Electronic Supplementary Material (ESI) for Materials Advances.

Supplementary information

Melanoma immunotherapy enabled by M2 macrophage targeted immunomodulatory cowpea mosaic virus

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Materials and Methods

Peptide synthesis – The sequences of peptides CD206 (azide-CSPGAKVRC) and CD206s (CSPGAK)[1] were provided to GenScript Biotech for solid-phase synthesis and analytics. We resuspended CD206s in MiliQ water and CD206 in ultrapure DMSO (VWR) both at concentrations of 5 mg/mL.

Production of CPMV and CPMV conjugates – CPMV was propagated in black eyed pea no. 5 plants and purified as previously reported[2, 3]. CPMV-DBCO, CPMV-Cy5, CPMV-DBCO-Cy5 and CPMV-SM(PEG)$_8$-Cy5 were synthesized by adding DBCO-PEG$_4$-NHS ester (649.7 g/mol, BroadPharm), sulfo-Cy5 NHS ester (777.95 g/mol, Lumiprobe) and SM(PEG)$_8$ (689.71 g/mol, Thermo Fisher Scientific), respectively, to the solvent-exposed surface lysine residues of CPMV. DBCO-PEG$_4$-NHS ester, sulfo-Cy5 NHS ester, and SM(PEG)$_8$ were prepared in ultrapure DMSO at 50 mg/mL. To synthesize CPMV-DBCO, we used a 1200-molar excess of DBCO-PEG$_4$-NHS ester. To synthesize CPMV-SM(PEG)$_8$, we used a 1200-molar excess of SM(PEG)$_8$. To synthesize CPMV-Cy5, we used a 1200-molar excess of sulfo-Cy5 NHS ester. To synthesize CPMV-DBCO-Cy5, we used a 1200-molar excess of DBCO-PEG$_4$-NHS ester and a 1200-molar excess of sulfo-Cy5 NHS ester. To synthesize CPMV-SM(PEG)$_8$-Cy5, we used a 1200-molar excess of SM(PEG)$_8$ and a 1200-molar excess of sulfo-Cy5 NHS ester. In each case, the other reaction component was 2 mg/mL (final concentration) of CPMV (molecular weight = 5.6 x 10^6 g/mol) in 10 mM KP buffer (pH 7). All reactions were incubated for 2 h at room temperature. To remove unconjugated linkers, the reaction mixtures were loaded onto a 40% sucrose cushion and centrifuged at 52,000 rpm for 70 min using an Optima-Max TL tabletop ultracentrifuge (Beckman Coulter). Purified CPMV conjugates were then resuspended in 10 mM KP buffer (pH 7). To produce CPMV-CD206 and CPMV-CD206-Cy5, a 1200-molar excess of CD206 was mixed with 2 mg/mL CPMV-DBCO or CPMV-DBCO-Cy5 in 10 mM KP buffer (pH 7), and incubated at room temperature for 4 h. For CPMV-CD206s and CPMV-CD206s-Cy5, a 1200-molar excess of CD206s was mixed with 2 mg/mL CPMV-SM(PEG)$_8$ or CPMV-SM(PEG)$_8$-Cy5 in 10 mM KP buffer (pH 7), and incubated at room temperature for 2 h. Unconjugated peptides were then removed using PD MidiTrap G-25 centrifugal columns (Cytiva). Purified CPMV-CD206, CPMV-CD206s, CPMV-CD206-Cy5 and CPMV-CD206s-Cy5 particles were stored at 4 °C.

Characterization of CPMV and its conjugates

UV-Vis spectrophotometry

Nanoparticle concentrations were determined by UV-Vis spectrophotometry using a NanoDrop 2000 instrument (Thermo Fisher Scientific) and the extinction coefficient (ε) of CPMV at 260 nm = 8.1 mL / (mg x cm). The number of conjugated Cy5 molecules per CPMV particle was determined using the molar extinction coefficient (ε) for sulfo-Cy5 (271000 L / (mol x cm)) at 647 nm.

