HHS Public Access

Author manuscript

Nano Lett. Author manuscript; available in PMC 2022 November 15.

Published in final edited form as:

Nano Lett. 2022 July 13; 22(13): 5348–5356. doi:10.1021/acs.nanolett.2c01328.

Cowpea Mosaic Virus and Natural Killer Cell Agonism for In Situ Cancer Vaccination

Edward C. Koellhoffer,

Department of Radiology, University of California, San Diego, La Jolla, California 92093, United States

Nicole F. Steinmetz

Department of Radiology, Department of NanoEngineering Department of Bioengineering, Moores Cancer Center, Center for Nano-ImmunoEngineering, and Institute for Materials Design and Discovery, University of California, San Diego, La Jolla, California 92093, United States

Abstract

We have previously shown the plant virus Cowpea mosaic virus (CPMV) to be an efficacious in situ cancer vaccine, providing elimination of tumors and tumor-specific immune memory. Additionally, we have shown that CPMV recruits Natural Killer (NK) cells within the tumor microenvironment. Here we aimed to determine whether a combination of CPMV and anti-4-1BB monoclonal antibody agonist to stimulate tumor-resident and CPMV-recruited NK cells is an effective dual therapy approach to improve NK cell function and in situ cancer vaccination efficacy. Using murine models of metastatic colon carcinomatosis and intradermal melanoma, intratumorally administered CPMV + anti-4-1BB dual therapy provided a robust antitumor response, improved elimination of primary tumors, and reduced mortality compared to CPMV and anti-4-1BB monotherapies. Additionally, on tumor rechallenge there was significant delay/prevention of tumor development and improved survival, highlighting that the CPMV + anti-4-1BB dual therapy enables potent and durable antitumor efficacy.

Graphical Abstract

Corresponding Author: **Nicole F. Steinmetz** – *Department of Radiology, Department of NanoEngineering, Department of Bioengineering, Moores Cancer Center, Center for Nano-ImmunoEngineering, and Institute for Materials Design and Discovery, University of California, San Diego, La Jolla, California 92093, United States;*.

Author Contributions

E.C.K. and N.F.S. designed experiments. E.C.K. performed experiments and data analysis. E.C.K. and N.F.S. wrote and edited the paper.

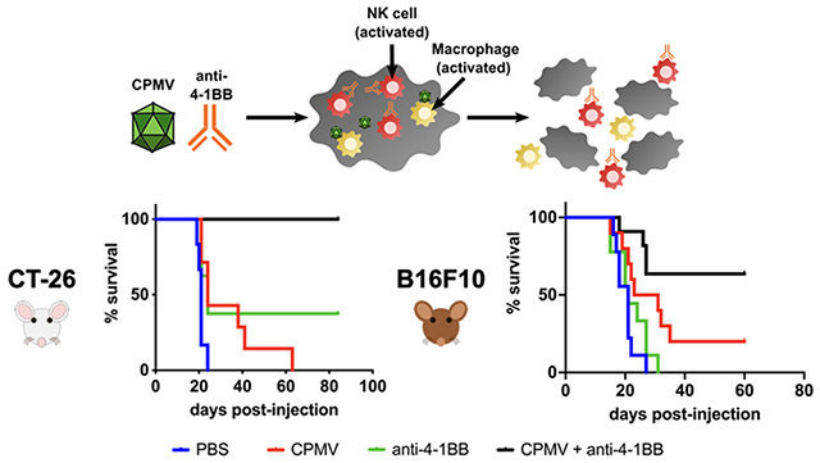
Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.nanolett.2c01328.

Additional experimental details for CPMV production and characterization, cell culture, and tumor models (PDF)

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.nanolett.2c01328

The authors declare the following competing financial interest(s): Dr. Steinmetz is a co-founder of, has equity in, and has a financial interest with Mosaic ImmunoEngineering Inc. Dr. Steinmetz serves as Director, Board Member, and Acting Chief Scientific Officer, and paid consultant to Mosaic. Dr. Koellhoffer declares no COI.



Keywords

In situ vaccine; Cowpea mosaic virus (CPMV); Natural Killer (NK) cell agonism; combination therapy; cancer immunotherapy

Cancer remains one of the leading causes of death in the United States, and can inflict devastating morbidity and costly challenges on patients and their families. The need to continue to develop cancer immunotherapies and cancer vaccines remains persistent. Cancer immunotherapies are a unique approach that are aimed to utilize the immune system in recognizing and eliminating transformed cancer cells.[1,2] However, malignant tumors themselves are poised to evade a robust immune response through a variety of mechanisms. Within the tumor microenvironment, cancers secrete a number of immunosuppressive cytokines and signaling molecules to suppress the innate immune cells and ultimately evade immunological response.[3–5]

We have previously shown the efficacy of the plant virus Cowpea mosaic virus (CPMV) as a potent immunotherapeutic in overcoming the immunosuppressive barrier of the cancer microenvironment in multiple tumor models including ovarian,[6,7] breast,[8] colon cancers,[8] dermal and metastatic melanoma,[8] as well as malignant glioma.[9] While noninfectious toward mammals, CPMV is recognized by pattern recognition receptors (PRRs) and elicits robust innate immune responses. Intratumoral administration of CPMV leads to innate immune cell recruitment and activation, including Natural Killer (NK) cells.[8,10,11] Activated NK cells phagocytose cancer cells and release tumor-associated and neoantigens that can then be presented by antigen presenting cells (APCs) such as polarized macrophages (M1) and neutrophils (N1) to the adaptive immune system.[6,10] This subsequently results in an adaptive immune response and tumor-specific immunological memory against the cancer, therefore protecting the subject from outgrowth or recurrence of metastatic disease. Notably, intratumoral CPMV has been shown to be effective at treating oral melanoma and mammary tumors of companion canines[12,13] with demonstrated tumor shrinkage of the treated and untreated tumors (i.e., abscopal effect) leading to increased survival. In this way, CPMV has the ability to be used as an in situ cancer vaccine in which an immunological response

is initiated in vivo without isolation of tumor-specific antigens and has demonstrated the potential to be translated for use in humans.

