Electronic Supplementary Material (ESI) for ChemComm.

Supplementary information

Synthesis and immunological evaluation of TLR1/2 ligand-conjugated receptor binding domains as self-adjuvanting vaccine candidates

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Table of Contents

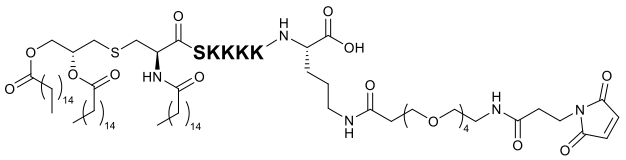
1. General

All reagents and solvents were obtained from commercial suppliers. They were used without further purification. All of the solvents used for experiment were reagent grade or HPLC grade. Manual solid phase peptide synthesis (SPPS) was carried out in Reservoir-2 FRITS (Agilent Technologies). Automated solid phase peptide synthesis (SPPS) was carried out by PurePep®Chorus Peptide Synthesizer (Gyros Protein Technologies). Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis was carried out by CLASS-VP system and LC solution system (SHIMADZU). The purity of synthesized peptide was evaluated by LC/MS with 1290 Infinity LC system (Agilent Technologies) connected to micrOTOF-QII-HR (ESI-TOF-MS, Bruker) or prominence LC system (SHIMADZU) connected to micrOTOF Compact (ESI-TOF-MS, Bruker). High-resolution mass spectra (HRMS) and tandem mass spectrometry (MS/MS) were obtained on a Q Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Massachusetts, US). MALDI-TOF-MS spectra were obtained by AXIMA Performance MALDI-TOF (SHIMADZU) with sinapinic acid as a matrix. All animal experiments were performed at Japan SLC. Inc. with following compliance with the Guidelines for the Care and Use of Laboratory Animals and approved by their ethics committees. Female wild-type BALB/c mice 6–8 weeks old used for immunological studies. ELISA experiments were performed using 96 well Microwell^{TM} MaxiSorp^{TM} microtiter plates (Sigma Aldrich). Horseradish peroxidase (HRP)-linked goat anti-mouse IgG (A4416, Sigma-Aldrich), IgM (ab97230, abcam, Cambridge, UK), IgG1 (ab97240, abcam), IgG2a (ab97245, abcam), IgG2b (ab97250, abcam), and IgG3 (ab97260, abcam) were used.

2. Materials

2-1. Synthesis of maleimide-/pyridyl disulfide-functionalized Pam_3CSK_4 1/2

<Synthesis of maleimide-functionalized Pam_3CSK_4 **1**>



The peptide Fmoc-Ser(^{t}Bu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(ivDde)-Wang-Resin was synthesized by automated SPPS on Fmoc-Lys(ivDde)-Wang-Resin (108.69 mg; 0.05 mmol, 0.46 mmol/g resin loading; Peptide Institute. Inc.) using Fmoc-based coupling reactions (6 equiv. of Fmoc amino acid). *O*-(1H-6-Chlorobenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HCTU, 6 equiv.) and *N*-methylmorpholine (NMM, 12 equiv.) solution in *N*,*N*-dimethylformamide (DMF, 10.5 mL) were used as condensation reagents for automatic peptide synthesis. Each condensation reaction was performed at room temperature for 30 minutes and repeated once. Deprotection of Fmoc was performed by 20% piperidine in DMF (10 mL) at room temperature for 4 minutes three times.

After construction of Fmoc-Ser(^{t}Bu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(ivDde)-Wang-Resin, the subsequent reactions were performed throughout manual SPPS in a 10 mL Agilent solid phase extraction (SPE) tube with an adapter cap. To the resultant resin was added 20% piperidine in DMF (1 mL) at room temperature. After being shaken for 30 minutes at room temperature, the resin was washed with dichloromethane (DCM) five times and DMF five times. To the resultant resin were added the mixture of Fmoc-Pam_2Cys-OH (1 equiv.), *N*-hydroxy-5-norbornene-2,3-dicarboximide (HONB, 2 equiv.), *N*,*N*′-Diisopropylcarbodiimide (DIC, 2 equiv.), and NMM (1.5 equiv.) in DMF/DCM (1/1) (1 mL) at room temperature. After being shaken for 1 hour at room temperature, the resin was washed with DCM five times and DMF five times. This coupling step was repeated, and the obtained resin was washed with DCM five times, and DMF five times. To the resultant resin was added 20% piperidine in DMF (1 mL) at room temperature. After being shaken for 30 minutes at room temperature, the resin was washed with DCM five times and DMF five times. To the resultant resin were added palmitic acid (5 equiv.), 1-[bis(dimethylamino)methylene]-1H-benzotriazolium 3-oxide hexafluorophosphate (HBTU, 5 equiv.), 1-hydroxybenzotriazole (HOBt, 5 equiv.), and NMM (10 equiv.) in DMF (2.5 mL) at room temperature. After being shaken for 1 hour, the resin was washed with DCM five times and DMF five times.

To the resultant resin was added 2% hydrazine in DMF (1 mL) at room temperature. After being shaken for 30 minutes, at room temperature, the resin was washed with DCM five times and DMF five times. To the resultant resin were added 19-Maleimido-17-oxo-4,7,10,13-tetraoxa-16-azanonadecanoic acid (1 equiv.), HBTU (1 equiv.), HOBt (1 equiv.), and NMM (2 equiv.) in DMF (1 mL) at room temperature. After being shaken for 1 hour at room temperature, the resin was washed with methanol five times and DCM five times and dried.

The peptide was deprotected and cleaved from the resin by treatment with a cocktail of trifluoroacetic acid (TFA) /triisopropylsilane (TIPS)/water = 95/2.5/2.5 (1 mL) at room temperature for 1 hour. The reaction mixture was filtered to remove resins. To the filtrate was added ice-cold diethyl ether to precipitate the crude product. The resultant precipitate was washed with ice-cold diethyl ether. The resultant crude product was purified by HPLC on a Cosmosil

5C4-AR-300 column (20 mm×250 mm) at a flow rate of 8 mL/min using a mobile phase of 0.1% TFA in water/acetonitrile/isopropanol (8/1/1: Solvent A) and 0.1% TFA in acetonitrile/isopropanol (1/1: Solvent B) on a 50 to 98% B gradient over 48 minutes, at UV detection at 220 nm, affording **1** (9.65 mg, 9% yield)) as a white solid after lyophilization.

