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In Situ Vaccination of Tumors Using Plant Viral Nanoparticles

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Abstract

Viral nanoparticles are self-assembling units that are being developed and applied for a variety of applications. While most clinical uses involve animal viruses, a plant-derived virus, *cowpea mosaic virus* (CPMV) has been shown to have antitumor properties in mice when applied as in situ vaccine. Here we describe the production and characterization of CPMVand its use as in situ vaccines in the context of cancer. Subsequent analyses to obtain efficacy or mechanistic data are also detailed.

Keywords

Cowpea mosaic virus (CPMV); In situ vaccine; Immunotherapy; Melanoma; B16F10; Flow cytometry; Luminex multiplex; Cytokine

1 Introduction

CPMV is a 30 nm-sized icosahedral virus with $T = 3$ symmetry, which has been extensively used as a biomaterial for various applications in biotechnology and medicine. In the context of cancer therapeutics, we have recently demonstrated potent efficacy of CPMV as an in situ vaccination platform. When introduced into the tumor microenvironment, the CPMV-based in situ vaccine functions as an immune activator to prime an antitumor immune response; the immune response is systemic and generates immune memory thus protecting from outgrowth or recurrence of the disease. We have demonstrated efficacy in mouse models of melanoma, breast cancer, ovarian cancer, and colon cancer [1].

Here we describe the methods for obtaining CPMV and its use as in situ vaccine. It should be noted that in our previous work, eCPMV, an RNA-free version of CPMV was used, while here we describe the application of native CPMV for in situ vaccination of melanoma. CPMV particles are obtained through infection of *Vigna unguiculata* plants followed by extraction and purification from the infected leaf tissue.

We describe the application of CPMV as in situ vaccine in a mouse model of melanoma. While tumor burden is the primary read-out to determine efficacy, we also provide protocols allowing the characterization of the immune cell profiles and chemo/cytokines. Multicolor flow cytometric analysis is used to determine cellular populations and changes amongst those populations in the tumor microenvironment. In this situation, this can be achieved by creating single cell tumor suspensions, which are probed with fluorescently labeled antibodies targeted towards specific cell differentiating surface markers. In addition, the in situ vaccination-mediated immunological changes are facilitated through communication via cytokines and chemokines. The interaction can be quantified by isolating the protein

mediators from all cellular components and quantifying their levels in a high-throughput approach using a Luminex assay.

In this chapter, we describe these steps in detail including propagation, purification, and characterization of CPMV, B16F10 tumor cell culture and dermal tumor establishment, in situ vaccination, and immunological analyses using the B16F10 model. It should be noted that these methods could be applied to other plant viruses and tumor models.

2 Materials

2.1 CPMV Propagation, Purification, and Characterization

1. *Vigna unguiculata* seeds (California Blackeye No. 5).
2. Pro Mix BX potting Soil.
3. Plant incubators, e.g. Geneva Scientific E-41L2 or Conviron A100.
4. Carborundum.
5. Avanti J-E centrifuge with JLA 10.500 rotor and JLA 16.25 rotor.
6. Optima L-90K ultracentrifuge with 50.2 Ti rotor and SW 32 Ti rotor.
7. Tabletop centrifuge.
8. Sucrose.
9. Chloroform.
10. 1-Butanol.
11. Sodium chloride (NaCl).
12. PEG [8000 MW].
13. Potassium phosphate dibasic.
14. Potassium phosphate monobasic.
15. Miracloth or cheesecloth.
16. Blender.
17. Spectrophotometer.
18. AKTA Explorer 100 chromatograph with Superose6 column.
19. NuPAGE SDS sample buffer (4×).
20. NuPAGE 4–12% Bis-Tris gel.
21. NuPAGE MOPS SDS running buffer (1×).
22. Novex SeeBlue Plus2 pre-stained protein standard.
23. Safestain.
24. 2% (w/v) uranyl acetate in water.