In tumor mouse models with intraperitoneal (i.p.) disseminated metastatic tumors, i.p. treatment with CPMV results in up to a 2-fold increase in the recruitment of NK cells to the tumor microenvironment within 6 h after treatment and approximately a 10-fold increase at 48 h.[6,10] NK cells are derived from common lymphoid progenitors but function as innate immune cells, and have the unique ability to recognize and destroy virally infected or transformed cells without costimulation, releasing foreign antigens to initiate an adaptive immune response.[4,14–16] NK cells may be activated through stimulation of several receptors, including 4-1BB (CD137).[17–19] However, exploiting NK cells in cancer immunotherapy is met by two major challenges: (1) recruiting NK cells to the tumor microenvironment, and (2) stimulating NK cells to overcome the immunosuppressive hurdle of the tumor microenvironment to enact NK cell function. Typically, NK cells are able to recognize virally infected or transformed cancer cells by their lack of major histocompatibility complex (MHC) Class I. However, within the tumor microenvironment, while cancer cells tend to have decreased expression of MHC Class I (which would make them recognizable by NK cells), the cancer cells can secrete a soluble form of MHC Class I that essentially saturates receptors on NK cells and suppresses them from functioning appropriately.[4] This overwhelming suppression outbalances stimulatory molecules and enables the tumor to evade immunological attack from NK cells. Nevertheless, stimulating intratumoral NK cells and enabling them to respond to transformed cells can prove to be advantageous in initiating an immunological response to cancer.[4,20–23]

NK cells may be stimulated via a variety of mechanisms such as stimulation with cytokines, e.g. IL-12, or stimulation of activation receptors, e.g. 4-1BB or NKG2D. Data indicate treatment with anti-4-1BB agonist to be NK cell-dependent and effective in treating murine tumor models;[4,20–23] therefore, we selected anti-4-1BB as a therapeutic approach to stimulate NK cells. It is of note though that 4-1BB is also expressed on lymphocytes, neutrophils, and macrophages; therefore, activation of immune cells may occur more broadly. In NK cells, agonism of the 4-1BB receptor activates NFkB, ERK, and P38-MAP kinase pathways,[19] thus priming recruited NK cells to escape the escape the immunosuppressive state and become activated to execute cytotoxic functions on tumor cells.[23]

Thus, as illustrated graphically in Scheme 1, we hypothesized that treatment with CPMV + anti-4-1BB would facilitate direct cytotoxicity of tumor cells, enhance innate immune cell processing and presentation of tumor antigens to the adaptive immune system, and improve in situ cancer vaccination efficacy.

CPMV was produced in black-eyed pea No. 5 plants and purified CPMV was characterized using several techniques to ensure quality control. UV–vis was used to determine the concentration of purified CPMV and the A260/280 ratio of 1.75 also indicates that pure CPMV was obtained (Figure 1A). CPMV was further characterized using native agarose and denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to characterize intact particles and coat proteins, respectively (Figure 1B). On the native

agarose gel, RNA comigrates with the capsid indicating the presence of intact CPMV particles. SDS-PAGE reveals the small (S-CP, ~24 kDa) and large (L-CP, ~42 kDa) coat protein subunits. Together, both agarose and SDS-PAGE methods indicate that pure CPMV preparations were obtained in the absence of free nucleic acids or protein contaminants. Fast protein liquid chromatography (FPLC) demonstrated uniform CPMV preparations with an elution of particles at 11.6 mL, characteristic for CPMV (Figure 1C); the 260 nm (RNA) and 280 nm (protein) peak overlap, again demonstrating that intact CPMV was eluted from the column. Additionally, aggregation, disassembly or contaminants were not apparent. This was also consistent with dynamic light scattering (DLS) showing a uniform population of particles with an average particle size of 36.82 nm (PDI 0.099) (Figure 1D). Monodisperse CPMV particles were also imaged by transmission electron microscopy (TEM) (Figure 1E). Together, this data demonstrates pure preparations of monodisperse and intact CPMV were obtained.

To test the efficacy of combined CPMV and NK cell agonist immunotherapy, we first utilized the CT-26 model of colon carcinomatosis. The i.p. disseminated tumors were established in BALB/c mice as previously described.[24,25] One week after tumor challenge, mice were assigned to one of four treatment groups: phosphate buffered saline (PBS) vehicle control, CPMV, anti-4-1BB NK cell agonist (BioXCell, clone LOB12.3), or dual therapy CPMV + anti-4-1BB (n = 6–8 per group). Treatments were initiated 7 days after tumor challenge and continued for 3 weeks. CPMV treatments (50 μg i.p.) were administered once weekly, anti-4-1BB treatments (5 μg i.p.) were administered twice weekly, and PBS vehicular control was administered twice weekly (see Figure 2A). Using the CT-26 i.p. tumor mouse model, we previously demonstrated potent and durable efficacy of CPMV at three weekly i.p. doses of 100 μg CPMV as a monotherapy. Therefore, to assess the efficacy of CPMV in combination with NK agonist therapy, we lowered the dose of CPMV to 50 μg. Tumor burden was followed using weight, abdominal circumference, and IVIS imaging of Luc-labeled CT-26 cells. After 1 week of treatment, mice treated with CPMV or anti-4-1BB alone demonstrated decreased luciferase bioluminescence compared to PBS vehicular controls. More importantly, mice treated with CPMV + anti-4-1BB dual immunotherapy demonstrated reduction of luciferase bioluminescence to near-background levels (Figure 2B). The negative control group of PBS-treated mice demonstrated a rapid increase in weight, abdominal circumference, and mortality (Figure 2C,D). Of mice receiving monotherapy treatment of CPMV or anti-4-1BB alone, there was significantly improved survival compared to the PBS control group (CPMV 24 days vs PBS 21 days, $p < 0.01$; anti-4-1BB 24 days vs PBS 21 days, $p < 0.05$). Impressively, mice treated with CPMV + anti-4-1BB dual therapy showed no signs of disease; although they gained physiological weight, there was no change in abdominal circumference (a measure of tumor burden). Most importantly, all mice treated with the dual therapy regimen remained alive at the end of the study.

Assessment of the raw data demonstrated two distinct groups of responders and nonresponders in CPMV and anti-4-1BB monotherapy groups. 4 of 7 CPMV and 5 of 8 anti-4-1BB monotherapy treated mice were nonresponders and demonstrated rapid increases in weight, abdominal circumference parameters, and early mortality (as per humane end points) comparable to those treated with PBS (Figure 3A,B). In contrast, 3 of 7 CPMV

and 3 of 8 anti-4-1BB monotherapy mice demonstrated at least partial response with delayed increases in weight and abdominal circumference parameters and improved survival compared to the respective nonresponder subgroup. CPMV responders had a median survival of 41 days compared to CPMV nonresponders with a median survival of 22.5 days (CPMV responders vs CPMV nonresponders, $p < 0.05$). Likewise, anti-4-1BB responders survived through the entirety of the study while anti-4-1BB nonresponders had a median survival of 21 days (anti-4-1BB responders vs anti-4-1BB nonresponders, $p < 0.05$). CPMV responders did ultimately succumb to tumor burden, which may be partially because of the intentional subtherapeutic dosing of CPMV used in these experiments. Interestingly, as anti-4-1BB responders demonstrated no signs of residual disease following treatment, this subgroup was comparable to the dual-therapy CPMV + anti-4-1BB group with no significant difference in mortality (anti-4-1BB responders vs anti-4-1BB, $p = 0.094$). This distinction of responders and nonresponders highlights that while monotherapy treatments may be partially effective on their own, the synergistic effect of dual therapy with CPMV and anti-4-1BB remains the most effective treatment to reduce tumor burden and improve survival.