NuPAGE

To prepare samples, CPMV and its conjugates were mixed with 4x lithium dodecylsulfate buffer (Thermo Fisher Scientific) with 1x reducing agent (Invitrogen) and then heated at 95 °C for 8 min. 20 µL of each sample was loaded onto a 4–12% NuPAGE gel (Thermo

Fisher Scientific), and run at 200 V, 120 mA, and 25 W for 35 min in 1x MOPS buffer (Thermo Fisher Scientific). For the Cy5-labeled samples, Cy5 fluorescence was first imaged using the MultiColor red filter, then all gels were stained with Coomassie Brilliant Blue for protein imaging. Images were captured using a ProteinSimple FluorChem R imager. To quantify the conjugated peptides per CPMV, ImageJ software was used.

Agarose gel electrophoresis

10 µg CPMV particles were first mixed with 6x Gel Loading Purple dye (Biolabs) and then loaded onto 1.2% (w/v) agarose gels containing GelRed nucleic acid gel stain (Gold Biotechnologies). The gels were run at 100 V and 400 mA for 35 min in 1x Tris acetate EDTA (TAE) buffer (Thermo Fisher Scientific). Prior to protein imaging using Coomassie, RNA was imaged using UV light and Cy5 was imaged using MultiColor Red filter. Gels were then stained with Coomassie Brilliant Blue to image proteins. Images were captured as described above.

Dynamic light scattering (DLS)

The samples were first diluted to 1 mg/mL. 100-µL aliquots of each sample were analyzed using a Zetasizer Nano ZSP/Zen5600 instrument (Malvern Panalytical) three times at room temperature.

Transmission electron microscopy (TEM)

Samples were diluted to 0.5 mg/mL in 10 mM KP buffer (pH 7). To prepare grids, 4-µL of each sample were applied to a glow-discharged carbon film with a 300-mesh Cu grid. After 30 s, the grids were blotted using filter paper, washed using Milli-Q water, blotted again, and then stained with 4 µL 1% (w/v) uranyl acetate (Electron Microscopy Sciences) for 30 s. After blotting on filter paper, the grids were air dried for imaging using a Talos Transmission Electron Microscope (Thermo Fisher Scientific) at a nominal magnification of 120,000x.

Cell culture

B16F10-OVA melanoma cells

B16F10-OVA murine melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Corning) supplemented with 10% (v/v) fetal-bovine serum (FBS; VWR), and 1% (v/v) penicillin/streptomycin (Pen/Strep; Cytiva) at 37 °C in a 5% CO_2 atmosphere. The cells were harvested in trypsin-EDTA (Corning), washed three times with PBS (Corning), and resuspended in PBS for inoculation.

RAW246.7, M1, and M2 macrophages

Murine RAW264.7 macrophages (ATCC TBI-71) were cultured in DMEM supplemented with 10% (v/v) FBS and 1% (v/v) Pen/Strep. To differentiate the cells into M1 and M2 macrophages, they were stimulated with 1 ng/mL LPS or 20 ng/mL IL-4 and 20 ng/mL IL-13 for 24 h, respectively. M2 macrophages were also serum starved for 48 h before uptake experiments[4]. All cells were maintained at 37 °C in a 5% CO_2 atmosphere.

Flow cytometry

To confirm successful stimulation of M1 and M2 macrophages and the expression of CD206 on M2 macrophages, RAW264.7, M1, and M2 macrophages were collected using Gibco Cell Dissociation Buffer (Thermo Fisher Scientific) and washed twice with PBS. Cells were first stained using LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) on ice for 15 min then blocked using anti-CD16/32 Fc block (Biolegend) on ice for 15 min, then stained using an Alexa Fluor 647 labeled anti-CD206 antibody (Biolegend) on ice for 30 min. To stain the intracellular inducible nitric oxide synthase (iNOS) and Arginase 1, cells were fixed and permeabilized using the BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit on ice for 20 min. After two washes with PBS, cells were stained using PE/Cy7 labeled anti-iNOS antibody (eBiosicences) and Alexa Fluor 488 labeled anti-Arginase 1 antibody (eBiosicences) on ice for 30 min. After washing, cells were resuspended in BD Pharmingen Stain Buffer (BD Biosciences) and analyzed using a BD LSR II flow cytometer (BD Biosciences). Data were analyzed using Flowjo_v10.7.

