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Cowpea Mosaic Virus (CPMV)-Based Cancer Testis Antigen NY-ESO-1 Vaccine Elicits an Antigen-Specific Cytotoxic T Cell Response

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Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acsabm.0c00259>.

Characterization of the CPMV-NY-ESO-1-TMR particles by native gel electrophoresis, denaturing gel electrophoresis and size exclusion chromatography indicating conjugation of TMR-tagged peptide with CPMV capsid; CPMV-OVA vaccine, immunization schedule, antigen-specific CD8⁺ T cell activation and cancer cell cytotoxicity (PDF)

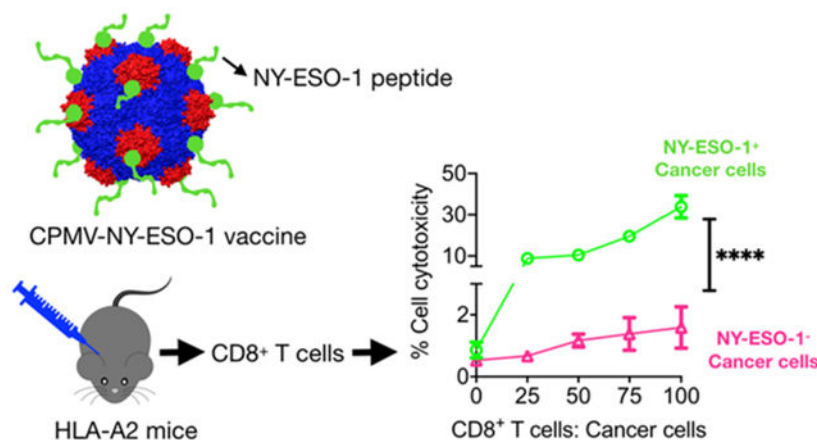
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Abstract

Cancer vaccines are promising adjuvant immunotherapies that can stimulate the immune system to recognize tumor-associated antigens and eliminate the residual or recurring disease. The aberrant and restricted expression of highly immunogenic cancer testis antigen NY-ESO-1 in several malignancies, including triple-negative breast cancer, melanoma, myelomas, and ovarian cancer, makes NY-ESO-1 an attractive antigenic target for cancer vaccines. This study describes a NY-ESO-1 vaccine based on a bio-inspired nanomaterial platform technology, specifically a plant virus nanoparticle. The 30 nm icosahedral plant virus cowpea mosaic virus (CPMV) displaying multiple copies of human HLA-A2 restricted peptide antigen NY-ESO-1_{157–165} exhibited enhanced uptake and activation of antigen-presenting cells and stimulated a potent CD8⁺ T cell response in transgenic human HLA-A2 expressing mice. CD8⁺ T cells from immunized mice exhibited antigen-specific proliferation and cancer cell cytotoxicity, highlighting the potential application of a CPMV-NY-ESO-1 vaccine against NY-ESO-1⁺ malignancies.

Graphical Abstract



Keywords

viral nanotechnology; cancer vaccine; plant virus; cowpea mosaic virus; NY-ESO-1 antigen; cancer testis antigen

INTRODUCTION

Cancer vaccines targeted to tumor-associated antigens can improve disease-free survival through immune system-mediated elimination of the residual or recurring disease.^{1,2} With its aberrant expression in several malignancies, including triple-negative breast cancer, melanomas, myeloma, and ovarian cancer, the cancer testis antigen NY-ESO-1 is an attractive target for immunotherapeutic intervention as evident from over 30+ ongoing vaccine clinical trials.^{3–5} NY-ESO-1⁺ malignancies are characterized by the presence of low levels of spontaneous antigen-specific humoral and cellular immune responses, including elevated CD8⁺ T cell infiltration and IFN- γ levels.^{3,6} However, these spontaneous responses are highly diminished by the immunosuppressive tumor microenvironment, which prevents

immunological clearance of cancer.^{7–10} Therefore, cancer vaccines provide an opportunity to initiate or amplify pre-existing antitumor immunity to clear primary tumors and metastasis and prime immune memory against recurring cancers.

Currently, several NY-ESO-1 vaccines are under evaluation, including dendritic cell (DC)-based vaccines, recombinant proteins or peptide subunit vaccines, and mammalian viral vectors. These approaches are hampered by numerous limitations: DC-based vaccines need to be custom made for each patient and can be technically challenging and expensive.^{11,12} Recombinant proteins may be costly to synthesize and suffer from inefficient or nonspecific responses arising due to misfolded proteins and inefficient presentation of epitopes.^{11,13,14} Mammalian viral vector expression systems carry antigen-coding RNA and depend on protein expression, proteolytic processing, and presentation of appropriate antigenic peptides to stimulate a cellular immune response.^{15,16} Subunit peptide vaccines offer the simplest form of vaccine that can incorporate a wide spectrum of antigenic determinants derived from tumor-associated antigens (TAAs). Short peptides derived from tumor antigens overcome the need for expression and processing of protein antigen prior to loading of epitopes on MHC-I/MHC-II molecules. Using rapidly evolving prediction methods, neoantigens can be identified and peptide fragments thereof can be selected for customized immune response.^{17–19} However, short peptides are poorly immunogenic and require adjuvants for enhanced and prolonged immune response.²⁰ In addition, short antigenic peptides also rely on delivery systems to protect against degradation and transport to desired tissues/cells.^{21,22} Many of conventional adjuvants are therefore delivered together with peptide antigens for improved vaccine efficacies.^{23,24}