After at least 30 days of tumor clearance, survivors (and naïve BALB/c control mice) were rechallenged to determine whether the treatment induced immunological memory. This time, we challenged mice with CT-26 cells subcutaneously (s.c.) and monitored tumor volume. Naïve mice demonstrated growth of CT-26 s.c. tumors and average mortality at 21 days (Figure 3C). In contrast, subcutaneous CT-26 tumors of dualtherapy-treated mice initially demonstrated mild tumor growth followed by resolution of tumor burden. All dual-therapy-treated mice remained alive after 42 days following tumor rechallenge, demonstrating that the CPMV + anti-4-1BB dual therapy elicits potent and durable antitumor immunity.

A valuable feature of CPMV cancer immunotherapy is that it is a single biologic that has the potential to induce a personalized tumor-specific immunotherapy approach. That is, the CPMV in situ vaccine is not tumor specific or tailored to a certain tumor type, but rather CPMV has demonstrated potent efficacy in multiple tumor types through innate immune stimulation and reversion of immunosuppression by turning cold into hot tumors.[9,10] Therefore, to validate the robustness of the CPMV + NK cell agonist dual immunotherapy, we next evaluated the efficacy of the dual-pronged therapeutic approach using a dermal melanoma mouse model using B16F10 cells in female C57Bl/6 mice. We established dermal melanoma tumors as previously described[24,26,27] ($n = 9–11$ per group) and began treatments when tumor volumes reached approximately 40 mm^3, approximately 7 days after tumor challenge and continued for 3 weeks (Figure 4A). To assess disease burden and efficacy, we monitored tumor volume and mortality. CPMV treatments (50 μg intratumorally (i.t.)) were administered once weekly, anti-4-1BB treatments (5 μg i.t.) were administered twice weekly, and PBS vehicular control was administered twice weekly. Again, similar to the CT-26 model, we designed the treatment plan for the CPMV monotherapy arm to be suboptimal from our previous work (where we showed 100 μg CPMV i.t. weekly to be an effective dose) to allow for a possible synergistic effect of the dual therapy to be able to be detected.

Mice treated with PBS demonstrated rapid tumor growth and an average mortality of 21 days (Figure 4B,C). Additionally, mice treated with anti-4-1BB alone demonstrated a similar

average mortality of 21 days (PBS vs anti-4-1BB, $p = 0.31$). As a group, CPMV mice demonstrated significantly improved survival compared to PBS-treated mice with an average mortality of 27 days (PBS vs CPMV, $p < 0.05$). In the dual-therapy CPMV + anti-4-1BB group, there were 7 of 11 mice surviving by the end of the study, demonstrating significantly improved survival compared to PBS-treated mice (PBS vs CPMV + anti-4-1BB, $p < 0.01$) albeit statistical significance between CPMV + anti-4-1BB and CPMV alone was not observed ($p = 0.053$).

Similar to the CT-26 model, there were responders and nonresponders in those treated with CPMV alone (Figure 5). In the CPMV monotherapy group, 3 of 10 mice demonstrated delayed tumor growth, with 1 of the responders eventually succumbing to the tumor burden following completion of treatment as outlined in Figure 4A. In contrast, 7 of 10 CPMV monotherapy treated mice did not demonstrate any substantial response to treatment with a median survival of 22 days, similar to a median survival of 21 for PBS-treated mice (PBS vs CPMV nonresponders, $p = 0.142$). In the CPMV + anti-4-1BB dual therapy group, 7 of 11 animals demonstrated a robust response to therapy and total or near-total resolution of their dermal melanoma tumors (Figure 5). In contrast, 4 of the 11 mice treated with dual therapy did not demonstrate any substantial response to treatment and had a median survival of 26.5 days, not significantly different from the median survival of PBS controls, 21 days. There was no significant difference in the median survival of CPMV responder and CPMV + anti-4-1BB responder subgroups ($p = 0.127$); however, detection of any statistical difference between these two subgroups may be limited by the decreased statistical power in comparing these smaller experimental subgroups.

After at least 30 days of tumor clearance, we then rechallenged survivors treated with CPMV + anti-4-1BB and naïve C57Bl/6 mice with B16F10 intradermal melanoma injected on the opposite flank and monitored tumor volume and overall mortality (Figure 5B). Compared to naïve mice, the previously treated CPMV + anti-4-1BB survivors demonstrated delayed tumor growth and significantly increased survival (21 days vs 17 days, $p = 0.042$, Figure 5B), again demonstrating that CPMV immunotherapy induces potent and long-lasting antitumor immunity and immunological memory.

In summary, here we demonstrate the efficacy of dual therapy approach of CPMV and anti-4-1BB in cancer in situ vaccination. In the CT-26-Luc model of disseminated colon carcinomatosis, CPMV and anti-4-1BB dual therapy demonstrated significantly improved survival compared to PBS controls and both CPMV and anti-4-1BB monotherapy groups. Remarkably, in the CT-26-Luc model, all mice receiving CPMV + anti-4-1BB dual therapy demonstrated rapid regression of tumor burden, and tumors were not detected throughout the remainder of the study. Furthermore, rechallenge with subcutaneous CT-26-Luc cells demonstrated immunological memory and elimination of tumor cells. Together, this data suggests that CPMV and anti-4-1BB dual therapy is highly effective at reducing tumor burden, improving survival, and eliciting a potent immunological memory response.

In the B16F10 dermal melanoma model, dual therapy with CPMV and anti-4-1BB demonstrated significantly improved survival compared to PBS control and anti-4-1BB monotherapy groups, with a trend toward significant improvement compared to CPMV

monotherapy. Rechallenge with intradermal melanoma tumors on the contralateral flanks demonstrated decreased tumor growth and improved survival compared to naïve animals, suggesting tumor-specific immunological memory. Future studies will focus on dosing and safety assessment of mono- and combination therapy arms.