HRMS (ESI-Orbitrap, positive): *m/z* for $C_{105}H_{194}N_{14}O_{22}S$ $[M+2H]^{2+}$ calculated 1018.7179; found 1018.7179, $[M+3H]^{3+}$ calculated 679.4811 found 679.4814.

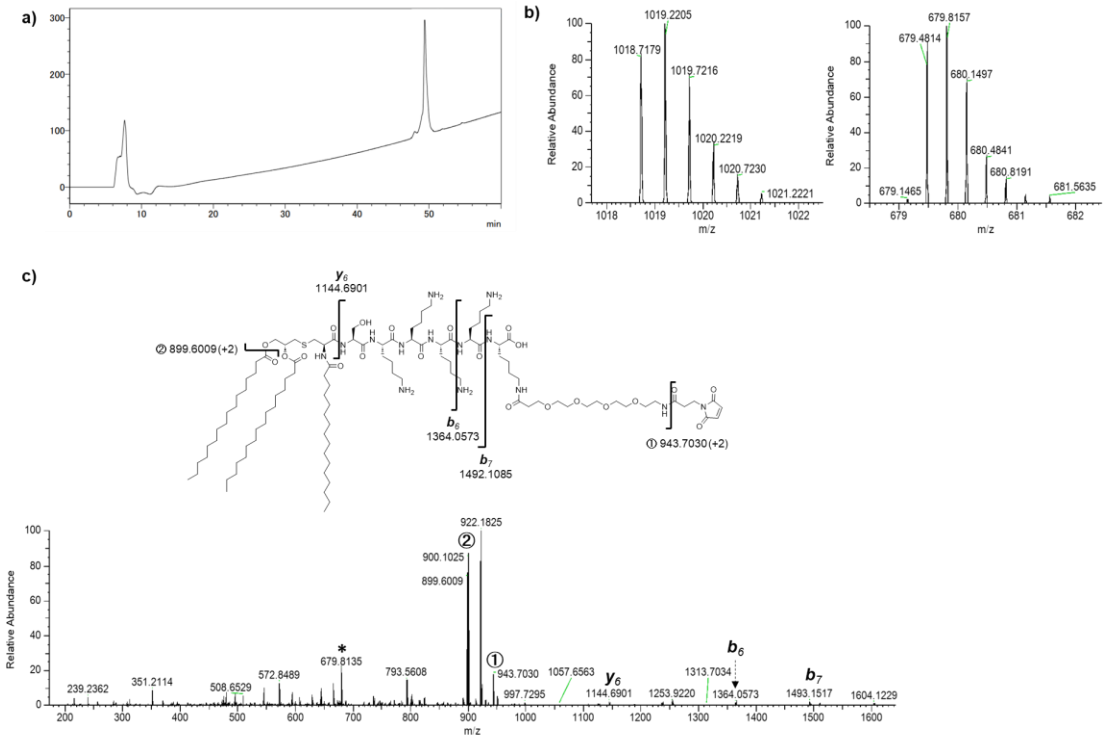
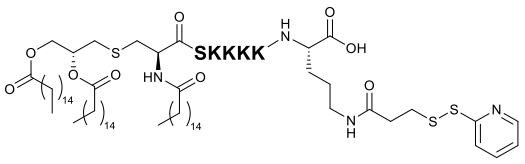


Figure S1. a) HPLC chart of maleimide-functionalized Pam_3CSK_4 **1**. Analytical column (5C4-AR300, 4.6×240 mm, Nacalai Tesque), eluent (0.1% TFA in water/acetonitrile/isopropanol (8/1/1, Solvent A) and 0.1% TFA in acetonitrile/isopropanol (1/1, Solvent B), 50 to 98% B gradient over 48 min, followed by isocratic elution of 98% B for 7 min), flow rate (0.5 mL/min); detection (220 nm UV). b) HRMS and b) MS/MS spectrum of maleimide-functionalized Pam_3CSK_4 **1**. Asterisk represents precursor ion (m/z = 679 ± 2.5). HCD = 20.

<Synthesis of pyridyl disulfide-functionalized Pam_3CSK_4 **2**>



The peptide Pam_3Cys-Ser(^{t}Bu)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(Boc)-Lys(ivDde)-Wang-Resin was synthesized as shown above. Half of the obtained resin was used for further reaction. To the resultant resin was added 2% hydrazine in DMF (1 mL) at room temperature. After being shaken for 30 minutes, at room temperature, the resin was washed with DCM five times and DMF five times. To the resultant resin were added 3-(2-pyridyl dithiol)-propionic acid (10 equiv.), HBTU (10 equiv.), HOBt (10 equiv.), and NMM (20 equiv.) in DMF (1 mL) at room temperature. After being

shaken for 1 hour at room temperature, the resin was washed with methanol five times and DCM five times and dried. The peptide was deprotected and cleaved from the resin by treatment with a cocktail of trifluoroacetic acid (TFA) /triisopropylsilane (TIPS)/water = 95/2.5/2.5 (1 mL) at room temperature for 1 hour. The reaction mixture was filtered to remove resins. To the filtrate was added ice-cold diethyl ether to precipitate the crude product. The resultant precipitate was washed with ice-cold diethyl ether. The resultant crude product was purified by HPLC on a Cosmosil 5C4-AR-300 column (20 mm×250 mm) at a flow rate of 7 mL/min using a mobile phase of 0.1% TFA in water/acetonitrile/isopropanol (8/1/1: Solvent A) and 0.1% TFA in acetonitrile/isopropanol (1/1: Solvent B) on a 50 to 98% B gradient over 48 minutes, at UV detection at 220 nm, affording **1**(3.8 mg, 8% yield) as a white solid after lyophilization.