25. Carbon-coated TEM grids.
26. TEM, e.g. FEI Tecnai F30 300 kV transmission electron microscope.

2.2 B10F10 Tumor Cell Culture and Dermal Tumor Establishment

1. B16F10 mouse melanoma cells.
2. Complete RPMI 1640 medium: RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM MEM nonessential amino acid, and 1% (v/v) Penicillin/Streptomycin, and 0.05 mM 2-Mercaptoethanol.
3. Phosphate-buffered saline.
4. Trypsin-EDTA (0.05%), phenol red.
5. Dual-chamber PolyPro bath.
6. Biological safety cabinet.
7. CO_2 incubator.
8. C57BL/6 mice (Jackson Labs, Bar Harbor, ME).
9. BD Lo-Dose™ U-100 insulin syringes.
10. Isoflurane.

2.3 In Situ Vaccination

1. BD Lo-Dose™ U-100 insulin syringes.

2.4 Tumor Homogenation for Cytokine Analysis

1. T-PER™ tissue protein extraction reagent.
2. Complete™ protease inhibitor cocktail.
3. HBSS, no calcium, no magnesium, no phenol red.
4. PBS.
5. Handheld homogenizer.
6. Laboratory balance.
7. Cryotube.
8. Clear 6-well plates—untreated; 6-well plate.
9. Clear polystyrene 96-well plates—untreated; well: V-shaped.

2.5 Flow Cytometry

1. 40 μm cell strainer
2. RPMI.
3. Ethanol.

4. Trypan blue.
5. Na_2-EDTA.
6. Potassium bicarbonate.
7. Ammonium chloride.
8. Purified anti-mouse CD16/32 Antibody to block Fc.
9. Pacific Blue™ anti-mouse CD45 antibody.
10. Pacific Blue™ Rat IgG2b, κ isotype ctrl antibody.
11. PE anti-mouse/human CD44 antibody.
12. PE Rat IgG2b, κ isotype ctrl antibody.
13. APC/Cy7 anti-mouse CD3ε antibody.
14. APC/Cy7 Armenian hamster IgG isotype ctrl antibody.
15. PE/Cy7 anti-mouse CD62L antibody.
16. PE/Cy7 Rat IgG2a, κ isotype ctrl antibody.
17. APC anti-mouse CD8a antibody.
18. APC Rat IgG2a, κ isotype ctrl antibody.
19. FITC anti-mouse CD4 antibody.
20. FITC Rat IgG2b, κ isotype ctrl antibody.
21. FITC anti-mouse/human CD11b antibody.
22. PE anti-mouse CD80 antibody.
23. PE Armenian hamster IgG isotype ctrl antibody.
24. PE/Cy7 anti-mouse CD86 antibody.
25. APC anti-mouse I-A/I-E antibody.
26. APC/Cy7 anti-mouse Ly-6G antibody.
27. APC/Cy7 Rat IgG2a, κ isotype ctrl antibody.
28. UltraComp eBeads.
29. Dead cell marker: fluorophore-conjugated viability dyes, propidium iodide, or 7-aminoactinomycin D.
30. LSR II flow cytometer or similar 8+ color flow cytometer.

3 Methods

3.1 CPMV Propagation, Purification, and Characterization

3.1.1 Plant Growth

1. Fill a plant tray with 3¾″ square pots, filling each pot with Pro-Mix BX Biofungicide + Mychorrhizae soil.
2. Place 3–4 California Blackeye No. 5 cowpea seeds into each pot, approximately 1½–2″ apart.
3. Water each plant pot enough to keep the soil moist (*see* Note 1).
4. Place plant trays into an incubator or plant room, providing ~15 h of sunlight with 25 °C with 50% humidity. For the night cycle, maintain the same temperature and humidity, with lights off.
5. Water every 2–3 days for approximately 10 days.

3.1.2 Plant Infection with CPMV

1. To work with or propagate CPMV or any other plant virus, USDA-approved protocols and facilities need to be established.
2. Prepare a 0.1 mg/mL CPMV in 0.1 M potassium phosphate (KP) buffer pH 7.0.
3. After approximately 10 days, when the seedlings have grown and the trifoliates leaves are starting to grow dust the primary leaves lightly with carborundum (*see* Note 2).
4. Pipette onto each leaf ~50 μL of 0.1 mg/mL CPMV in 0.1 M KP buffer pH 7.0 and gently spread the droplet over the leaf. Gently rubbing the leaves in combination with the carborundum dust will create lesions in the leaf tissue allowing CPMV to enter and start its replication process. Repeat for all primary leaves in the plant tray.
5. Continue watering the infected cowpea plants until the infection is established; at least an additional 10 days. The infection will be detectable based on the typical mosaic symptoms in the primary and trifoliate leaves.