Treatment with anti-4-1BB alone or CPMV + anti-4-1BB dual therapy was less effective in the B16F10 dermal melanoma model compared to the CT-26-Luc model of colon carcinomatosis. This may be because of the differences in the tumor microenvironments. In the intraperitoneal CT-26 model, treatments diffuse and spread throughout the peritoneal cavity enabling interaction with tumor cells which may also enable more efficient recruitment of NK cells by CPMV. In contrast, solid dermal B16F10 tumors may be more restrictive to CPMV diffusion, CPMV-mediated NK cell recruitment, and/or diffusion of the anti-4-1BB antibody. It is possible this may be overcome with modification, such as using multiple injection sites or delivery of therapeutics via microneedle patches. Indeed, we have previously demonstrated that efficacy of immunotherapy can be improved in solid tumors through microneedle patch delivery.[28]

Ultimately, this work demonstrates synergy between CPMV and anti-4-1BB immunotherapy treatments. Our previous research has shown that CPMV stimulates PRRs on tumor-resident innate immune cells, which then become activated and subsequently secrete pro-inflammatory cytokines and chemokines.[8,10,11] This results in recruitment of NK cells to the tumor microenvironment where they have the potential to perform potent cytotoxic antitumor functions. In this work, we demonstrate that addition of anti-4-1BB potentiates the therapy with improved efficacy and survival (see Scheme 1).

Oncolytic cancer immunotherapies are undergoing development and several are being used clinically. While the plant virus in situ vaccine and the oncolytic approach are conceptually distinct (oncolytic viruses target and kill tumor cells directly, while the plant virus targets innate immune cells to active cell killing function by the immune system), data suggest that oncolytic viruses have the potential to be further modified to further recruit and activate NK cells.[29,30] For example, this may be accomplished by creating recombinant oncolytic viruses expressing various cytokines promoting NK cell activation, such as IL-2[31,32] and IL-15.[33] While promising, such avenues require further recombinant engineering and optimization whereas CPMV is inherently capable of recruiting NK cells to the tumor microenvironment. Given the unique potency and potential to combine CPMV with NK agonists as well as other immunotherapies such as checkpoint inhibitors,[34] chemotherapeutic agents,[35] and radiation,[36] CPMV immunotherapy makes an attractive platform technology for multimodal cancer immunotherapy.

As we move forward to the potential use of CPMV in human patients, biosafety profiles and production methods must be considered. As CPMV is not inherently infectious toward humans, we anticipate CPMV will have an improved biosafety profile for both the patient and the administering healthcare professionals compared to oncolytic viral treatments. Additionally, another favorable attribute is its high yielding production through molecular farming in plants; 1 plant yields ~1 mg of pure CPMV,[37] which is sufficient to complete the treatment schedule for 1 canine patient.[38] As canine patients present with tumors

comparable in size to human tumors, we project the human dose to be equivalent to the canine dose. However, clinical trials remain necessary to determine the dose and safety profile of CPMV for use in human patients.

Being able to recruit NK cells to the tumor microenvironment and take advantage of their innate cytolytic functions in cancer immunotherapies has been a challenge for many years. The ability to enable NK cells to function appropriately against tumor cells despite strong immunosuppressive signals within the tumor microenvironment would enable improved processing of tumor antigens and a robust antitumor immunologic response. Our work shows that a dual-therapy approach using CPMV and a NK cell agonist improves tumor-specific immunological response and improved survival. This approach may be essential to fully utilize NK cell capabilities in the development of new and potent cancer immunotherapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding

This work was supported in part through NIH grants R01 CA224605, R01 CA253615, and U01 CA218292 (to N.F.S.). E.C.K. was supported by the NIH T32EB005970 institutional training grant through the Clinician-Scientist Radiology Residency Program. N.F.S. acknowledges support through the Shaughnessy Family Fund for Nano-ImmunoEngineering (nanoIE) at UCSD.

ABBREVIATIONS

CPMV	Cowpea mosaic virus
NK	Natural Killer

REFERENCES

(1). Goto T Radiation as an in situ auto-vaccination: Current perspectives and challenges. Vaccines 2019, 7, 100.

(2). Hammerich L; Bhardwaj N; Kohrt HE; Brody JD In situ vaccination for the treatment of cancer. Immunotherapy 2016, 8, 315–330. [PubMed: 26860335]

(3). Derynck R; Turley SJ; Akhurst RJ TGFβ biology in cancer progression and immunotherapy. Nat. Rev. Clin. Oncol 2021, 18, 9–34. [PubMed: 32710082]

(4). Au KM; Park SI; Wang AZ Trispecific natural killer cell nanoengagers for targeted chemoimmunotherapy. Sci. Adv 2020, 6, No. eaba8564.

(5). Cao Y; Wang X; Jin T; Tian Y; Dai C; Widarma C; Song R; Xu F Immune checkpoint molecules in natural killer cells as potential targets for cancer immunotherapy. Signal Transduct. Target. Ther 2020, 5, 250. [PubMed: 33122640]

(6). Wang C; Beiss V; Steinmetz NF Cowpea Mosaic Virus Nanoparticles and Empty Virus-Like Particles Show Distinct but Overlapping Immunostimulatory Properties. J. Virol 2019, 93, e00129–19. [PubMed: 31375592]

(7). Czapar AE; Tiu BDB; Veliz FA; Pokorski JK; Steinmetz NF Slow-Release Formulation of Cowpea Mosaic Virus for In Situ Vaccine Delivery to Treat Ovarian Cancer. Adv. Sci 2018, 5, 1700991.

(8). Lizotte PH; et al. In situ vaccination with cowpea mosaic virus nanoparticles suppresses metastatic cancer. Nat. Nanotechnol 2016, 11, 295–303. [PubMed: 26689376]

(9). Kerstetter-Fogle A; et al. Plant Virus-Like Particle In Situ Vaccine for Intracranial Glioma Immunotherapy. Cancers (Basel). 2019, 11, 515.

(10). Wang C; Fiering SN; Steinmetz NF Cowpea Mosaic Virus Promotes Anti-Tumor Activity and Immune Memory in a Mouse Ovarian Tumor Model. Adv. Ther 2019, 2, 1900003.

(11). Albakri MM; Veliz FA; Fiering SN; Steinmetz NF; Sieg SF Endosomal toll-like receptors play a key role in activation of primary human monocytes by cowpea mosaic virus. Immunology 2020, 159, 183–192. [PubMed: 31630392]

(12). Hoopes PJ; et al. Treatment of canine oral melanoma with nanotechnology-based immunotherapy and radiation. Mol. Pharmaceutics 2018, 15, 3717.