In this study, we exploited a plant virus epitope display platform technology—cowpea mosaic virus (CPMV)—to develop an NY-ESO-1 vaccine. Here, CPMV serves the dual purpose of a delivery system and an adjuvant. The 30 nm icosahedral ssRNA viral nanoparticle of CPMV has been previously established as a highly potent antigenic carrier²⁵ and immune stimulant.²⁶ The potency of CPMV as an immune stimulant is derived from its highly organized three-dimensional (3D) protein architecture with its encapsidated nucleic acid and an intrinsic immune cell tropism.²⁷ We have previously established that CPMV can facilitate efficient delivery of tumor antigens to antigen-presenting cells (APCs) and provide the additional immune stimulus for effective processing and presentation of these antigens.²⁸ Based on these features, we have previously developed a human epidermal growth factor receptor 2 (HER2) breast cancer vaccine using the CPMV platform. The CPMV-HER2 vaccine primed an effective anti-HER2 antibody response leading to delayed tumor progression and improved survival in mice challenged with HER2⁺ tumors.^{25,29} Here, the motivation was to develop a CPMV-based vaccine to stimulate an antigen-specific cellular immune response. To generate an effective cytotoxic T cell response against cancer antigen using a cancer vaccine, exogenous peptide epitopes must be delivered to the cytosol of antigen-presenting cells (APCs) for cross-presentation.^{30,31} Particulate antigen delivery platforms including virus-like particles (VLPs) and plant viruses are known to facilitate such antigen cross-presentation of exogenous antigens.^{32–34} Here, CPMV was chemically modified to display multiple copies of the HLA-A2-restricted NY-ESO-1_{157–165} peptide. The potency CPMV-NY-ESO-1 vaccine to stimulate an antigen-specific CTL response was then tested in transgenic human HLA-A2 expressing mice.

RESULTS AND DISCUSSION

CPMV is a 30 nm icosahedral viral particle with a pseudo $T = 3$ symmetry and consists of 60 copies of a 24 kDa small coat protein (S-CP) and 42 kDa large coat protein (L-CP) (Figure 1A). A CPMV capsid offers 300 reactive lysines available for bioconjugation using the *N*-hydroxysuccinimide (NHS) chemistry. CPMV was produced in *Vigna unguiculata* plants with yields of 50–100 mg of virus/100 g of infected leaves. NY-ESO-1_{157–165} (SLLMWITQC) is a validated immunodominant MHC-I epitope that has been extensively studied for the development of subunit vaccines. NY-ESO-1 peptide with a C165V substitution (as previously described³⁵), flexible LSPG linker, and a terminal cysteine (SLLMWITQV-LSPG-C) was conjugated to CPMV using a two-step protocol through a bifunctional NHS-maleimide (SM-PEG₁₂) linker (Figure 1B). Conjugation of NY-ESO-1 peptide on CPMV was confirmed by denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of purified CPMV-NY-ESO-1 particles, which revealed the presence of higher molecular weight protein bands for both the S and L proteins. This is as expected because the Lys side chains are available on both CPMV capsid proteins. The higher molecular weight band above the 24 kDa small coat protein (S-CP) band of CPMV was better resolved, which allowed us to quantify the bands using the ImageJ band analysis tool (Figure 1C). Density analysis indicated ~45% of S-CP modified with the NY-ESO-1 peptide (or 27 NY-ESO-1 peptides per S-CP); the measurement on the L-CP was not conclusive because the bands of free and modified L-CP were not sufficiently separated. Therefore, we estimate that on an average 30–60 peptides were displayed per CPMV. While using over a thousand fold molar excess of reacting ligands maximizes uniform conjugation densities, VNP heterogeneity cannot be totally ruled out. Alternatively, genetic engineering methods can be used for the expression of epitopes on CPMV.^{36,37} However, genetic engineering and chemical bioconjugation each have their advantages: for initial development, chemical bioconjugation is the preferred method because of its ease and speed. While some degree of heterogeneity cannot be ruled out, there is no indication that this is a barrier for clinical development as several VLPs formulated using these principles have been in clinical trials.^{38,39} Genetic engineering may allow for a greater degree of homogeneity; however, the development timeline is longer and more cumbersome. Finally, homogeneity is not guaranteed, as previous studies indicated that inserted peptides could be cleaved resulting in loss of the peptides.⁴⁰

Fluorescent NY-ESO-1_{TMR} peptide was similarly conjugated to CPMV and characterized using native and denaturing gel electrophoresis and size exclusion chromatography (Figure S1, Supporting Information). The association of fluorescent peptide with the capsid protein was confirmed by the native gel and SDS-PAGE, where CPMV capsid/coat protein bands are fluorescently tagged. The co-elution of 550 nm fluorescent peak with the 260/280 peaks representing the CPMV also confirmed the association of NY-ESO-1-TMR peptide with CPMV capsid. TEM images confirmed the structural integrity of the purified CPMV-NY-ESO-1 particles (Figure 1D).

As an epitope display platform, the highly ordered 3D architecture of plant virus CPMV offers several unique features. The proteinaceous scaffold allows for a multivalent and repetitive display of the antigenic epitope, which activates pathogen-associated molecular

pattern (PAMP) recognition pathways leading to the induction of stronger and longer-lasting antigen-specific immune responses. The viral nucleocapsid itself engages several pattern recognition receptors (PRRs) on immune cells, thereby enhancing the immunological visibility of the vaccine and providing additional immune stimulus.⁴¹ Based on these features, we anticipated that CPMV would be a suitable platform technology for the NY-ESO-1 display to launch a potent CD8⁺ CTL response.

We first evaluated the delivery of the NY-ESO-1 peptide antigen to APCs with and without the CPMV carrier and compared their activation. RAW 264.7 macrophages were incubated with CPMV-NY-ESO-1_{TMR} or equivalent concentration of soluble NY-ESO-1_{TMR} peptide, and cellular uptake was compared using confocal microscopy. As evident from imaging data, a significantly higher uptake of CPMV-conjugated NY-ESO-1_{TMR} was observed over soluble non-conjugated NY-ESO-1_{TMR} peptide (Figure 2A,B). The data corroborated previously observed enhanced delivery of epitopes to APCs by CPMV nanocarriers.²⁸ Earlier studies have revealed a natural tropism of CPMV for APCs.^{42,43} We have also demonstrated that subcutaneously injected CPMV particles form local depots at the site of administration for sustained trafficking to the draining lymph nodes, thereby extending antigen sampling by peripheral APCs and improving the delivery of antigen to the draining lymph nodes.²⁸ Together with efficient trafficking, the cellular uptake of the CPMV carrier improves the delivery of tumor antigens to APCs for effective processing and presentation.