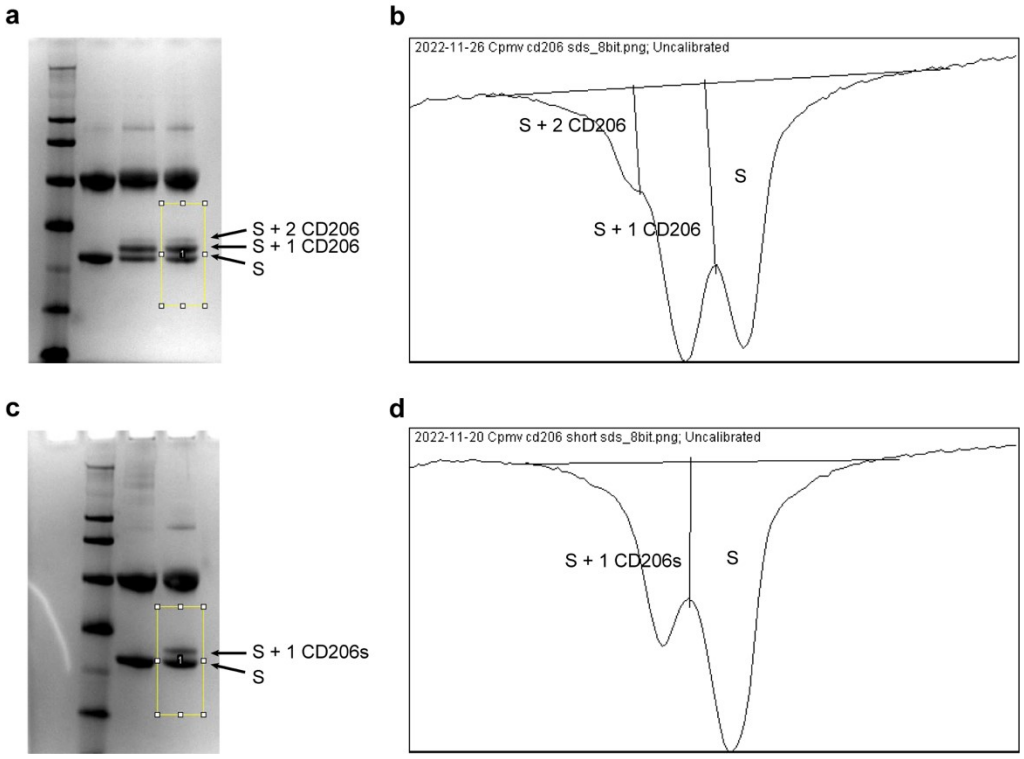
RAW264.7, M1, and M2 macrophages were harvested using cell scrapers (Thermo Fisher Scientific) and 5 x 10^5 cells were then seeded in 12-well plates (Corning). We added 1 x 10^6 per cell of CPMV-Cy5, CPMV-CD206-Cy5 or CPMV-CD206s-Cy5 and incubated for 24 h. Cells were harvested in Gibco Cell Dissociation Buffer (Thermo Fisher Scientific), washed twice with PBS, and fixed using Stabilizing Fixative 3x Concentrate (BD Biosciences). After washing, cells were resuspended in BD Pharmingen Stain Buffer (BD Biosciences) and analyzed using a BD Accuri C6 Plus flow cytometer (BD Biosciences). Data was processed using Flowjo_v10.7 software.