(13). Alonso-Miguel D; Valdivia G; Guerrera D; Perez-Alenza MD; Pantelyushin S; Alonso-Diez A; Beiss V; Fiering S; Steinmetz NF; Suarez-Redondo M; vom Berg J; Pena L; Arias-Pulido H Neoadjuvant in situ vaccination with cowpea mosaic virus as a novel therapy against canine inflammatory mammary cancer. J. Immunother. Cancer 2022, 10, No. e004044. [PubMed: 35277459]

(14). Paul S; Lal G The Molecular Mechanism of Natural Killer Cells Function and Its Importance in Cancer Immunotherapy. Front. Immunol 2017, 8, 1124. [PubMed: 28955340]

(15). Vivier E; Tomasello E; Baratin M; Walzer T; Ugolini S Functions of natural killer cells. Nat. Immunol 2008, 9, 503–510. [PubMed: 18425107]

(16). Chiossone L; Dumas P-Y; Vienne M; Vivier E Natural killer cells and other innate lymphoid cells in cancer. Nat. Rev. Immunol 2018, 18, 671–688. [PubMed: 30209347]

(17). Halstead ES; Mueller YM; Altman JD; Katsikis PD In vivo stimulation of CD137 broadens primary antiviral CD8+ T cell responses. Nat. Immunol 2002, 3, 536–541. [PubMed: 12021777]

(18). Kohrt HE; et al. Stimulation of natural killer cells with a CD137-specific antibody enhances trastuzumab efficacy in xenotransplant models of breast cancer. J. Clin. Invest 2012, 122, 1066–75. [PubMed: 22326955]

(19). Zapata JM; Perez-Chacon G; Carr-Baena P; Martinez-Forero I; Azpilikueta A; Otano I; Melero I; et al. CD137 (4-1BB) Signalosome: Complexity Is a Matter of TRAFs. Front. Immunol 2018, 9, 2618. [PubMed: 30524423]

(20). Wilcox RA; et al. Provision of antigen and CD137 signaling breaks immunological ignorance, promoting regression of poorly immunogenic tumors. J. Clin. Invest 2002, 109, 651–9. [PubMed: 11877473]

(21). Gauttier V; et al. Agonistic anti-CD137 antibody treatment leads to antitumor response in mice with liver cancer. Int. J. Cancer 2014, 135, 2857–2867. [PubMed: 24789574]

(22). Vinay DS; Kwon BS Therapeutic potential of anti-CD137 (4-1BB) monoclonal antibodies. Expert Opin. Ther. Targets 2016, 20, 361–373. [PubMed: 26428392]

(23). Guillerey C; et al. Immunosurveillance and therapy of multiple myeloma are CD226 dependent. J. Clin. Invest 2015, 125, 2077–2089. [PubMed: 25893601]

(24). Wang C; Steinmetz NF A Combination of Cowpea Mosaic Virus and Immune Checkpoint Therapy Synergistically Improves Therapeutic Efficacy in Three Tumor Models. Adv. Funct. Mater 2020, 30, 2002299. [PubMed: 34366758]

(25). Cai H; Shukla S; Steinmetz NF The Antitumor Efficacy of CpG Oligonucleotides is Improved by Encapsulation in Plant Virus-Like Particles. Adv. Funct. Mater 2020, 30, 1908743. [PubMed: 34366757]

(26). Murray AA; Sheen MR; Veliz FA; Fiering SN; Steinmetz NF In Situ Vaccination of Tumors Using Plant Viral Nanoparticles. Methods Mol. Biol 2019, 2000, 111–124. [PubMed: 31148013]

(27). Murray AA; Wang C; Fiering S; Steinmetz NF In Situ Vaccination with Cowpea vs Tobacco Mosaic Virus against Melanoma. Mol. Pharmaceutics 2018, 15, 3700–3716.

(28). Boone CE; et al. Active Microneedle Administration of Plant Virus Nanoparticles for Cancer in situ Vaccination Improves Immunotherapeutic Efficacy. ACS Appl. nano Mater 2020, 3, 8037–8051. [PubMed: 33969278]

(29). Bommareddy PK; Kaufman HL Unleashing the therapeutic potential of oncolytic viruses. J. Clin. Invest 2018, 128, 1258–1260. [PubMed: 29504947]

(30). Marotel M; Hasim MS; Hagerman A; Ardolino M The two-faces of NK cells in oncolytic virotherapy. Cytokine Growth Factor Rev. 2020, 56, 59–68. [PubMed: 32586674]

(31). Wu Y; et al. Recombinant Newcastle disease virus (NDV/Anh-IL-2) expressing human IL-2 as a potential candidate for suppresses growth of hepatoma therapy. J. Pharmacol. Sci 2016, 132, 24–30. [PubMed: 27174862]

(32). Zamarin D; Vigil A; Kelly K; García-Sastre A; Fong Y Genetically engineered Newcastle disease virus for malignant melanoma therapy. Gene Ther. 2009, 16, 796–804. [PubMed: 19242529]

(33). Hock K; et al. Oncolytic influenza A virus expressing interleukin-15 decreases tumor growth in vivo. Surgery 2017, 161, 735–746. [PubMed: 27776794]

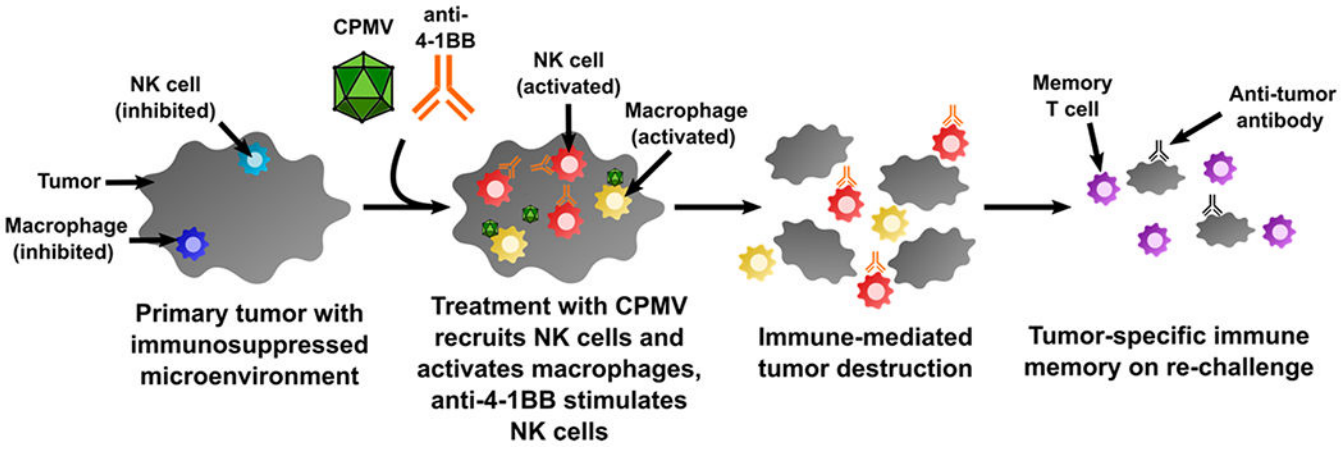
(34). Gautam A; Beiss V; Wang C; Wang L; Steinmetz NF Plant Viral Nanoparticle Conjugated with Anti-PD-1 Peptide for Ovarian Cancer Immunotherapy. Int. J. Mol. Sci 2021, 22, 9733. [PubMed: 34575893]

(35). Aljabali AAA; Shukla S; Lomonossoff GP; Steinmetz NF; Evans DJ CPMV-DOX delivers. Mol. Pharmaceutics 2013, 10, 3–10.