To investigate the potency of the CPMV-NY-ESO-1 vaccine to activate APCs, we measured the release of cytokines TNF- α , IL-1 β , IL-6, and IL-12p70 by mouse BMDCs upon stimulation with CPMV-NY-ESO-1 versus soluble NY-ESO-1 peptide antigen. Following 24 h incubation with the CPMV-NY-ESO-1 vaccine or equivalent amount of soluble peptide, culture supernatant from mouse BMDCs was analyzed for above-mentioned cytokines using ELISAs. NY-ESO-1 peptide and the CPMV-NY-ESO-1 vaccine both resulted in increased production of cytokines over nonstimulated BMDCs, highlighting the potency of the NY-ESO-1 antigen. However, CPMV-NY-ESO-1 significantly enhanced the cytokine levels, with nearly 2-fold higher levels of TNF- α , 1.5-fold higher levels of IL-1 β , 1.7-fold higher IL-6, and ~1.2-fold higher levels of IL-12p70 over the soluble NY-ESO-1 peptide (Figure 2C).

DCs play a central role in initiating an antigen-specific immune response by presenting antigens to T cells and providing the required immune stimulus through cell-to-cell contact and secreted cytokines. TNF- α plays an important role in maturation and migration of DCs to lymph nodes after sampling antigens and thus is critical for subsequent presentation of the antigens to T cells.⁴⁴ TNF- α also enhances local inflammatory responses and plays an essential role in the inhibition of tumor growth. IL-1 β release by DCs is induced by the activation of the NLRP3 inflammasome, an intracellular multiprotein signaling complex assembled as an inflammatory response to internalization of certain types of particulate antigens in dendritic cells.⁴⁵ IL-1 β signaling activates innate immune cells and is critical for T cell priming by dendritic cells.⁴⁶ Proinflammatory cytokines IL-6 and IL-12 produced by activated DCs provide the necessary signal to induce the development of CTL effector functions. IL-6 plays a key role in promoting T cell trafficking to lymph nodes for activation and to tumor sites for effector functions.⁴⁷ IL-12 links innate and adaptive immune responses. IL-12 released by APCs induces activation and proliferation of NK cells and T

cells, polarizes T cells to a type 1 helper T (Th1) effector cell phenotype, and induces production of IFN- γ as primary antitumor response.⁴⁸

We next investigated the efficacy of the CPMV-NY-ESO-1 vaccine to elicit an antigen-specific T cell response using transgenic mice expressing human HLA-A2, as NY-ESO-1_{157–165} is an HLA-A2-restricted antigen. Soluble peptides are poorly immunogenic in vivo, therefore the efficacy of the CPMV-NY-ESO-1 vaccine was compared to the equivalent amount of NY-ESO-1 peptide administered with a commercial adjuvant Complete Freund's Adjuvant (CFA). Female HLA-A2 mice were immunized subcutaneously with CPMV-NY-ESO-1 or CFA + NY-ESO-1 at day 0 and with a booster dose of CPMV-NY-ESO-1 or IFA (Incomplete Freund's Adjuvant) + NY-ESO-1 on day 14 (Figure 3A). Two weeks after the second immunization, spleens were harvested from immunized mice and CD8⁺ T cells were isolated from the splenocytes. To probe antigen specificity, CD8⁺ T cell proliferation and activation were evaluated in the presence of NY-ESO-1 peptide-pulsed BMDCs isolated from naïve HLA-A2 mice (Figure 3B). CD8⁺ T cells incubated with nonpulsed BMDCs or with NY-ESO-1 peptide were used as controls. Improved antigen trafficking, APC uptake, and activation facilitated by CPMV translated into an effective cellular immune response. Thus, immunizations with the CPMV-NY-ESO-1 vaccine significantly increased the NY-ESO-1-specific CD8⁺ T cell population in spleens as evident from the enhanced proliferation and elevated IFN- γ secretion by CD8⁺ T cells cultured with peptide-pulsed BMDCs as compared to CD8⁺ T cells incubated with peptide alone or with nonpulsed BMDCs (Figure 3C,D). We also observed high-peptide-specific CD8⁺ T cell proliferation and IFN- γ secretion for the group immunized with NY-ESO-1 peptide + CFA, an adjuvant which is known to induce a strong Th1-dominated inflammatory response.⁴⁹ However, the CD8⁺ T cells from CPMV-NY-ESO-1-immunized mice displayed significantly higher proliferation and IFN- γ secretion compared to those from CFA + NY-ESO-1-immunized mice, suggesting enhanced potency of the CPMV-based vaccine. Furthermore, CD8⁺ T cell from CPMV-NY-ESO-1 also showed ~6-fold higher IFN- γ levels when incubated with the NY-ESO-1-pulsed BMDCs as compared to an irrelevant HER2-derived P4 peptide-pulsed BMDCs, suggesting antigen specificity (Figure 3E).

We next evaluated the cytotoxicity of CD8⁺ T cells toward NY-ESO-1-expressing cancer cells. The elevated levels of peptide-specific IFN- γ translated into significantly higher effector/target cell ratio-dependent cancer cell cytotoxicity. Thus, CD8⁺ T cells from immunized mice (both the CPMV-NY-ESO-1 vaccine and CFA + NY-ESO-1 groups) showed significant cancer cell cytotoxicity when co-cultured with NY-ESO-1⁺/HLA-A2-expressing A375 human melanoma cells as compared to NY-ESO-1[−] mouse B16F10-OVA melanoma cells, which also did not express the HLA-A2 antigen (Figure 4). Similar studies with a CPMV-OVA vaccine formulation revealed an antigen-specific cellular immune response in C57BL6 mice. Here, CD8⁺ T cells from immunized mice displayed antigen-specific IFN- γ secretion when co-cultured with OVA-pulsed BMDCs from naïve C57BL6 mice and resulted in B16F10-OVA cell lysis (Figure S2, Supporting Information).