3.1.3 Harvest and Storage of Infected Cowpea Leaves

1. Once the typical mosaic patterns are detectable on the leaves, collect the leaves and place in a Ziploc bag. Discard the pots with stems into biohazard waste.
2. Weigh the bag with leaves and note the date and weight. Once a bag reaches 100 g of leaves, begin collecting leaves in a new Ziploc bag.
3. Infected cowpea leaves should be stored at −80 °C (*see* Note 3).

3.1.4 Purification

1. By hand, pulverize the leaves by squeezing the bag (*see* Note 4).

2. Homogenize the pulverized leaves in a blender with approximately 3× volumes of ice-cold 0.1 M KP buffer pH 7.0. Filter homogenate through 2–3 layers of miracloth or cheesecloth into an autoclaved, sterile beaker. To improve filtration and recovery, carefully squeeze the miracloth or cheesecloth to force the filtered homogenate through. Discard miracloth or cheesecloth into biohazard waste (*see* Note 5).

3. Centrifuge the filtered plant homogenate using an Avanti J-E Centrifuge and JLA 10.500 rotor at 18,000 × *g* for 20 min at 4 °C. Collect the supernatant into autoclaved, sterile beaker.

4. Add a stir bar to the plant sap and place the beaker into an ice bath sitting on a stir plate in a fume hood. Into the beaker, add 0.7 volumes of 1:1 (v/v) chloroform:1-butanol. Stir the mixture for 20–30 min, avoiding the formation of bubbles from turbulent mixing (*see* Note 6).

5. Centrifuge the mixture using an Avanti J-E Centrifuge and a JLA 10.500 rotor at 6600 × *g* for 10 min at 4 °C. Remove the centrifuge bottles carefully as to not mix the separated aqueous and organic phases. Collect the upper aqueous phase using a 20 mL syringe and transfer the aqueous phase into an autoclaved, sterile beaker (*see* Note 7). Discard organic waste in liquid chemical waste.

6. Add NaCl to the aqueous phase to give a final molarity of 0.2 M NaCl. In addition, add 8% (w/v) PEG 8000 to the solution. Mix the solution in the beaker with NaCl and PEG 8000 in an ice bath using a stir plate for a minimum of 30 min and store the beaker at 4 °C for at least 2 h (*see* Note 8).

7. Place solution in an autoclaved, sterile 250 mL centrifuge bottle and centrifuge the solution in a JLA 16.25 rotor at 30,000 × *g* for 15 min at 4 °C. Discard supernatant and resuspend pellet(s) with 10 mM KP buffer pH 7.0 by pipetting up down repeatedly (*see* Note 9).

8. Centrifuge resuspended pellet in a JLA 16.25 rotor at 13,500 × *g* for 15 min at 4 °C and collect supernatant.

9. Purify the sample over a 10–40% sucrose gradient using ultra clear tubes for a SW32 rotor. Run SW32 rotor at 133,000 × *g* for 3 h at 4 °C. In a dark room, shine a light through the tube to visualize the CPMV bands. Remove the light scattering bands using a pipette and place in ultracentrifuge tube (Part number 337901, Beckman—polycarbonate tubes with cap assembly).

10. Fill the remaining volume of the tube with 0.1 M KP, if needed. Centrifuge in a Type 50.2 Ti ultracentrifuge rotor at 210,000 × *g* for 3 h at 4 °C. Discard supernatant and resuspend pellet with 1 mL 0.1 M KP. Recover resuspended pellet into a sterile 1.5 or 2 mL microcentrifuge tube.

11. Clearing spin: Centrifuge resuspended pellet in a tabletop centrifuge at 10,000 × *g* for 10 min. Recover supernatant in a new, sterile microcentrifuge tube.

3.1.5 Characterization by UV-Vis Absorbance

1. Determine concentration of purified CPMV using UV-Vis absorbance (e.g. using a Nanodrop instrument). Measure the absorbance (A) of the CPMV solution at 260 nm (RNA) and 280 nm (protein). The ratio of A_{260}/A_{280} is a good indication of purity and should be as close to 1.8 as possible (*see* Notes 10 and 11).
2. Using the Beer-Lambert law ($A = \varepsilon cl$), the concentration can be determined. A is absorbance at 260 nm, ε is the extinction coefficient of CPMV (8.1 mL·cm^{-1}·mg^{-1}), c is concentration (mg/mL), and l is path length (cm).

