples.

Enzyme-linked immunosorbent assay (ELISA)

The production of RBD-specific IgG, IgG1, IgG2a, IgG2b, IgG3, IgM in mice serum was tested by ELISA. RBD antigen (0.25 μg per well) were coated in a 96-well plate at 4°C overnight. The plate was blocked with 2% BSA for 2 h at 37°C. By washing the plate three times with PBS (Phosphate buffer saline (PBS) with 0.05% Tween 20),100 μL mouse serum was added into per well and incubated at 37°C for 1 h. The plate was washed with PBS three times. Each well of 96-well plate was added with 100 μL HRP-labeled goat anti-mouse IgG, gC1, IgG2a, IgG2b, IgG3, IgM binding antibody at 37°C for 1 h. After washing the plate three times with PBS, 50 μL3,3′,5,5′-tetramethylbenzidine (TMB; Sigma) was added into per well. The reaction was stopped with 50 μL0.05% H₂SO₄. The absorbance value at 450 nm and 630 nm wavelength was determined by ELISA plate reader (Tecan, San Jose, CA).

The detection of neutralizing antibody against SARS-CoV-2

The detection of neutralizing antibody was carried out by pseudovirus infection platform. Three types of SARS-CoV-2 pseudovirus (Wuhan-Hu-1, B.1.1.7, B.1.351 and B.1.617.2) were from National Institutes for Food and Drug Control. 50 μL pseudovirus was incubated with serially diluted serum at 37°C for 1 h and was added into ACE2-high expression 293 T cells (5 × 10⁶ cells per well). 48 h later, the supernatant solution was removed 0.100 μL luciferase substrate (Promega) was added into per well. The relative luciferase activity was measured using the luminometer (Bio-Tech).

Flow cytometric analysis

Fresh splenocytes were collected by grinding mouse spleen. 1 × 10⁶ cells were stained with Zombie NIR™ (Biolegend) to evaluate cell viability. Both CD3 and CD4 were stained with anti-CD3-Brilliant Violet 510 and anti-CD4-FITC monoclonal antibodies (Biolegend) to assess the counts of CD4+ T cells. For the detection of B cell activation and dendritic cell maturation, 1 × 10⁶ cells were stained with Zombie NIR™ (Biolegend) to evaluate cell viability. CD45R, CD40 and CD86 were stained with anti-CD45R-Brilliant Violet 510, anti-CD40-FITC and anti-CD86-PE/Cyanine 7 monoclonal antibodies (Biolegend) to assess DC cell maturation. CD11c, CD40 and CD86 were stained with anti-CD11c-Alexa Fluor 700, anti-CD40-FITC and anti-CD86-PE/Cyanine 7 monoclonal antibodies (Biolegend) to assess B cell activation. At least 50,000 cells were collected by BD LS Fortessa (BD Biosciences). The data was analyzed with FlowJo software (Tree Star, Ashland, OR).

Western blot analysis

2 μg dimeric RBD protein or 2 μg monomeric RBD protein was added into 5 ×loading buffer. After 10 min boiling, RBD protein samples were performed by SDS-PAGE gel electrophoresis. RBD protein was transferred on a PVDF membrane and incubated with anti-RBD rabbit monoclonal antibody (targeting antibody) at 4°C overnight. HRP-conjugated goat-anti-rabbit antibody was incubated with PVDF membrane 2 h. RBD protein was finally visualized by adding 1 mL chemiluminescent substrate (Bio-Rad).

The analysis of weight and organ coefficient

Mice were randomly divided into two groups (n = 5). Mice in AAS group and control group received 100 μg AAS and 0.9% saline by tail vein injection. All mice were monitored every 4 h. The weight of mice was recorded every 2 days. At day 14, the survival rate of mice was recorded. The heart, liver, spleen, lung, kidney and brain in mice with or without 100 μg AAS injection were harvested. All organs were weighted on a digital balance. The organ coefficient was calculated as the following formula: organ coefficient (%) = (organ weight/body weight) × 100%.

The detection of biochemical indicators in serum samples

The mice blood was centrifuged at 3500 rpm for 10 min to obtain serum. The expression of alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (CRE) and urea nitrogen (UREA) in serum samples was analyzed by a fully automatic animal blood biochemical analyzer (BS-200, Mindray).

Immunohistochemical analysis

The mice organs (heart, liver, spleen, lung, kidney) were harvested and fixed with 4% (v/v) formalin at room temperature. These organs were embedded in paraffin and were cut into the slices with 5 μm thickness. These slices were stained with hematoxylin-eosin and were observed under an optical microscope (Zeiss).
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. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nantod.2022.101393.

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Statistical analysis

Statistical analysis was carried out using GraphPad Prism 9 Software. One-way analysis of variance (ANOVA) and T-test were used to test for statistical significance. All p values were marked in the figures.

Author contributions

Ye Liu conceived and designed the experiments. Ye Liu and Ye Zhang designed and produced immunogens. Ye Zhang, Chunyan He, Jia Luo, Yu-Fang Zhang, Chunyan He, the figures.

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Declaration of Competing Interest

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.nantod.2022.101393.
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