Our results are comparable to those obtained with the protein nanoparticle pyruvate dehydrogenase (E2 nanoparticle)-based NY-ESO-1 vaccine that has been shown to improve DC activation and antigen cross-presentation.^{50,51} However, while the E2 nanoparticle-based

NY-ESO-1 vaccine required an additional immune stimulant in the form of the TLR9 agonist CpG oligonucleotide (ODN) 1826 for efficacy, the CPMV viral nanoparticle itself was able to achieve comparable outcomes.

We also observed a comparable antigen-specific cancer cell cytotoxicity with the CFA + NY-ESO-1 vaccine, suggesting similar potency of CPMV and CFA as adjuvants. Similar to other adjuvants, CFA is likely to stimulate the immune system via multiple mechanisms including depot effect, recruiting APCs to the site of injection, enhancing antigen uptake, APC activation, and migration of activated APCs to draining lymph nodes.⁵² While CFA has been proven an effective Th1 adjuvant for preclinical research, its use is associated with strong long-lasting and possibly painful local inflammation.⁵³ Therefore, the application of CFA as an adjuvant is restricted by numerous regulatory guidelines.^{20,54} IFA, used for booster immunizations, has been tested in clinical trials but was discontinued as a vaccine adjuvant in humans due to associated severe side effects.^{55,56} Therefore, there is a need for the development of novel adjuvants, and our data support the need for further development of the CPMV platform for cancer immunotherapy applications. Other clinically approved adjuvants such as monophosphoryl lipid A (MPL), MF59, and alum have been previously evaluated for cancer vaccines, often requiring additional immunostimulatory molecules to achieve a Th1 immune response (for example, CpG ODN).^{23,57,58} CPMV, on the other hand, serves the dual purpose of a carrier and an adjuvant, with established Th1 immune response.²⁹ Thus, the CPMV-delivering platform obviates the need for additional immunostimulants.

CONCLUSIONS

In conclusion, we have developed an NY-ESO-1 cancer vaccine based on the plant viral nanoparticle CPMV as the epitope display platform. The CPMV platform enhanced uptake of NY-ESO-1 peptides into antigen-presenting cells and led to improved activation of immune cells. The CPMV-NY-ESO-1 vaccine triggered a potent CD8⁺ T cell response in transgenic HLA-A2 mice and demonstrated antigen-specific lysis of NY-ESO-1⁺ cancer cells. The efficacy of this vaccine can be attributed to the inherent immunogenicity, cellular tropism toward immune cells, and efficient lymphatic trafficking as described previously.²⁸

We incorporated the adjuvant CFA in our studies to demonstrate the feasibility and comparable efficacy of the CPMV-based vaccine approach. Future studies comparing the CPMV-based cancer vaccine with other adjuvants such as MPL or MF59^{23,59} will help us further evaluate the mechanism and efficacy of our approach. A vaccine-based immunotherapy approach could particularly benefit patients with NY-ESO-1⁺ malignancies such as triple-negative breast cancer (TNBC) that frequently exhibits local and regional recurrence and metastatic relapse within 5 years following surgical resection of the primary tumor and have no tumor-specific treatment options.^{60,61}

The plant virus-based approach to vaccines offers several advantages. Genetic engineering could be used to express epitopes on the viral capsid leading to a homogenous formulation and mitigate heterogeneity of the chemical conjugation methods.⁶² Such genetically engineered vaccines can also be propagated in and purified from host plants using molecular farming, thereby reducing downstream processing and cost.^{63,64} Furthermore, plant virus-

based vaccines could also be incorporated into polymeric implants and devices, which improves the shelf life of the product and will enable the extended release of the antigen.⁶⁵ Along with its immunostimulatory properties and added layer of safety associated with plant viruses, such advantages make CPMV an attractive plant viral platform for vaccine applications.

EXPERIMENTAL SECTION

Production of Cowpea Mosaic Virus (CPMV).

CPMV was propagated in *V. unguiculata* plants and purified from infected leaves using previously described methods.⁶⁶ Post-purification CPMV concentrations were determined by ultraviolet/visible (UV/vis) spectroscopy (CPMV specific extinction coefficient $\epsilon_{260\text{nm}} = 8.1 \text{ mg}^{-1} \text{ mL cm}^{-1}$). Particle integrity was verified by the elution profile determined by size exclusion chromatography using a Superose6 column on the ÄKTA Explorer chromatography system (GE Healthcare, Pittsburgh, PA) and the 260:280 ratio (for intact CPMV the 260:280 ratio is 1.8).

Synthesis of CPMV-NY-ESO-1 Vaccine.

NY-ESO-1 peptide (NY-ESO-1_{157–165}) with a terminal cysteine and a flexible LSPG linker—SLLMWITQV-LSPG-C, or its fluorescent version—tetramethylrhodamine (TMR)-conjugated peptide NY-ESO-1_{TMR} (Genscript, Piscataway, NJ), was conjugated to CPMV using a two-step protocol through a bifunctional *N*-hydroxysuccinimide-PEG₁₂-maleimide (SM-PEG₁₂) linker (Thermo Fisher Scientific, Waltham, MA). CPMV in 0.1 M sodium phosphate (KP) buffer pH 7.4 with constant mixing was first reacted with 2000 molar excess of the SM-PEG₁₂ linker at room temperature for 2 h at 1 mg/mL protein concentration. Next, 3000 molar excess of the peptide was reacted overnight with SM-PEG₁₂-modified CPMV. The CPMV-NY-ESO-1 formulation was purified by spin filtration (10 000 rpm/5 min; 100 kDa molecular weight cutoff filters, Amicon Ultra, Millipore Sigma, Burlington, MA).