Animal studies

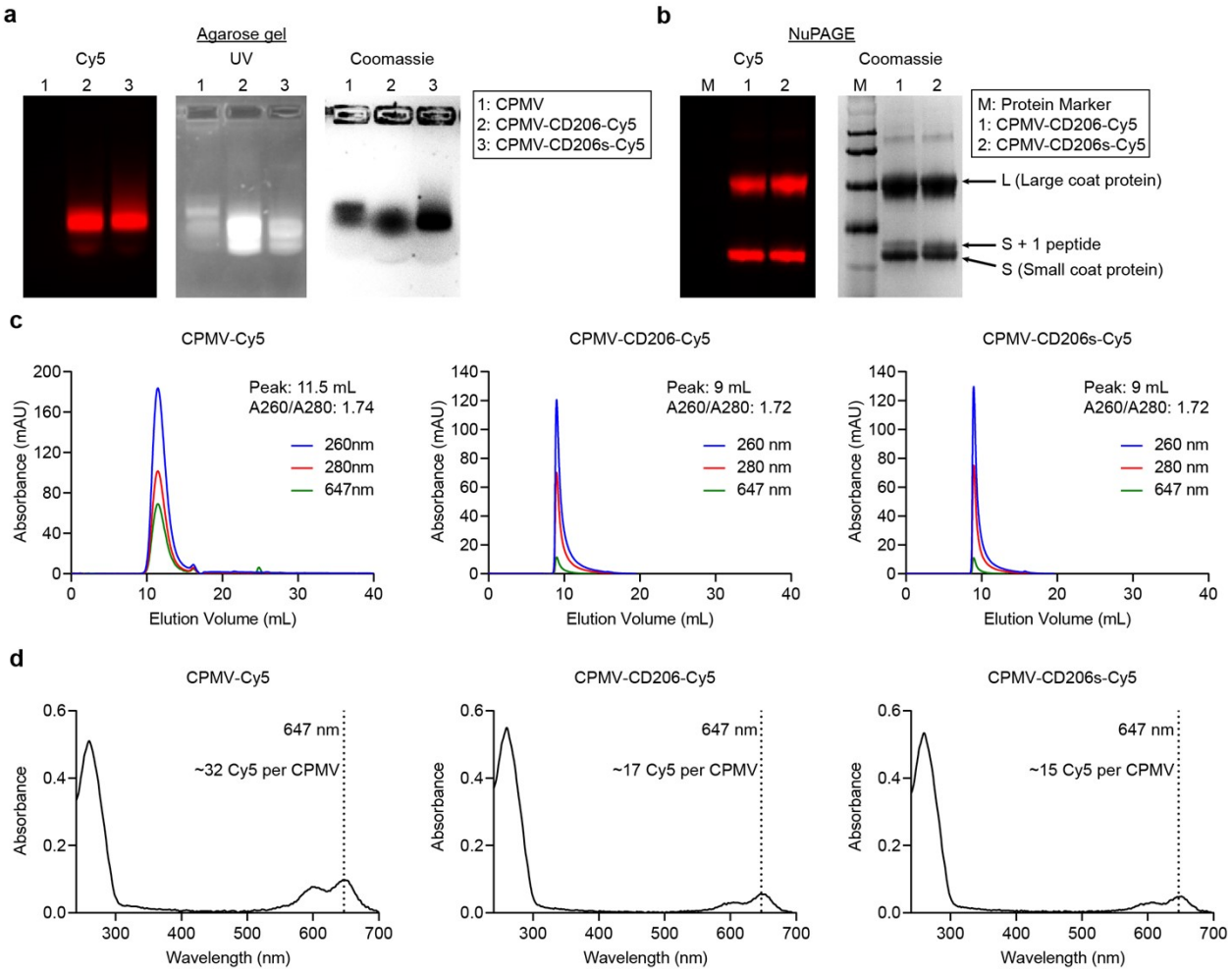
Experiments on mice were carried out in accordance with the guidelines of the Institutional Animal Care and Use Committee (IACUC) of the University of California, San Diego (UCSD) and were approved by the Animal Ethics Committee of UCSD. We acquired 7-week-old female C57BL/6J mice from Jackson Laboratories.

There were six treatment groups (n = 7): PBS, CD206 peptide, CD206s peptide, CPMV, CPMV-CD206, and CPMV-CD206s. On Day 0, 2 x 10^5 B16F10-OVA cells in 20 µL PBS were injected intradermally (i.d.) into the right flank to establish dermal melanomas. Treatments were started when the tumors reached ~30 mm^3 on day 9. All mice received three intra-tumoral (i.t.) doses of weekly on days 9, 16 and 23. Each dose comprised 100 µg CPMV in 30 µL PBS or 30 µL PBS as a control. The CD206 and CD206s free peptides were matched to the peptide doses in the CPMV-CD206 and CPMV-CD206s treatments, respectively. Mice were monitored every 2 days to record the tumor volume. Mice were killed humanely when the tumor volume reached 1000 mm^3.