3.1.6 Characterization by Size Exclusion Chromatography (SEC)

1. Prepare 200 μL of 0.5 mg/mL CPMV in 0.1 M KP pH 7.0.
2. Inject CPMV solution into an AKTA explorer FPLC system using a Superose 6 10/300 GL size exclusion column (GE Lifesciences).
3. Run the AKTA explorer FPLC system at 0.5 mL/min, setting the absorbance readings at 260 nm and 280 nm. Intact CPMV elutes in the 10–15 mL fraction on a Superose6 column (*see* Note 12).

3.1.7 Characterization by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS PAGE)

1. Prepare samples by mixing: 2 μL of 3 mg/mL CPMV, 7 μL of 0.1 M KP pH 7.0, and 3 μL of NuPage LDS sample buffer (4×). Denature the proteins by boiling for 5–7 min at 100 °C.
2. Prepare a NuPAGE 4–12% Bis-Tris gel in a gel tank. For this particular gel, fill the inner chamber completely with 1 MOPS and fill a small volume of the 1 × MOPS on the outer chamber.
3. To serve as a molecular weight reference, load 10 μL of the Novex SeeBlue Plus2 pre-stained protein standard into a well.
4. Next to the well containing the protein standard, load the CPMV samples along with any known control sample in the remaining wells.
5. Run the gel electrophoresis for ~45 min at 200 V.
6. Once gel run is complete, remove the gel from the plastic casing and stain the gel using Safetstain according to the manufacturer's instructions (*see* Note 13).

3.1.8 Characterization by Transmission Electron Microscopy

1. Dilute CPMV sample to a concentration of 0.1 mg/mL in DI water (*see* Note 14).
2. On a piece of parafilm, place a 20 μL droplet of each of the following: CPMV dilution, water, water, 2% (w/v) uranyl acetate, and water.
3. Place TEM grid on CPMV dilution droplet for 2 min. Dry the TEM grid by carefully wicking away the liquid on filter paper.

4. Very briefly, wash the TEM grid by placing the TEM grid on the two water droplets and dry by wicking with filter paper.
5. Place TEM grid on the 2% (w/v) uranyl acetate droplet for 2 min.
6. Rinse the TEM grid by briefly placing on the last water droplet. Store TEM grid in grid holder until analysis.
7. Image the TEM grids using a FEI Tecnai F30 300 kV transmission electron microscope.

3.2 B16F10 Tumor Cell Culture and Dermal Tumor Establishment

1. Acquire approval from the institutional animal care and use committee (IACUC) prior to initiating any studies involving animals. The procedures described here were approved by IACUC at Case Western Reserve University.
2. Thaw cryopreserved B16F10 cells from liquid nitrogen tank, wash with PBS or complete culture medium to remove DMSO, and transfer cells into an appropriate culture vessel in relation to the number of cells thawed (for one million cells use a T175 tissue culture flask containing 20 mL of complete RPMI medium).
3. Place a culture flask with cells into 37 °C gassed (5% CO_2) cell incubator and culture cells, changing the media every 2–3 days.
4. Maintain cells until confluent (usually takes 4–5 days when cells seeded at 1/10 ratio).
5. Once cells form a confluent monolayer, split, and expand as follows: every 4–5 days, remove old media and rinse cells with 10 mL of PBS. Discard PBS, then add 3 mL of 0.25% Trypsin/2.21 mM EDTA, and place the flask in the incubator at 37 °C for 2–5 min to allow for cell dissociation. Observe cells under the inverted microscope to confirm that cells are released. Then immediately add fresh complete RPMI medium containing FBS to stop the trypsin and collect the dissociated cells from the flask (*see* Note 15). Gently mix cell suspension by pipetting the solution up and down, place cells in 50 mL tube, spin the cells down by centrifugation at 500 × *g* for 5 min. Wash cells once with 10 mL of PBS or complete culture medium. Resuspend cells in complete culture medium, and then distribute the cells at the desired dilution into new tissue culture flask using complete RPMI for the dilution (*see* Note 16).
6. For tumor inoculation, harvest tumor cells when cells reach no more than 85% confluence to ensure good viability.
7. Prepare cells in plain RPMI without serum at a concentration of 1.25×10^5 cells in 30 μL per mouse, and aliquot cells in 1.5 mL tubes for easy loading of syringe and to avoid over-mixing of cells while tumor challenging. Keep cells on ice during tumor cell inoculation and invert or pipet to mix prior to drawing into syringe.