Conjugation of NY-ESO-1 peptides on CPMV was quantified using SDS-PAGE gels. Ten micrograms of CPMV and CPMV-NY-ESO-1 was mixed with SDS running buffer (Thermo Fisher Scientific), heated at 100 °C for 5 min, and then loaded on precast Nu-PAGE 4–12% Bis–Tris protein gels (Thermo Fisher Scientific). Electrophoresis was performed for 40 min at 200 V. Gels were stained using GelCode Blue Safe protein stain (Thermo Fisher Scientific) to visualize the protein bands corresponding to molecular weight ladders; the degree of peptide modification was quantified using lane density analysis (Fiji software). Particle integrity was verified by transmission electron microscopy (TEM). CPMV-NY-ESO-1 particles (0.5 mg/mL) were loaded on 400-mesh copper grids bearing the Formvar support film, stained with 2% (w/v) uranyl acetate, and visualized using the FEI Tecnai Spirit G2 BioTWIN microscope (FEI, Hillsboro, OR). CPMV-NY-ESO-1-TMR formulation was characterized using denaturing and native gel electrophoresis. Native gel electrophoresis (100 V for 40 min) of CPMV and CPMV-NY-ESO-1-TMR particles (10 μg in 6 \times loading dye) was performed on agarose gel (1.2% w/v) containing 1 μL of GelRed Nucleic Acid Stain for RNA visualization (GoldBio, St Louis, MO) in Tris borate EDTA (TBE) buffer.

The native gel was visualized under UV light for nucleic acid, using 534 nm light source for TMR dye and after staining with Coomassie Brilliant Blue (0.25% w/v) (Sigma, St Louis, MO) to visualize the capsid protein; denaturing gel was visualized under 534 nm for fluorescence and white light for stained proteins. CPMV-NY-ESO-1-TMR was also characterized using size exclusion chromatography using a Superose6 column on the ÄKTA Explorer chromatography system (GE Healthcare, Marlborough, MA).

A CPMV-OVA vaccine was similarly synthesized by conjugating the H2-K^b-restricted OVA peptide with the GPSL linker and a terminal cysteine C-LSPG-SIINFEKL (Genscript) to CPMV via the SM-PEG₁₂ linker.

Mice.

All animal experiments were carried out in accordance with Case Western Reserve University's Institutional Animal Care and Use Committee (IACUC). Transgenic C57BL/-Mcph1 Tg (HLA-A2.1)-1Enge/J mice, expressing the human HLA-A2 gene (HLA-A2 mice), were obtained from the Jackson Laboratory (Bar Harbor, ME). Six–eight week old female HLA-A2 mice were immunized subcutaneously twice at 14 days intervals with 50 µg of CPMV-NY-ESO-1 vaccine in 100 µL of phosphate-buffered saline (PBS) or equivalent dose of the NY-ESO-1 peptide mixed with Complete Freund's Adjuvant (CFA) for first injection/Incomplete Freund's Adjuvant (IFA) for booster injection (Invivogen, San Diego, CA) in 100 µL of PBS. To evaluate the CPMV-OVA vaccine, 8-week-old female C57BL6 mice (Jackson Laboratory) were immunized with 50 µg of CPMV-OVA or 1 µg of OVA peptide, as described for the NY-ESO-1 antigen. Two weeks after the last immunization, mice were sacrificed and spleens were harvested to isolate CD8⁺ T cells.

Cell Lines.

A375, an HLA-A2⁺ NY-ESO-1⁺ human malignant melanoma cell line, was purchased from ATCC (Manassas, VA) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Atlanta Biologicals, Minneapolis, MN) and 1% (v/v) penicillin/streptomycin (Pen/Strep) (Thermo Fisher Scientific). Murine macrophage cell lines RAW 264.7, mouse melanoma cells B16F10 (ATCC), and B16F10-OVA (gift from Dr. Steve N. Fiering, Dartmouth College, NH) were maintained on the DMEM medium described above.

Macrophage Uptake.

Macrophage uptake of CPMV-ESO-1_{TMR} particles and soluble ESO-1_{TMR} peptides was compared using confocal microscopy. Thirty thousand RAW 264.7 macrophage cells in 0.5 mL media were seeded overnight in 24-well plates on circular glass coverslips. Cells were incubated with 5 µg of CPMV-NY-ESO-1_{TMR} particles or 1 µg of NY-ESO-1_{TMR} peptides for 2 h at 37 °C. Cells were washed, fixed in 5% (v/v) paraformaldehyde/0.3% (v/v) glutaraldehyde in Dulbecco's phosphate-buffered saline (DPBS) for 10 min, stained for the cell membrane with Wheat Germ Agglutinin-AlexaFluor 488 (Thermo Fisher Scientific), and diluted 1:1000 in 5% (v/v) goat serum (Thermo Fisher Scientific) in DPBS. Nuclei were stained with DAPI in the mounting medium (Vector Laboratories, Burlingame, CA). The

stained cells were imaged on a Leica TCS SPE confocal microscope with a 63× oil immersion objective and images were analyzed with Fiji software.

Bone Marrow-Derived Dendritic Cells (BMDCs).

BMDCs were isolated from a single-cell suspension of whole bone marrow cells harvested from the femurs and tibias of female HLA-A2 or C57BL6 mice. The cells were washed with PBS, and red blood cells were lysed using RBC lysis buffer (Thermo Fisher Scientific) at 37 °C for 5 min. The cells were then centrifuged, washed, and resuspended at 3×10^6 cells/mL in the T cell medium: Roswell Park Memorial Institute (RPMI) (Thermo Fisher Scientific) supplemented with 10% (v/v) GemCell FBS (Gemini Bio-Products, West Sacramento, CA), 1% (w/v) Pen/Strep, 1 mM sodium pyruvate (Thermo Fisher Scientific), and 50 mM β -mercaptoethanol (Millipore Sigma) supplemented with 10 ng/mL mouse IL-4 and 15 ng/mL mouse GM-CSF (both Peprotech, Rocky Hill, NJ). The media was removed and replaced with fresh T cell media supplemented with IL-4 and GM-CSF on day 3 and then again on day 5. Cells were harvested on day 7 and used for BMDC activation and antigen-presentation studies.