HRMS (ESI-Orbitrap, positive): *m/z* for $C_{95}H_{175}N_{13}O_{15}S_3[M+2H]^{2+}$ calculated 918.1310; found 918.1324, $[M+3H]^{3+}$ calculated 612.4237; found 612.4243, $[M+4H]^{4+}$ calculated 459.5696; found 459.5699.

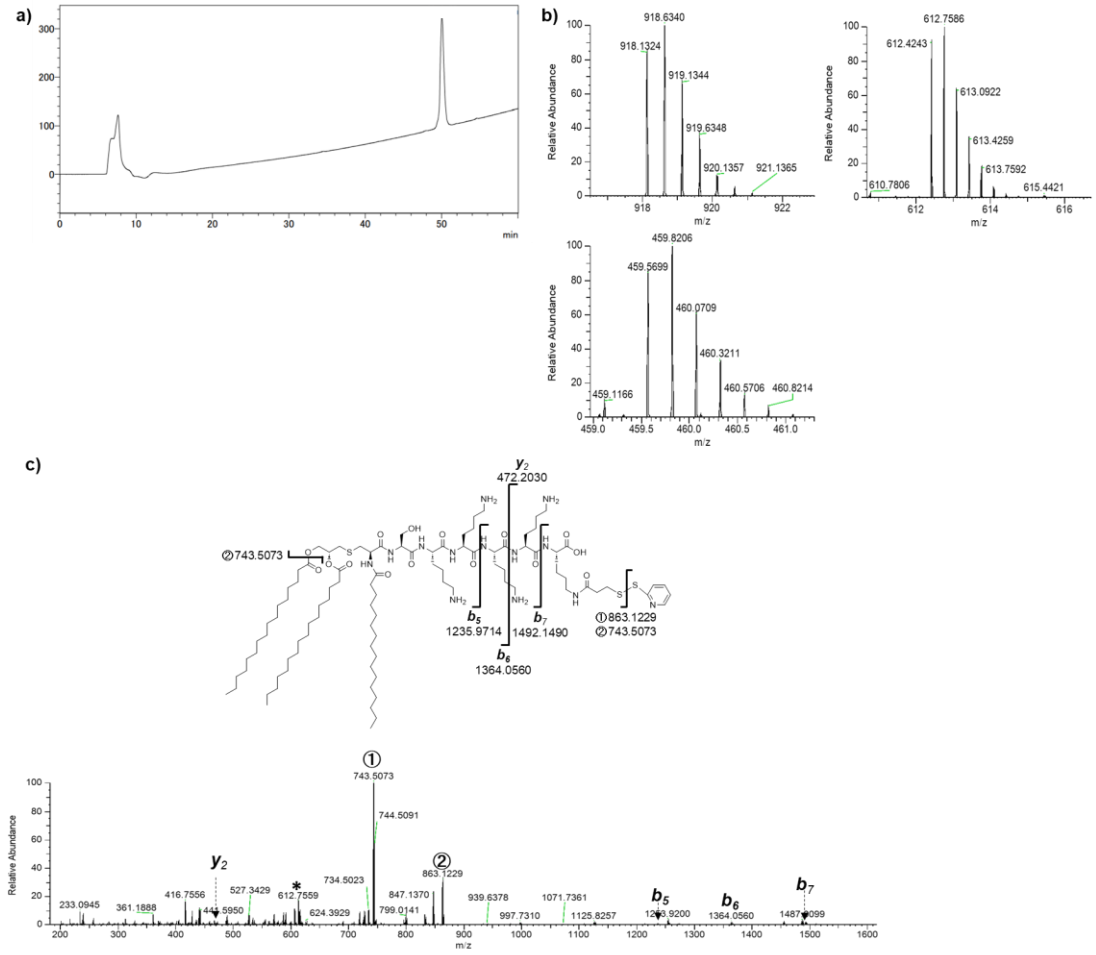


Figure S2. a) HPLC chart of pyridyl disulfide-functionalized Pam_3CSK_4 **2**. Analytical column (5C4-AR300, 4.6×240 mm, Nacalai Tesque), eluent (0.1% TFA in water/acetonitrile/isopropanol (8/1/1, Solvent A) and 0.1% TFA in acetonitrile/isopropanol (1/1, Solvent B) , 50 to 98% B gradient over 48 min, followed by isocratic elution of 98% B for 7 min), flow rate (0.5mL/min); detection (220 nm UV).HRMS and b) MS/MS spectrum of pyridyl disulfide-functionalized Pam_3CSK_4 **2**. Asterisk represents precursor ion (m/z = 612 ± 2.5). HCD = 20.

2-2. Preparation of RBD and deglyRBD

The purification of the receptor binding domain (RBD) of the spike protein was described earlier.[1]

For the production of the deglycosylated RBD (deglyRBD), the protein was treated with endoglycosidase-H (EndoH) at a 1:80 enzyme-to-substrate ratio to remove *N*-glycans. Following a 20-hour reaction at 18 ºC, SDS-PAGE analysis confirmed successful cleavage. Subsequent to EndoH removal using an MBP-Trap Column (GE Healthcare), the deglyRBD was further purified on a HiLoad 26/60 Superdex 75 Column (GE Healthcare), pre-equilibrated with 25 mM TRIS and 150 mM NaCl pH 7.5. Protein quantification was performed via absorbance at 280 nm, applying a theoretical extinction coefficient, $\varepsilon_{280\ nm}$ = 33,350 $M^{-1}cm^{-1}$ for both RBD and degly RBD.

2-3. Conjugation with Pam_3CSK_4

<Preparation of RBD-mal-Pam_3CSK_4/deglyRBD-mal-Pam_3CSK_4>

To RBD/deglyRBD (30 μg/45 μL) in phosphate-buffered saline (PBS) was added 50 mM tris(2-carboxyethyl)phosphine in water (TCEP, 5 μL, final concentration of TCEP: 5 mM) at room temperature. After being shaken for 1 hour at room temperature, the reaction solution was transferred to a 10 kDa ultrafiltration tube (Amicon® Ultra-0.5mL Centrifugal Filters, Ultracel®-10K), and 350 μL PBS was added. The solution was centrifuged under 10,000 rpm for 15 minutes at 4 ºC. To the higher molecular weight fraction was added 350 μL PBS. The steps of centrifugation and PBS addition were repeated four times.