(36). Patel R; Czapar AE; Fiering S; Oleinick NL; Steinmetz NF Radiation Therapy Combined with Cowpea Mosaic Virus Nanoparticle in Situ Vaccination Initiates Immune-Mediated Tumor Regression. ACS omega 2018, 3, 3702–3707. [PubMed: 29732445]

(37). Chung YH; Church D; Koellhoffer EC; Osota E; Shukla S; Rybicki EP; Pokorski JK; Steinmetz NF; et al. Integrating plant molecular farming and materials research for next-generation vaccines. Nat. Rev. Mater 2022, 7, 372–388. [PubMed: 34900343]

(38). Hoopes PJ; et al. Treatment of Canine Oral Melanoma with Nanotechnology-Based Immunotherapy and Radiation. Mol Pharmaceutics 2018, 15, 3717–3722.



Scheme 1. Hypothesis: Treatment with CPMV Recruits NK Cells to the Tumor Microenvironment, and anti-4-1BB Antibody Activates Tumor-Resident and Recruited Intratumoral NK Cells[a]

[a]Together with activation of macrophages (M1) or neutrophils (N1, not shown), this leads to enhanced immune-mediated tumor destruction. Subsequent presentation of antigen to T cells then initiates an adaptive immune response and subsequent immunological memory.

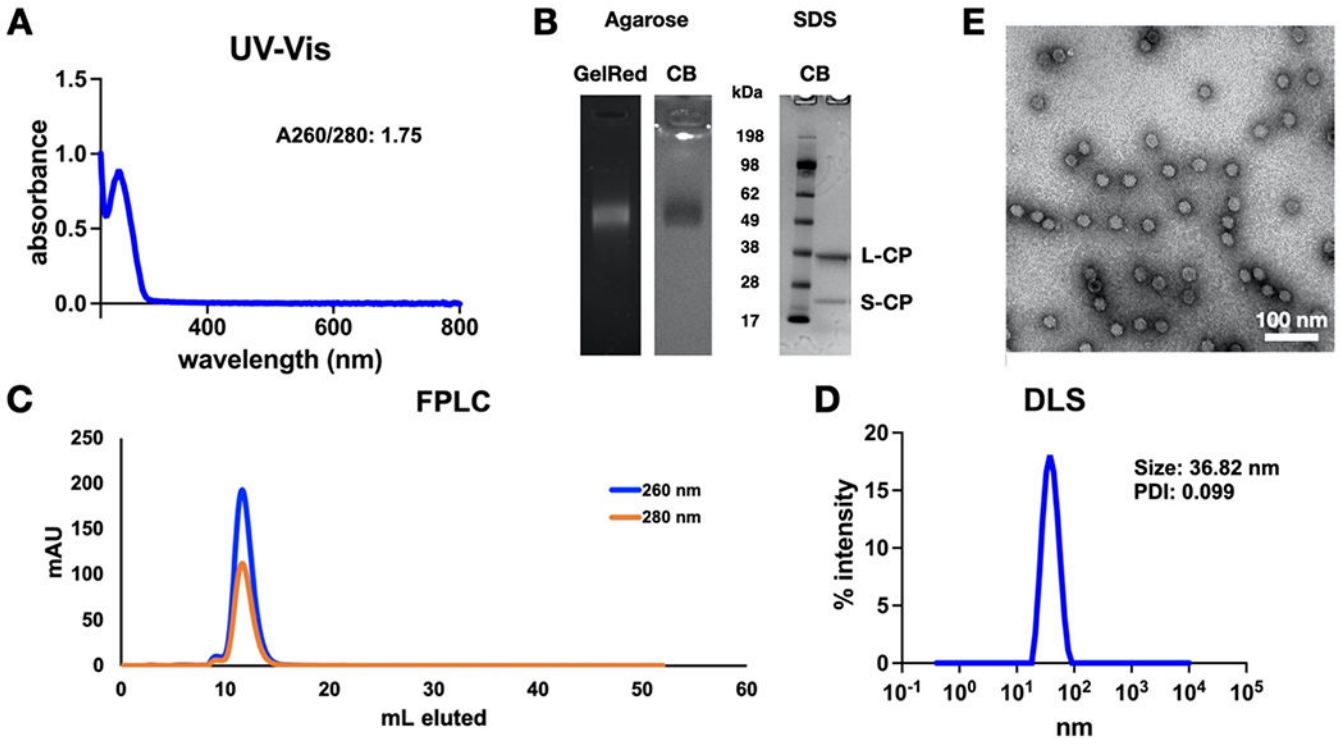


Figure 1.

Isolation of CPMV yields monodisperse nanoparticles. (A) UV–vis spectroscopy spectrum of purified CPMV. (B) Native agarose gel (0.8% w/v) of intact CPMV stained with nucleic acid stain GelRed and protein stain Coomassie blue (CB). Denaturing SDS-PAGE (4–12%) of the coat proteins stained with Coomassie blue (CB) identifies the small coat protein (S-CP) of approximately 24 kDa and large coat protein (LCP) of approximately 42 kDa. (C) FPLC, (D) DLS, and (E) TEM of purified CPMV (negatively stained with 2% (w/v) uranyl acetate).