BMDC Activation and Cytokine Secretion.

BMDCs harvested on day 7 were plated at 1×10^6 cells/100 μ L medium and incubated with 10 μ g of CPMV-NY-ESO-1 particles and 2 μ g of the NY-ESO-1 peptide (10× equivalent NY-ESO-1 peptide compared to CPMV-NY-ESO-1) at 37 °C for 24 h in cytokine-free T cell media. Bacterial LPS (100 ng/mL, eBioscience, Thermo Fisher Scientific) was used as a positive control. Following incubation, cell supernatants were collected and analyzed for cytokines TNF- α , IL-6, IL-12, and IL-1 β using ELISA kits (BioLegend, San Diego, CA) as per instructions from the manufacturer.

CD8⁺ T Cell Proliferation and Stimulation.

CD8⁺ T cells were isolated from single-cell suspension obtained from the spleens of mice immunized with CPMV-NY-ESO-1 and CFA + NY-ESO-1 peptides. Spleens were homogenized and passed through a 40 μ m cell strainer in ice-cold PBS and centrifuged at 500g for 5 min. RBCs were depleted with RBC lysing buffer (eBioscience, Thermo Fisher Scientific), and CD8⁺ T cells were isolated using the RoboSep CD8⁺ T cell negative isolation kit (STEMCELL Technologies, Cambridge, MA) according to the manufacturer's instructions.

CD8⁺ T cells were co-cultured with antigen-pulsed BMDCs to measure proliferation and IFN- γ secretion. Specifically, BMDCs isolated from naïve HLA-A2 mice were pulsed with increasing concentrations of NY-ESO-1 peptide (10, 20, 30 μ g/mL) for 4 h at 37 °C. Cells were washed twice with PBS to remove excessive peptides. CD8⁺ T cells isolated from immunized mice (CPMV-NY-ESO-1 and CFA + NY-ESO-1 groups) were co-cultured with antigen-pulsed and nonpulsed BMDCs (stimulating cells) at a 1:10 (T cells/BMDCs) ratio in a 96-well plate in triplicate at 37 °C for 48 h in an atmosphere of 5% CO₂. CD8⁺ T cells incubated with CD8⁺ T cells incubated with the NY-ESO-1 peptide alone were used as controls. T cell proliferation was measured using MTT assays, performed as per the manufacturer's recommendation. A Tecan microplate reader was used for readout. The %

cell proliferation was calculated as experimental proliferation/control proliferation $\times 100$, where experimental proliferation is the proliferation of co-cultured cells minus proliferation of BMDCs only minus proliferation of T cells only and control proliferation is the proliferation of T cells only. For measurements of secreted IFN- γ , culture supernatants were collected and assayed with the Mouse IFN- γ ELISA kits (BioLegend) as per instructions from the manufacturer.

To determine the epitope specificity, CD8⁺ T cells from CPMV-NY-ESO-1-immunized mice were co-cultured with BMDCs pulsed with NY-ESO-1 peptide or irrelevant HER2 peptide P4 (PESFDGD-PASNTAPLQPEQLQ). Secreted IFN- γ levels were compared, as described above.

CD8⁺ T cells from CPMV-OVA-immunized mice were similarly harvested and secreted IFN- γ levels were measured by co-culturing CD8⁺ T cells with OVA/ P4-pulsed BMDCs from naïve C57BL6 mice.

CD8⁺ T Cell-Mediated Cancer Cell Cytotoxicity.

CD8⁺ T cells from immunized mice were plated in 96-well plates in RPMI at 5×10^6 cells/mL and incubated overnight at 37 °C. On day 1, 10 μ g/mL NY-ESO-1 was added to the cells and incubated at 37 °C. Cells were collected on day 3 to perform the cytotoxicity assay. To examine antigen-specific cytotoxicity, NY-ESO-1⁺ target cancer cells A375 and NY-ESO-1⁻ control cell line B16F10-OVA were co-cultured with CD8⁺ T cells at effector-to-target ratios of 100:1, 75:1, 50:1, 25:1, 0:1 and cytotoxicity evaluated using the MTT assay. Percentage cytotoxicity was determined as experimental cytotoxicity/control toxicity $\times 100$, where experimental cytotoxicity is cytotoxicity for co-culture (cytotoxicity of CD8⁺ T cells only + Cytotoxicity of cancer cells only) and control cytotoxicity is the cytotoxicity of cancer cells only under identical culture conditions. Cytotoxicity of CD8⁺ T cells from CPMV-OVA/CFA + OVA-immunized mice was similarly evaluated against OVA⁺B16F10-OVA and OVA⁻B16F10 cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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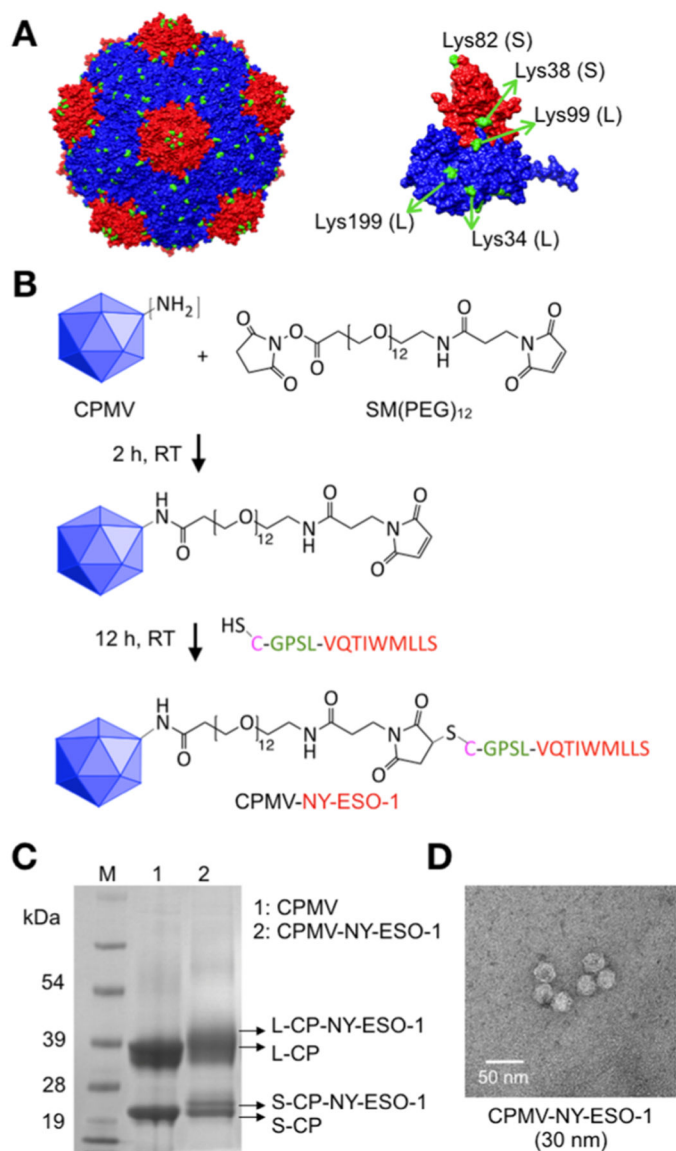