To the resultant disulfide-cleaved RBD/deglyRBD in PBS (30 μg/40 μL) was added 10 mM maleimide-functionalized Pam_3CSK_4 **1** in DMSO (10 μL, final concentration of **1**: 2 mM) at room temperature. After being incubated for 1 hour at 37 ºC, the reaction solution was dialyzed with PBS with a 14 kDa dialysis membrane (042-30913, FUJIFILM Wako Chemicals Corp.) for 48 hours at 4 ºC to give RBD-mal-Pam_3CSK_4/dglyRBD-mal-Pam_3CSK_4.

<Results of MALDI-MS analysis>

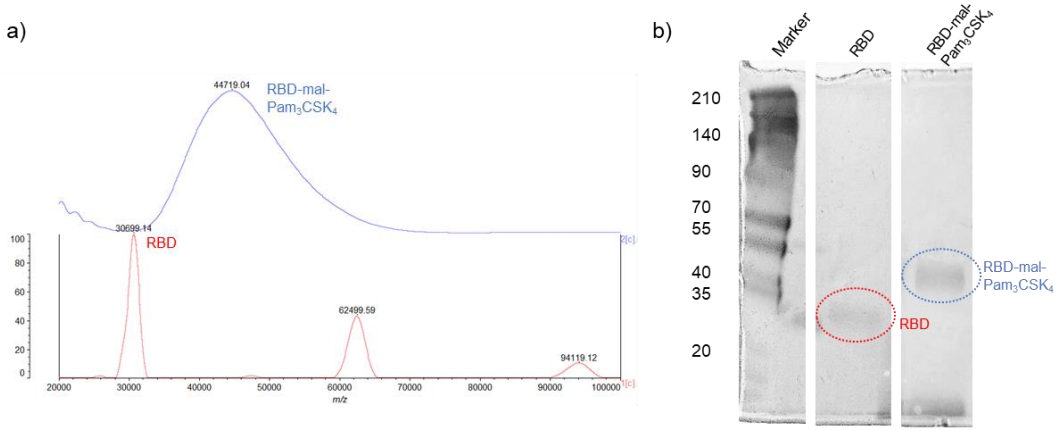


Fig. S3. a) MALDI-MS analysis. b) SDS-PAGE analysis. Red: RBD, Blue: RBD-mal-Pam_3CSK_4. The average loading ratio of Pam_3CSK_4 was estimated to 7.

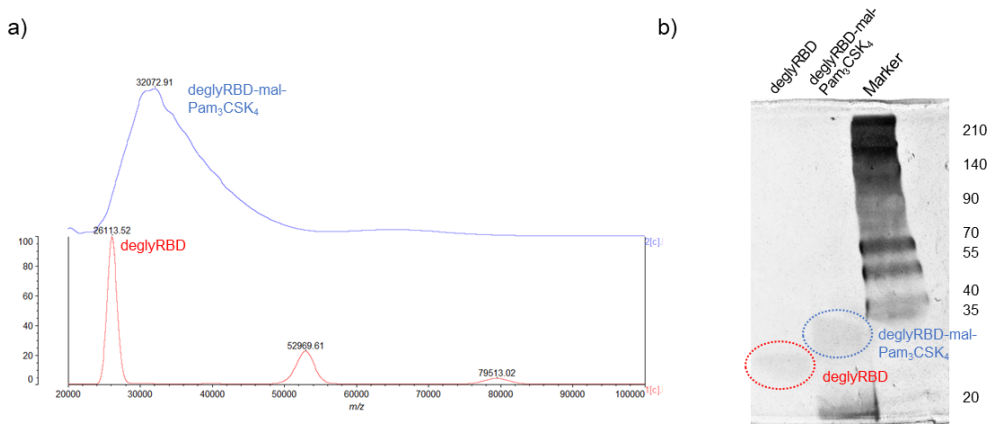


Fig. S4. a) MALDI-MS analysis. b) SDS-PAGE analysis. Red: deglyRBD, Blue: deglyRBD-mal-Pam_3CSK_4. The average loading ratio of Pam_3CSK_4 was estimated to 3.

<Preparation of RBD-SS-Pam_3CSK_4/deglyRBD-SS-Pam_3CSK_4>

To RBD/deglyRBD (30 μg/45 μL) in phosphate-buffered saline (PBS) was added 50 mM tris(2-carboxyethyl)phosphine in water (TCEP, 5 μL, final concentration of TCEP: 5 mM) at room temperature. After being shaken for 1 hour at room temperature, the reaction solution was transferred to a 10 kDa ultrafiltration tube (Amicon® Ultra-0.5mL Centrifugal Filters, Ultracel®-10K), and 350 μL PBS was added. The solution was centrifuged under 10,000 rpm for 15 minutes at 4 ºC. To the higher molecular weight fraction was added 350 μL PBS. The steps of centrifugation and PBS addition were repeated four times.

To the resultant disulfide-cleaved RBD/deglyRBD in PBS (30 μg/60 μL [RBD], deglyRBD: 30 μg/50 μL [deglyRBD]) was added 10 mM pyridyl disulfide-functionalized Pam_3CSK_4 **2** in DMSO (10 μL, final concentration of **1**: 1.4 mM [RBD], 1.7 mM [deglyRBD]) at room temperature. After being incubated for 1 hour at 37 ºC, the reaction solution was dialyzed with PBS with a 14 kDa dialysis membrane (042-30913, FUJIFILM Wako Chemicals Corp.) for 48 hours at 4 ºC to give RBD-SS-Pam_3CSK_4/dglyRBD-SS-Pam_3CSK_4.