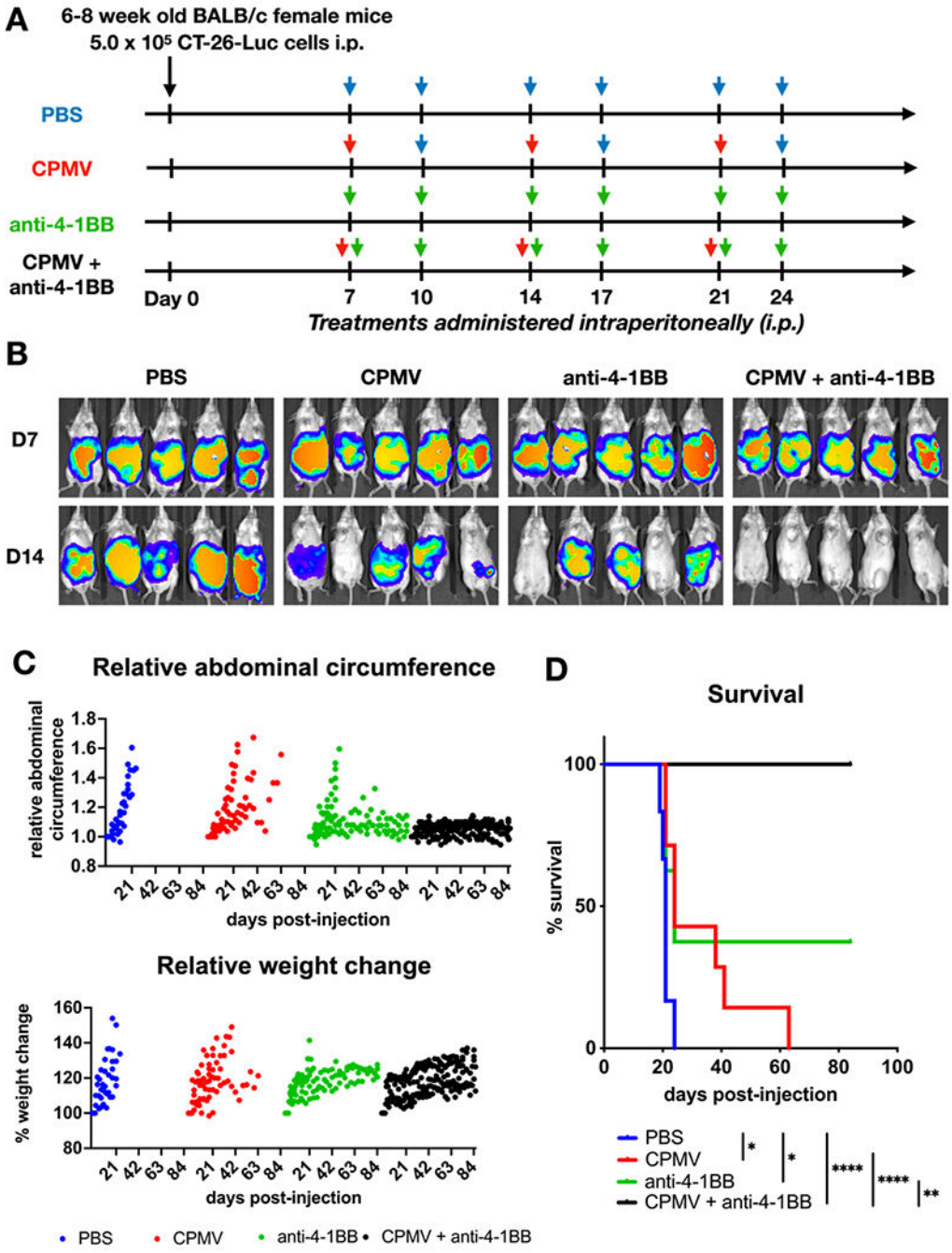


Figure 2.

Dual therapy with CPMV and anti-4-1BB leads to significantly decreased tumor burden and improved survival in a CT-26 model of colon carcinomatosis. (A) Schematic outline of treatment regimen for PBS ($n = 5$), CPMV ($n = 7$), anti-4-1BB ($n = 8$), and CPMV + anti-4-1BB ($n = 8$) groups in the CT-26-Luc disseminated intraperitoneal colon cancer model. (B) IVIS of PBS, CPMV, anti-4-1BB, and CPMV + anti-4-1BB treatment groups at 7 days post-tumor challenge and following 1 week of treatment at 14 days. (C) Relative percentage weight change and relative abdominal circumference of treatment groups. (D)

Survival of treatment groups was plotted and statistical analysis was performed using a Mantel–Cox test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$, ****$p < 0.001$). There was a median survival of 21 days (PBS), 24 days (CPMV), 24 days (anti-4-1BB), and indeterminate (CPMV + anti-4-1BB). Data are combined from two independent experimental repeats.

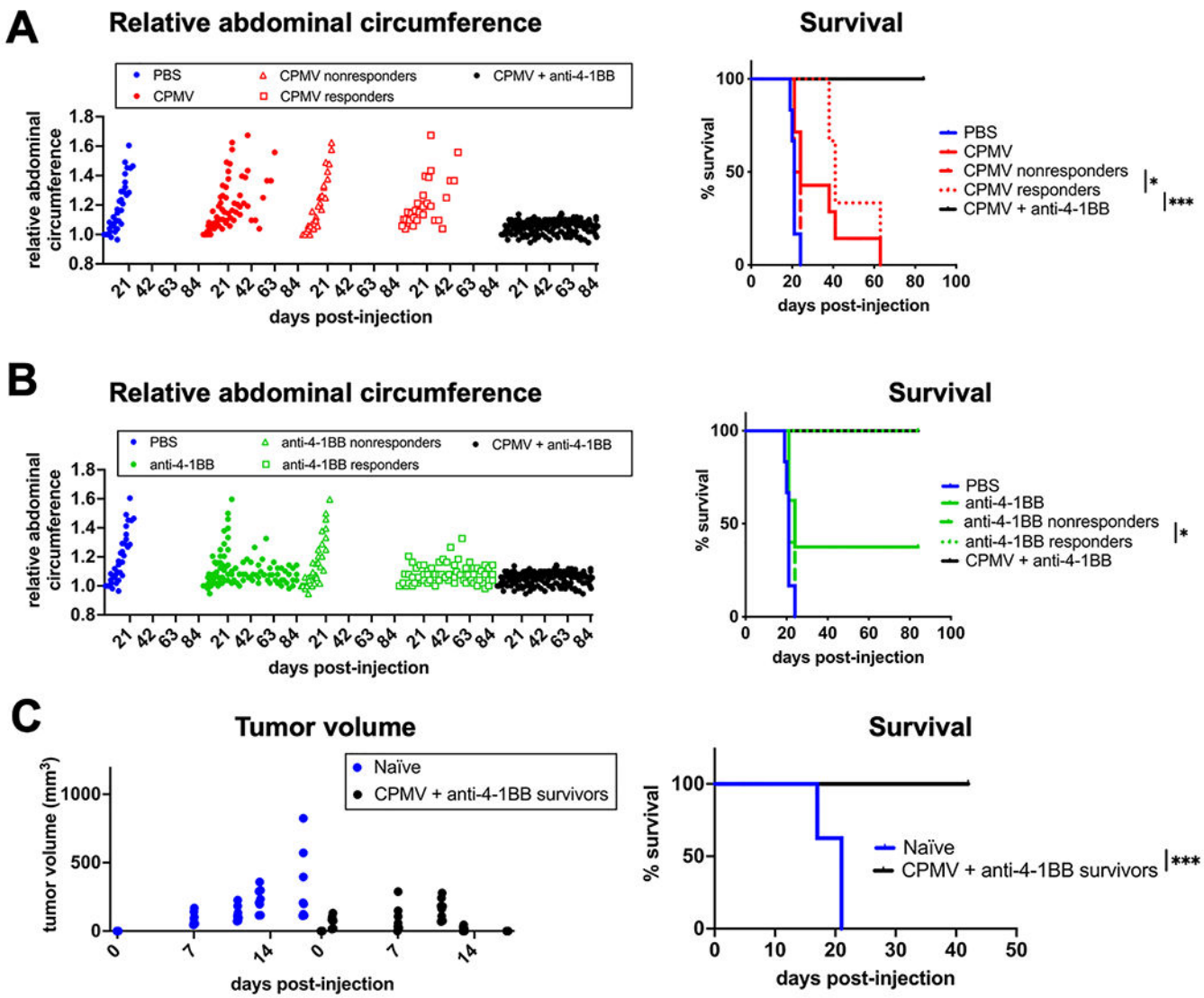


Figure 3.