Figure 1.

Synthesis and characterization of the CPMV-NY-ESO-1 nanoparticles. (A) Structure of CPMV created with UCSF Chimera (v1.12) using the Protein Data Bank entry 1NY7 shows icosahedral morphology. The two protein subunits are colored red (S) and blue (L). The reactive Lysine residues on the capsid and the asymmetric unit are highlighted in green. (B) NY-ESO-1 peptide with the flexible LSPG linker with a C-terminal Cys was conjugated through the solvent-exposed Lys residues using NHS-chemistry via the bifunctional linker SM (PEG)₁₂. (C) Denaturing 4–12% Nu-PAGE gel stained with GelCode Blue Safe protein stain was used to confirm the successful conjugation of the peptide to the CPMV coat proteins (S-CP and L-CP). (D) Transmission electron microscopic (TEM) images of the negatively stained CPMV-NY-ESO-1 nanoparticles show particle integrity post-conjugation.

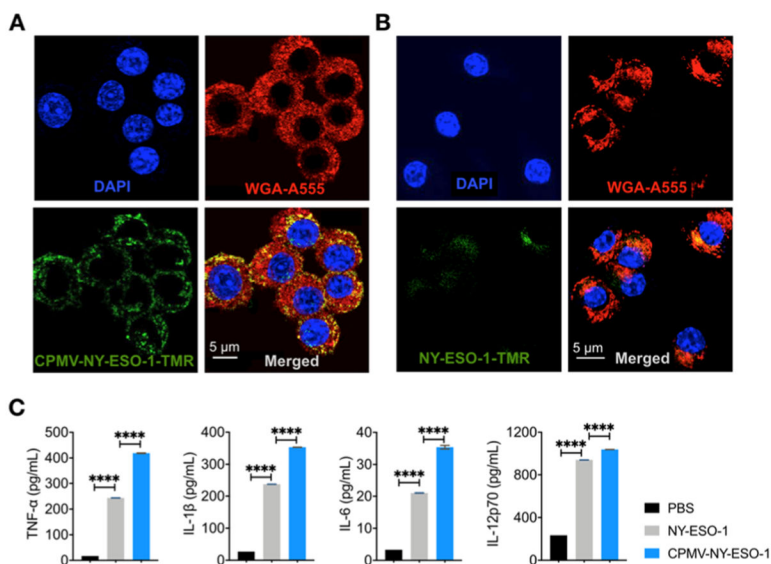
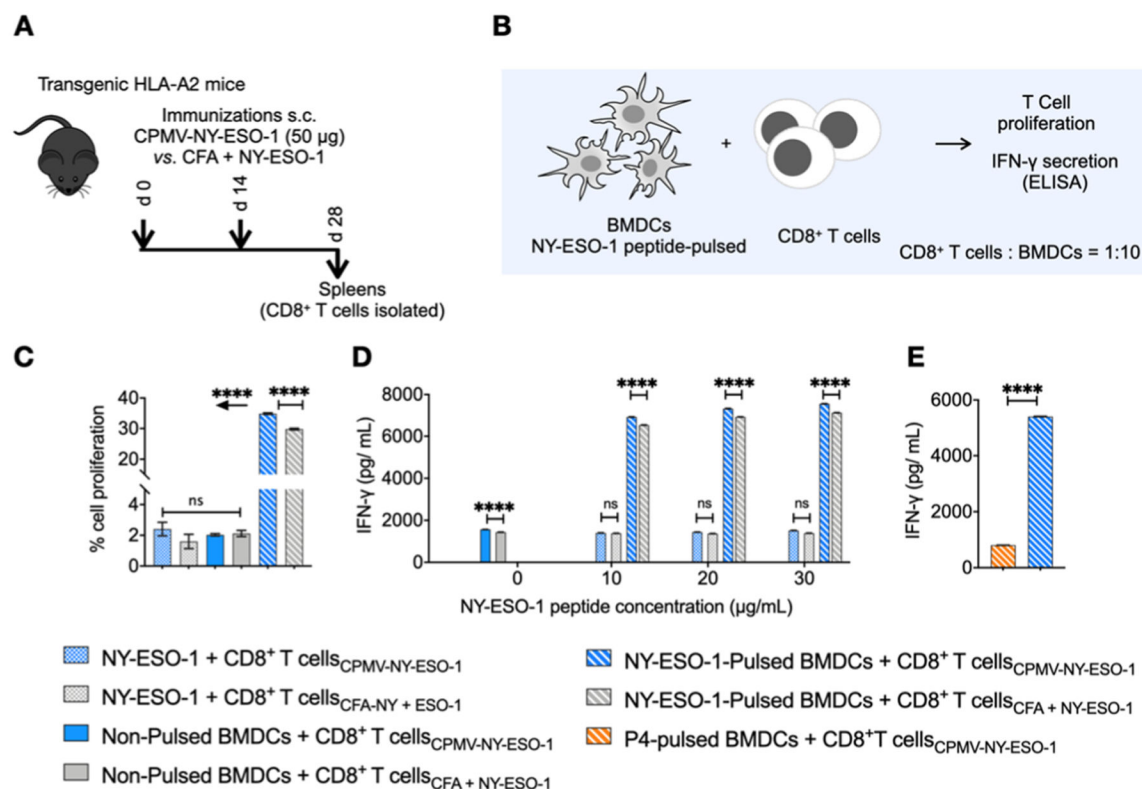