<Results of MALDI-MS analysis>

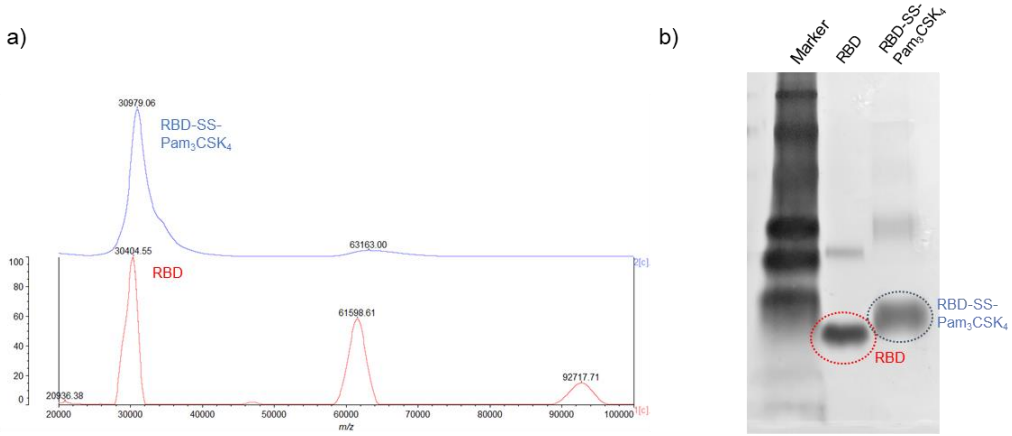


Fig. S5. a) MALDI-MS analysis. b) SDS-PAGE analysis. Red: RBD, Blue: RBD-SS-Pam_3CSK_4. The average loading ratio of Pam_3CSK_4 was estimated to 1.

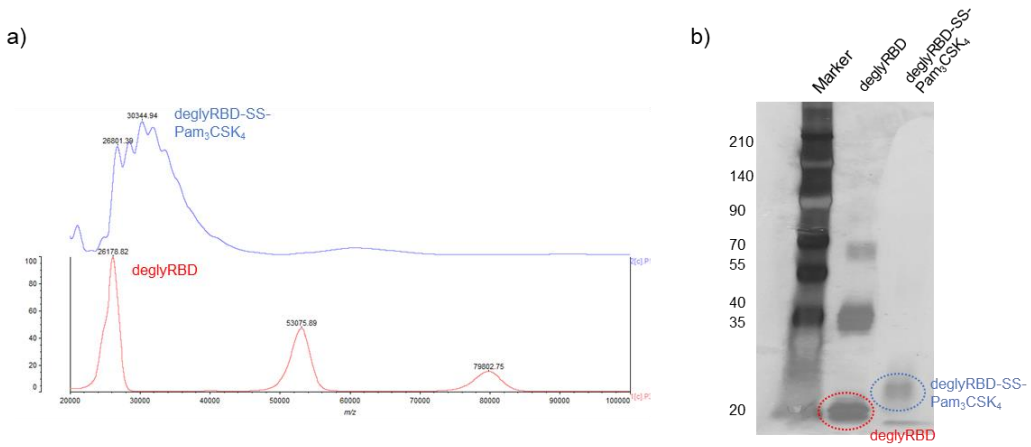


Fig. S6. a) MALDI-MS analysis. b) SDS-PAGE analysis. Red: deglyRBD, Blue: deglyRBD-SS-Pam_3CSK_4. The average loading ratio of Pam_3CSK_4 was estimated to 2.

3. Mice immunization

Experiments using BALB/c mice were approved in compliance with the Guidelines for the Care and Use of Laboratory animals, and all animal studies were approved by the animal experiment committee of Japan SLC, Inc. The numbers of approval are #BT22017 and #BT22138. Each group of five female wild-type BALB/c mice (8 weeks age) was inoculated with subcutaneous (s.c.) injection of V1-V8 listed in Table 1 on day 0. The immunization schedule included boosting each mouse two times on days 14, 28, respectively. Blood was collected from each mouse before immunization (blank controls) and on day 7, 21, 35, and was clotted to obtain plasmas that were stored at -80 °C before use.

4. ELISA

<Protocol>

Nunc™ Microwell™ MaxiSorp™ 96-well ELISA microtiter plates (Thermo Fisher Scientific)were coated with a solution of the RBD/deglyRBD (1 μg /mL, 50 μL per well) in the coating buffer (50 mM carbonate, pH 9.5) at 4 ºC for 16 h. Nonspecific sites were blocked with 1% (w/v) BSA (9018A3733-50G, Sigma Aldrich) in coating buffer at 37 ºC for 2 h, then washed three times with PBS at pH 7.4. Subsequently, an individual mouse plasma with serial half-log dilutions from 1:100 to 1:102400 in PBS containing 1% BSA was added to the coated plates (50 μL per well). The plates were incubated at 25 ºC for 2 h and then washed three times with PBS containing 0.05 % Tween-20 (PBST). It was incubated at room temperature for 1 h with a 1:1000 diluted solution of HRP-linked goat anti-mouse IgG (A4416, Sigma-Aldrich), IgM (ab97230, abcam) antibody, IgG1 (ab97240, abcam), IgG2a (ab97245, abcam), IgG2b (ab97250, abcam), or IgG3 (ab97260, abcam) antibody (50 μL per well), respectively. After the plates were washed five times with 0.05% PBST, 0.4 mg/mL *o*-phenylenediamine dihydrochloride (158-02151, OPD.2HCl, 5 mg/Tablet) in 0.05 M phosphate-citrate buffer at pH 5.0 with 0.4 μL/mL 30% H_2O_2 was added to the plates (100 μL per well) for 25 min at room temperature, 2.5 M aqueous sulfuric acid (50 μL per well) was subsequently added to stop the colorimetric reaction. Optical density (OD) was measured at 492 nm on an BioTek Cytation 5 plate reader (Agilent Technologies Inc.). For titer analysis, the OD values were plotted against the serum dilution numbers to obtain a best-fit a logarithm line. The equation of this line was used to calculate the dilution number at which an OD value of 0.1 for IgG, 0.2 for IgG1 and IgG2a, and 0.3 for IgG2b, IgG3, and IgM was achieved, and this dilution number was defined as the antibody titer.