8. After mice were anesthetized with isoflurane. Inject 30 μL of the cell suspension intradermally using 0.5 mL insulin syringes (*see* Notes 17 and 18). Intradermal tumor growth is autochthonous for melanoma and facilitates tumor observation. The injections must be done very slowly with excellent control to make sure the inoculation is within the dermis and tumor cells do not leak out. Practice is advised.

9. Monitor mice for development of tumors (*see* Note 19).

3.3 In Situ Vaccination

1. Tumor volume is measured using caliper and calculated using the following formula, $V = (\text{length} \times \text{width}^2)/2$, where V is tumor volume, W is tumor width (shorter dimension) and L is tumor length.

2. After 7–10 days, when tumors a volume of 40–80 mm^3 prepare for in situ vaccination. CPMV in PBS is injected intratumorally at a concentration of 100 μg CPMV in 20 μL. Control groups are treated with 20 μL of PBS. Again, patience and good control is required to make sure the treatment is fully incorporated into the tumor and does not leak out.

3. Monitor tumor growth at regular intervals. Euthanize mice once tumors reach 1000 mm^3 or according to IACUC-approved protocols.

3.4 Euthanasia

1. Euthanize mice according to IACUC-approved protocol.

2. CO_2 inhalation is described here. Briefly, place mice in a CO_2 box for 5 min with CO_2 infusion.

3. Turn off CO_2 and leave mice for another 5 min in the chamber.

4. Remove mice from chamber and conduct cervical dislocation to assure of euthanasia.

3.5 Tumor Homogenation for Cytokine Analysis

1. For follow-up immunological investigation, remove tumors and homogenize using the following protocol (*see* Note 20).

2. Extract tumor by cutting with surgical scissors along the base/margin of the tumor making sure to remove connective tissue and overlying skin (*see* Note 21). Weigh and note tumor mass.

3. Dissolve one tablet of complete protease inhibitor in 8 mL of T-PER buffer at room temperature before use.

4. Add a collected tumor sample to tissue grinder or a well on a six well plate on ice.

5. Add 1 mL of the tissue extraction reagent (made in step three) to the well per 100 mg of tissue sample.

6. Homogenize the tissues on ice using a homogenizer (*see* Note 22).
7. Collect the tissue lysate into a 1.5 mL microcentrifuge tube. Maintain tubes on ice.
8. Add 0.5 mL of HBSS buffer to rinse tissue homogenizer and collect HBSS buffer to the microcentrifuge tube in the previous step.
9. Centrifuge the sample at 9000 × *g* for 10 min at 2–8 °C to pellet the tissue debris.
10. Collect the supernatant, taking care to avoid the fat layer floating on the top (*see* Note 23).
11. Aliquot the cleared lysate into clean microcentrifuge tubes noting the aliquot volume (*see* Note 24).
12. Between the samples, run tissue homogenizer sequentially in 70% ethanol (v/v) and 1× PBS to prevent contamination between the samples (*see* Note 25).
13. Measure protein concentration of the cleared lysate using BCA or Bradford assay.
14. To store samples, freeze aliquots at −80°C (*see* Note 26).

3.6 Luminex

1. Transfer 30 μL of the supernatant to 96-well plate (s*e*e Note 27). Include blank tissue extraction reagent as a negative control.
2. Take plate to core providing Luminex services. A typical starting place is the Mouse Cytokine/Chemokine 32plex panel. Panels can also be customized for individual needs.
3. Luminex results will be in pg/mL and should be analyzed taking the total protein concentration of the tumor as determined by the BCA assay.