Figure 2. CPMV-NY-ESO-1 is taken up by the antigen-presenting cells and triggers activation. (A, B) Confocal microscopy was used to verify the uptake of fluorescent CPMV-NY-ESO-1-TMR vaccine particles and soluble NY-ESO-1-TMR peptides by murine macrophage RAW 264.7 cells. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue), the cell membrane was stained with WGA-A555 (red), and fluorescent TMR (green) was used for monitoring the peptide/CPMV-peptide. (C) Immunostimulation of bone marrow-derived dendritic cells (BMDCs) derived from the transgenic HLA-A2 mice by the CPMV-NY-ESO-1 vaccine was examined by incubating 500 000 BMDCs with the CPMV-NY-ESO-1 vaccine and the soluble NY-ESO-1 peptide for 24 h and quantifying the cytokines (IL β 1, TNF- α , IL-6, and IL-12p70) released in the culture supernatant using enzyme-linked immunosorbent assays (ELISAs). The results were compared using one-way analysis of variance (ANOVA) (with **** = $p < 0.0001$).

**Figure 3.**

Immunogenicity of the CPMV-NY-ESO-1 vaccine. (A) Transgenic HLA-A2 mice ($n = 5$) were immunized subcutaneously 2 \times biweekly with 50 μ g of the CPMV-NY-ESO-1 vaccine or equivalent amount of NY-ESO-1 peptide mixed with the CFA adjuvant (CFA + NY-ESO-1); the IFA adjuvant was used for the booster dose on day 14. Spleens were harvested from immunized mice 2 weeks following the booster dose and CD8⁺ T cells were isolated. (B) Schematic representation of assays used to determine antigen-specific CD8⁺ T cell proliferation. (C, D) T cell proliferation was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays (C) and quantification of secreted IFN- γ (D) using ELISAs to determine epitope-specific immunostimulation. CD8⁺ T cells harvested from immunized mice were co-cultured with NY-ESO-1 peptide-pulsed BMDCs from naïve HLA-A2 mice. Nonpulsed BMDCs and NY-ESO-1 peptide (without BMDCs) were used as controls. (E) Antigen specificity of T cell response was determined by co-culturing CD8⁺ T cells from CPMV-NY-ESO-1-immunized mice with BMDCs pulsed with NY-ESO-1 peptide or irrelevant HER2 peptide P4 and comparing IFN- γ secretion. The results were compared using one-way ANOVA (with **** = $p < 0.0001$).

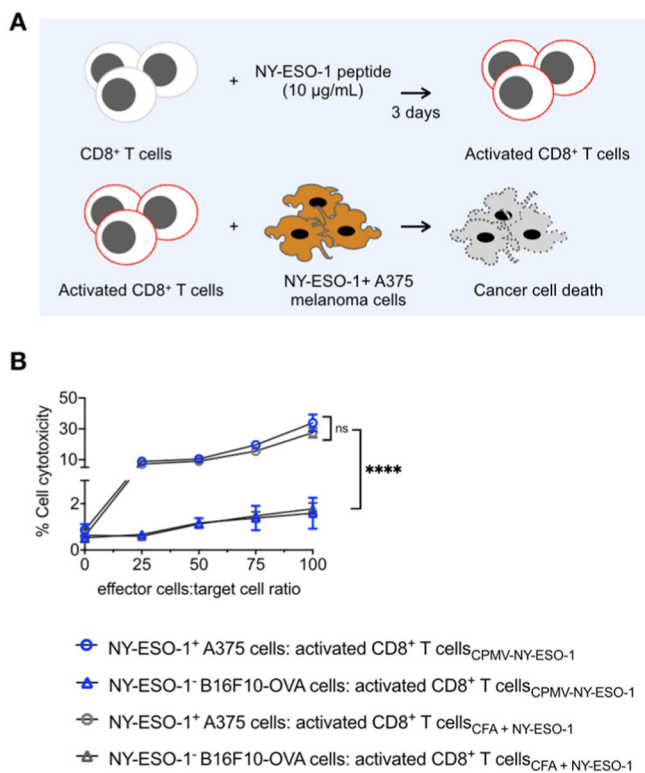


Figure 4.

Antigen-specific cancer cell lysis. (A) CD8⁺ T cells from CPMV-NY-ESO-1 and CFA + NY-ESO-1-immunized mice were cultured for 3 days with NY-ESO-1 peptide. Activated CD8⁺ T cells were then co-cultured at varying effector/target cell ratios with NY-ESO-1⁺ A375 cancer cells or NY-ESO-1⁻ B16F10-OVA cells. (B) Cytotoxicity of CD8⁺ T cells was determined using MTT assays. The results were compared using one-way ANOVA (with **** = $p < 0.0001$).