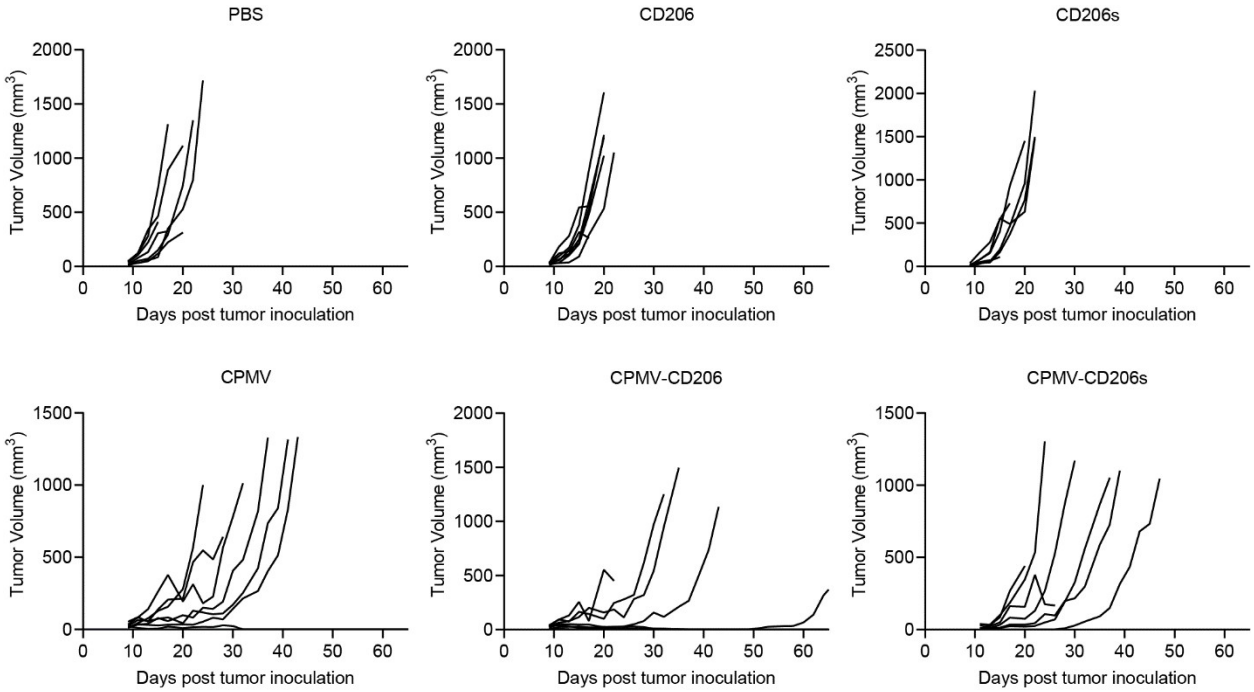
Supplemental Figures



Supplementary Figure 1. Quantification of conjugated peptides by gel densitometry using ImageJ software. (a) NuPAGE of CPMV-CD206. Lane 1: protein ladder; lane 2: CPMV; lane 3 and 4: CPMV-CD206. (b) Plotted gel density of the boxed area in (a). (c) NuPAGE of CPMV-CD206s. Lane 1: protein ladder; lane 2: CPMV; lane 3: CPMV-CD206s. (d) Plotted gel density of the boxed area in (c).



Supplementary Figure 2. Analysis of Cy5-labeled particles. (a,b) Analysis of (a) CPMV-CD206-Cy5 and (b) CPMV-CD206s-Cy5 by agarose gel electrophoresis and NuPAGE. (c) Analysis of CPMV-CD206-Cy5 and CPMV-CD206s-Cy5 by dynamic light scattering. (d) UV-Vis spectra of CPMV-CD206-Cy5 and CPMV-CD206s-Cy5.



Supplementary Figure 3. Individual tumor growth curves for all treatment groups.

References

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