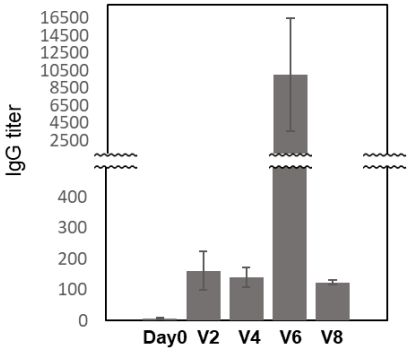


Fig. S7 IgG antibody titters against deglyRBD after third immunizations with V2, V4, V6, and V8. Data represent the results from five experiments (n = 5). The error bars represent the standard error of the mean value.

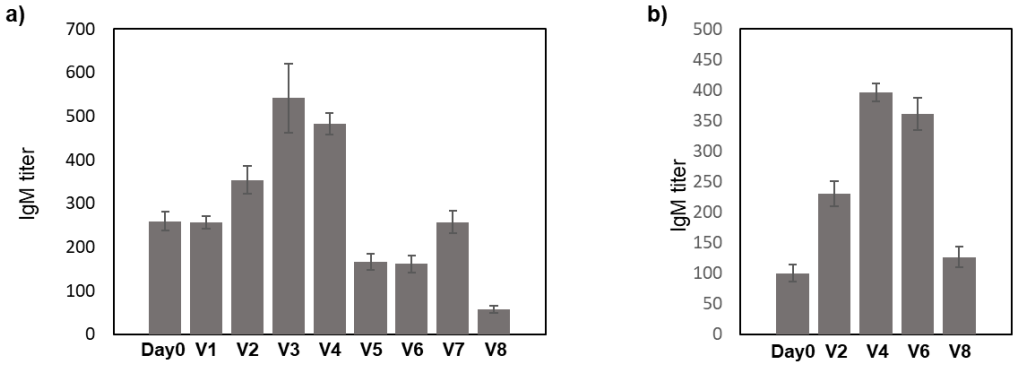


Fig. S8 IgM antibody titters against (a) RBD after third immunizations with V1–V8 and (b) deglyRBD after

third immunizations with V2, V4, V6, and V8. Data represent the results from five experiments (n = 5). The error bars represent the standard error of the mean value.

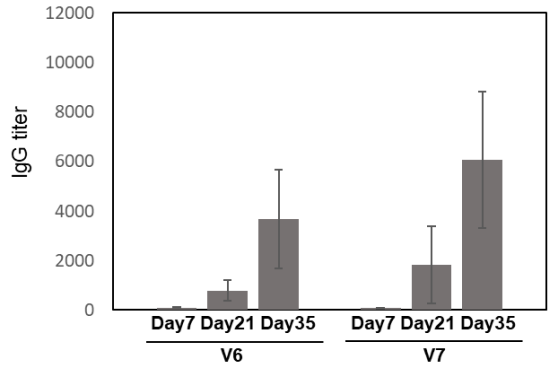


Fig. S9 IgG antibody titters against RBD of V6 and V7 on day 7, day 21, and day 35. Data represent the results from five experiments (n = 5). The error bars represent the standard error of the mean value.

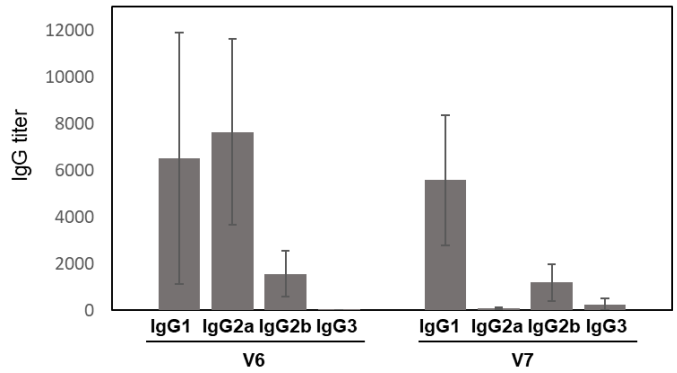


Fig. S10 IgG1, IgG2a, IgG2b, IgG3 antibody titters against RBD after third immunizations with V6 and V7. Data represent the results from five experiments (n = 5). The error bars represent the standard error of the mean value.

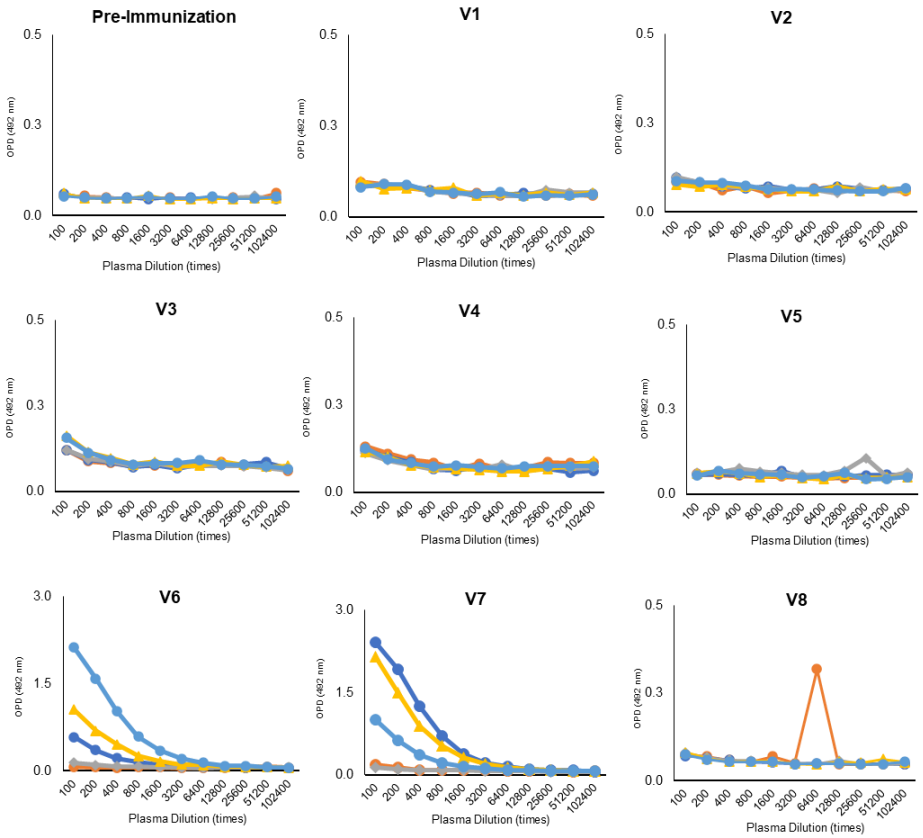


Fig. S11 IgG antibody titers against RBD of plasma immunized with V1-V8 on day 35 and pre-immunization.