3.7 Preparation of Single Cell Suspensions for Flow Cytometric Analysis

1. Euthanize and remove tumor as described above.
2. Excise established intradermal tumor mass, remove connective tissue and fat around tumor mass, and cut the tumor into smaller pieces using surgical scissors.
3. Place pieces of tumor mass into a 40 μm cell strainer and add 1 mL of HBSS or tissue culture media such as complete RPMI medium to wet the tumor sample.
4. Using the plunger end of a syringe (*see* Note 28), mash the tumor mass through the cell strainer into the 50 mL falcon tube.
5. Rinse strainer with 5 mL of HBSS or complete culture medium (*see* Note 29) and then discard the strainer.
6. Spin cells down by centrifugation at 200 × *g* for 5 min at 4 °C.
7. Discard supernatant and resuspend pellet of tumor cells in 5 mL of ACK lysis buffer allowing for red blood cell (RBC) lysis.

8. Incubate for 5 min on ice with occasional shaking.
9. Stop the reaction by diluting the ACK lysis buffer with 10–20 mL of 1× PBS and spin cells down at 200 × *g* for 5 min.
10. Repeat RBC lysis procedure until all RBCs are completely lysed and the supernatant is clear (*see* Note 30).
11. Resuspend pellet in 1× PBS and volume for use in the next step of experimental procedure.
12. Count cells using an automatic cell counter or hemocytometer and aliquot 1 × 10^6 cells per well for flow cytometry analysis (*see* Note 31).
13. To limit counting time, dilute10 μL cell suspension in 90 μL of trypan blue and incubate for 5 min. Load 10 μL of the sample into the hemocytometer for counting.

$$\text{Total cells} = \text{mL}\left(\frac{\sum \text{Cells in quadrants}}{\text{Number of quadrants counted}}\right) \times 10,000 \times \text{Dilution factor}$$

3.8 Flow Cytometry

1. Aliquot 1 × 10^6 cells for each condition (*see* Notes 32 and 33).
2. Wash cells with 500 μL of ice-cold 1× PBS and centrifuge at 400–600 × *g* for 5 min at 4 °C.
3. Discard supernatant and disperse the cell pellet by tapping and resuspending in 50 μL of FACS staining buffer (*see* Note 34) or 1× PBS.
4. Prepare controls including the unstained control, single color controls, isotype controls, compensation controls (using beads), and fluorescence minus one (FMO) controls.
5. For Fc receptor blocking, pre-incubate the cells with purified anti-CD16/CD32 antibody (≤ 1.0 μg per 10^6 cells) for 15–30 min on ice and in the dark prior to immunostaining.
6. Wash cells with 500 μL of ice-cold 1× PBS and centrifuge at 400–600 × *g* for 5 min at 4 °C.
7. Discard supernatant and disperse the cell pellet by tapping and resuspending in 50 μL of chilled PBS.
8. Prepare the stain solutions with recommended titer of each antibody (*see* Note 35). We have included a list of a useful panel we have used in the past, but it is just one option as an example.
9. Add antibodies to cells and incubate with slow rock at 4 °C and in the dark for 30 min (*see* Note 36).

10. Wash cells with twice 500 μL of ice-cold 1 PBS and centrifuge at 400–600 × *g* for 5 min at 4 °C.
11. Discard supernatant and disperse the cell pellet by tapping and resuspending in 300 μL of FACS buffer or ice-cold 1× PBS (*see* Note 37).
12. If needed, fix cells after staining by adding cell fixation buffer (*see* Note 38) and incubate for 10–15 min at room temperature.
13. Analyze stained cells using a multicolor flow cytometer. Number of colors needed depend on number of receptor targets. Here we used the LSR II Flow Cytometer.
14. Analyze cell populations with preferred flow cytometry analysis software. Here we used FlowJo®. Cell population filtration and exclusion strategies are dependent on receptors being probed.