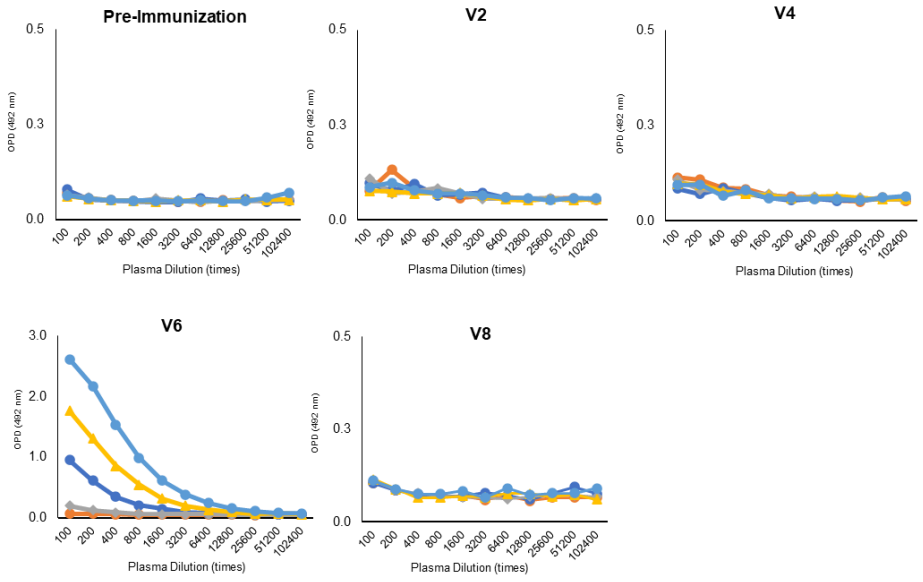


Fig. S12 IgG antibody titers against deglyRBD of plasma immunized with V2, V4, V6, and V8 on day 35 and pre-immunization.

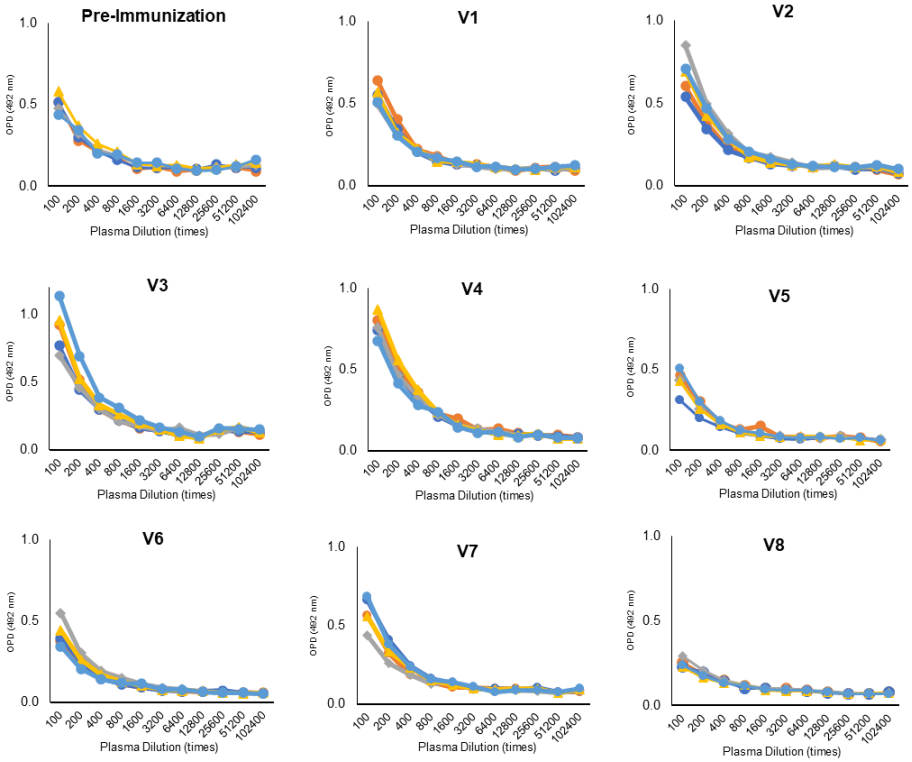


Fig. S13 IgM antibody titers against RBD of plasma immunized with V1-V8 on day 35 and pre-immunization.

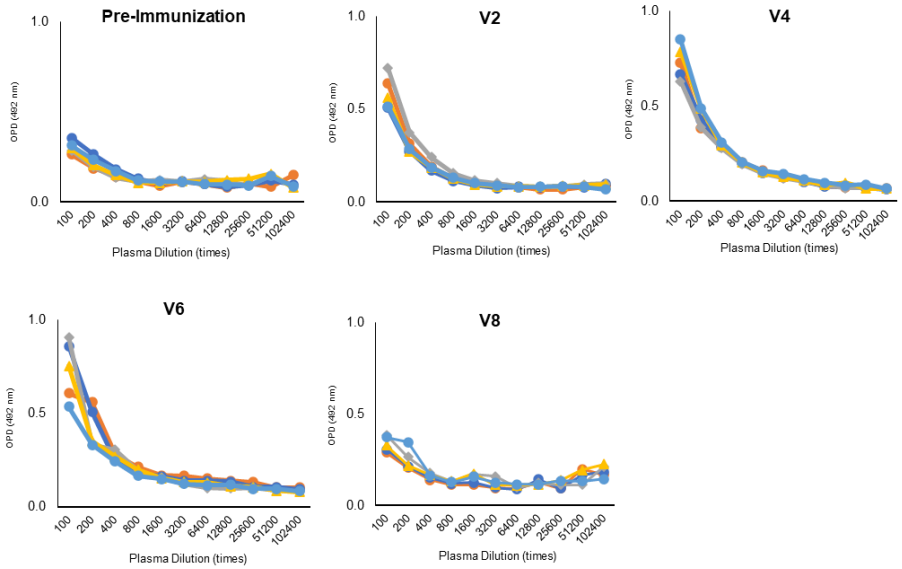


Fig. S14 IgM antibody titers against deglyRBD of plasma immunized with V2, V4, V6, and V8 on day 35 and pre-immunization.