4 Notes

1. Avoid waterlogging as this can stunt the growth of the plants.
2. After mechanical inoculation keep the plants in the dark for several hours, then wash the leaves with tap water to remove carborundum and avoid “burning” of the leaves, prior to placing the plants into a plant incubator.
3. Leaves stored in −80 °C can be stored indefinitely.
4. Pulverizing the leaves by hand is most efficient when the leaves are frozen.
5. Filtering through cheesecloth may indicate a false positive of LPS if using the *Limulus* Amebocyte Lysate (LAL) assay. This is due to cheesecloth being made of cotton, a cellulose-based material.
6. Due to the dangerous fumes given off by chloroform, this step should be performed in a fume hood.
7. Extract as much aqueous phase as possible near the interface between aqueous and organic phases as this is where the majority of CPMV particles may rest following centrifugation. If organic phase is extracted, simply wait for the two phases to separate while in the syringe, and discard the organic phase once it has settled.
8. Alternatively, this step can be done inside of a cold room.
9. Additional resuspension can take place overnight at 4 °C on a nutator if needed.
10. A_{260}/A_{280} ratio that is lower than 1.8 may indicate that there is less RNA packaged within the CPMV particles. A_{260}/A_{280} ratio that is higher than 1.8 may indicate the presence of contaminating proteins. A deviating A_{260}/A_{280} ratio may also indicate presence of solvent or plant material contaminants.
11. Protein concentration of the purified CPMV sample can also be determined by Bradford Assay or Lowry Assay.

12. A single absorbance peak for the 260 nm and 280 nm settings should form at an elution volume around 10–15 mL. Additional peaks at a lower elution volume may indicate aggregates and peaks at larger elution volumes, such as around 20 mL, may indicate broken particles.

13. Due to the denaturing step, the CPMV sample should show two distinct bands. The “L” capsid protein should show a band at approximately 42 kDa, and the “S” capsid protein should show a band at approximately 24 kDa.

14. Diluting CPMV in a buffer, such as phosphate buffer, will create precipitates on the TEM grid.

15. 6 mLs of fresh DMEM are usually added to yield at ~10 mLs of cells. This allows for easier splitting to the desired ratio.

16. A 1:10 split is recommended for cell maintenance. Cells should be monitored daily and are typically split every 4–5 days when grown in a T175 flask.

17. Flank of mice should be shaved at least 1 day prior to allow for injections of tumor cells.

18. To assure uniform tumor growth, gently mix the cell suspension prior to loading of the syringe, and by gently tapping the syringe in between injections.

19. Tumors are usually visible and palpable about 8 days post inoculation.

20. Homogenation of tumors should be done soon after harvesting to prevent cytokine degradation. Freezing of tissues prior to homogenation is not recommended.

21. Tumor is encapsulated in fascia, which should not be punctured.

22. 5 min of homogenation on a medium setting is sufficient.

23. If there is fat contamination, centrifuge again at 9000 × *g* for 10 min at 2–8 °C to pellet the remaining tissue debris.

24. Total protein concentration of the lysate can be determined using a bicinchoninic acid assay (BCA assay), Bradford assay, or by measuring protein at A280. All cytokine levels should be normalized to total protein levels.

25. Depending on the number of samples, these solutions will need to be replaced with fresh solutions.

26. Avoid multiple freeze thaw cycles.

27. Consult with core to determine actual volumes required. Depending on concentration ranges, samples might need to be diluted.

28. Diameter of plunger should be less than diameter of the strainer. Five cc syringes work well for 40 μm cell strainer.

29. Make sure to collect all cells; cells may be stuck on the sidewalls of the strainer.

30. For dermal tumor, 1–2 times is usually enough.

31. Higher cells/well might be needed if a rare population is being studied.

32. Cells can be aliquoted in a variety of tubes or plates as long as an appropriate centrifuge is available. This is also dependent on the capabilities of the flow cytometer as to which tubes it can handle. For multiple samples, untreated v-bottom or u-bottom 96-well plates are preferred.

33. Make sure not to have any clumps. If cell clumps are visible, filter the cells through a 40 μm cell strainer.

34. Buffer made with 1× PBS, 1% (v/v) BSA (fraction IV, protease free) or 5–10% (v/v) FBS and 0.01% (w/v) sodium azide.

35. Dilutions should be made in PBS or FACS staining buffer.

36. Propidium Iodide (PI) (0.1–10 μg/mL) or 7-Aminoactinomycin D) (1 mg/mL) can be added here for live/dead staining and exclusion.

37. Make sure not to have any clumps. If cell clumps are visible, filter the cells through a 40 μm cell strainer.

38. Chilled PBS supplemented with 1–2% (v/v) paraformaldehyde, 1% BSA (w/v) (fraction IV, protease free) or 3% (v/v) FBS and 0.01% (w/v) sodium azide. Aim for final paraformaldehyde concentration between 0.5% and 1% (v/v).

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