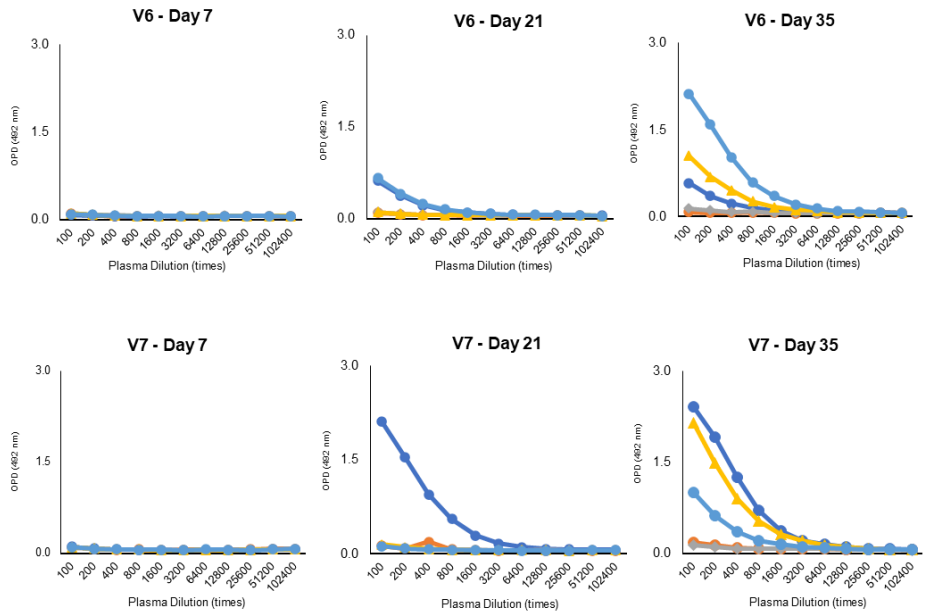


Fig. S15 IgG antibody titers against RBD of plasma immunized with V6 and V7 on day 7, 21, and 35.

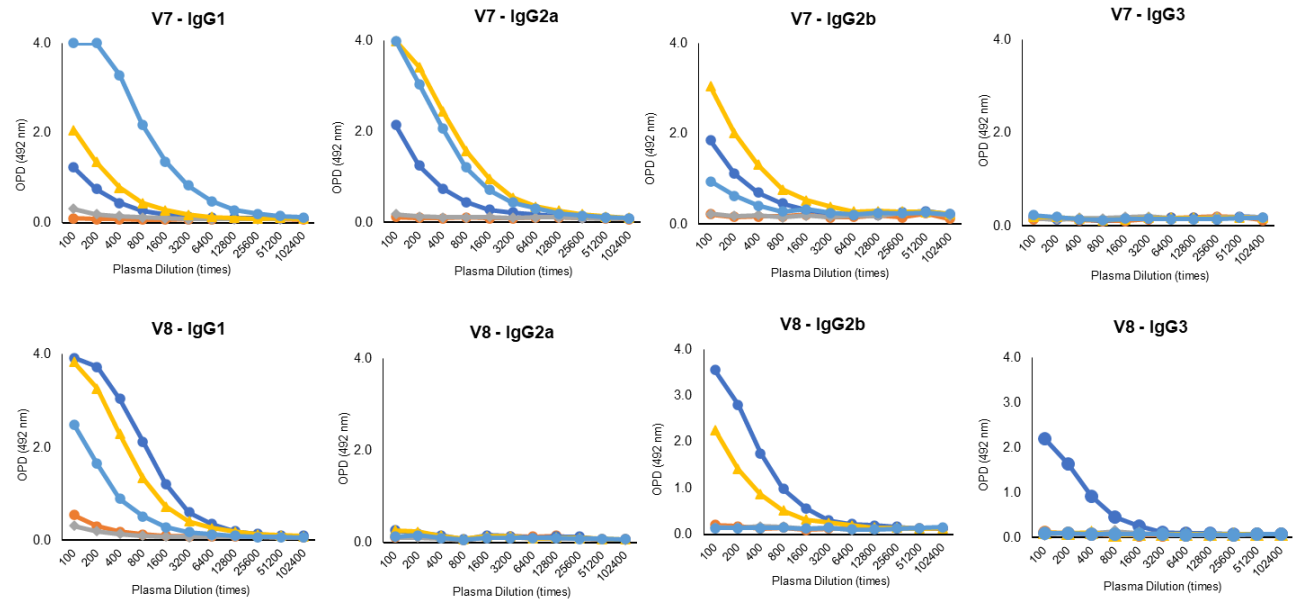


Fig. S16 IgG1, IgG2a, IgG2b, IgG3 antibody titters against RBD of plasma immunized with V6 and V7 on day 35.

5. References

1. S. Villanueva-Saz, J. Giner, A. Fernández, D. Lacasta, A. Ortín, J. J. Ramos, L. M. Ferrer, M. Ruiz de Arcaute, A. P. Tobajas, M. D. Pérez, M. Verde, D. Marteles, R. Hurtado-Guerrero, J. Pardo, L. Santiago, A. M. González-Ramírez, J. Macías-León, A. García-García, V. Taleb, E. Lira-Navarrete, J. R. Paño-Pardo and H. Ruíz, *Animals*, 2021, **11**, 1984.