CPMV and anti-4-1BB monotherapy groups demonstrate distinct subgroups of responders and nonresponders to treatment in a CT-26 model of colon carcinomatosis. Survival of treatment groups was plotted and statistical analysis was performed using a Mantel–Cox test (*$p < 0.05$, **$p < 0.01$). (A) In the CPMV monotherapy group, out of 8 mice there were 4 responders and 4 nonresponders. CPMV nonresponders demonstrated similar survival compared to PBS control (PBS vs CPMV nonresponders, $p = 0.184$). There was a median survival of 21 days for PBS, 24 days for CPMV, and indeterminate for CPMV + anti-4-1BB groups. For the CPMV subgroups, there was a median survival of 22.5 days for CPMV nonresponders and 41 days for CPMV responders. Data are combined from two independent experimental repeats. (B) In the anti-4-1BB monotherapy group, out of 8 mice there were 3 responders and 5 nonresponders. For the anti-4-1BB subgroups, there was a median survival of 21 days for anti-4-1BB nonresponders and indeterminate for anti-4-1BB responders. Data are combined from two independent experimental repeats. (C) Tumor rechallenge in

CPMV + anti-4-1BB treated survivors and naïve BALB/c mice using a CT-26 subcutaneous model. Tumors were measured with calipers and volumes were estimated as [length × (short width)^2]/2. There was a median survival of 21 days (naïve BALB/c, $n = 8$) vs indeterminate (CPMV + anti-4-1BB survivors, $n = 8$). Statistical analysis was performed using a Mantel–Cox test (***$p < 0.005$).

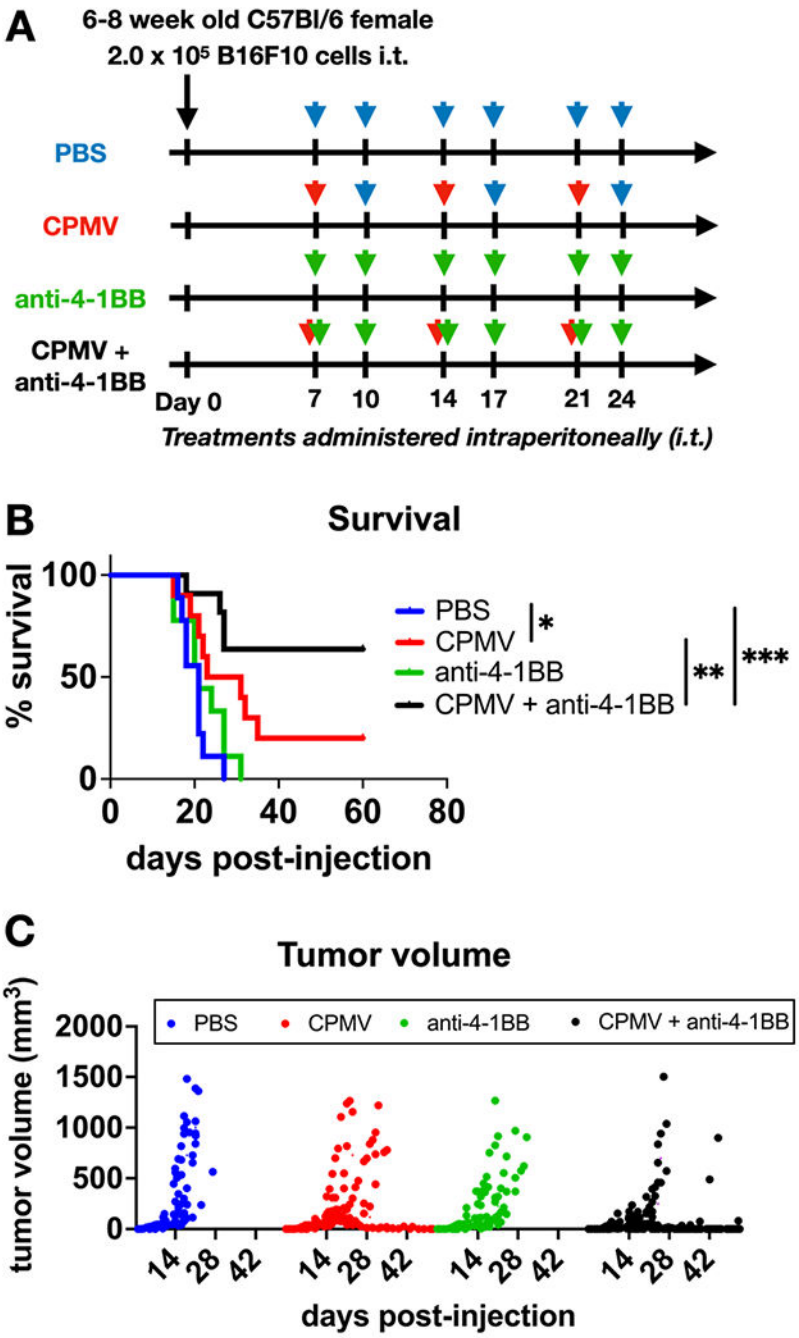


Figure 4.

Dual therapy with CPMV and anti-4-1BB leads to significantly decreased tumor burden and improved survival in a B16F10 model of dermal melanoma. (A) Schematic outline of treatment regimen for PBS ($n = 9$), CPMV ($n = 10$), anti-4-1BB ($n = 9$), and CPMV + anti-4-1BB ($n = 11$) groups in the B16F10 dermal melanoma model. Treatments began when tumors reached ~40 mm^3, and the end point was defined as tumors exceeding 1500 mm^3. (B) Estimated tumor volume as calculated by volume = [(short length)^2 × (long length)]/2. (C) Survival of treatment groups was plotted and statistical analysis was performed using

a Mantel–Cox test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$). There was a median survival of 21 days (PBS), 27 days (CPMV), 21 days (anti-4-1BB), and indeterminate (CPMV + anti-4-1BB). Data are combined from two independent experimental repeats.

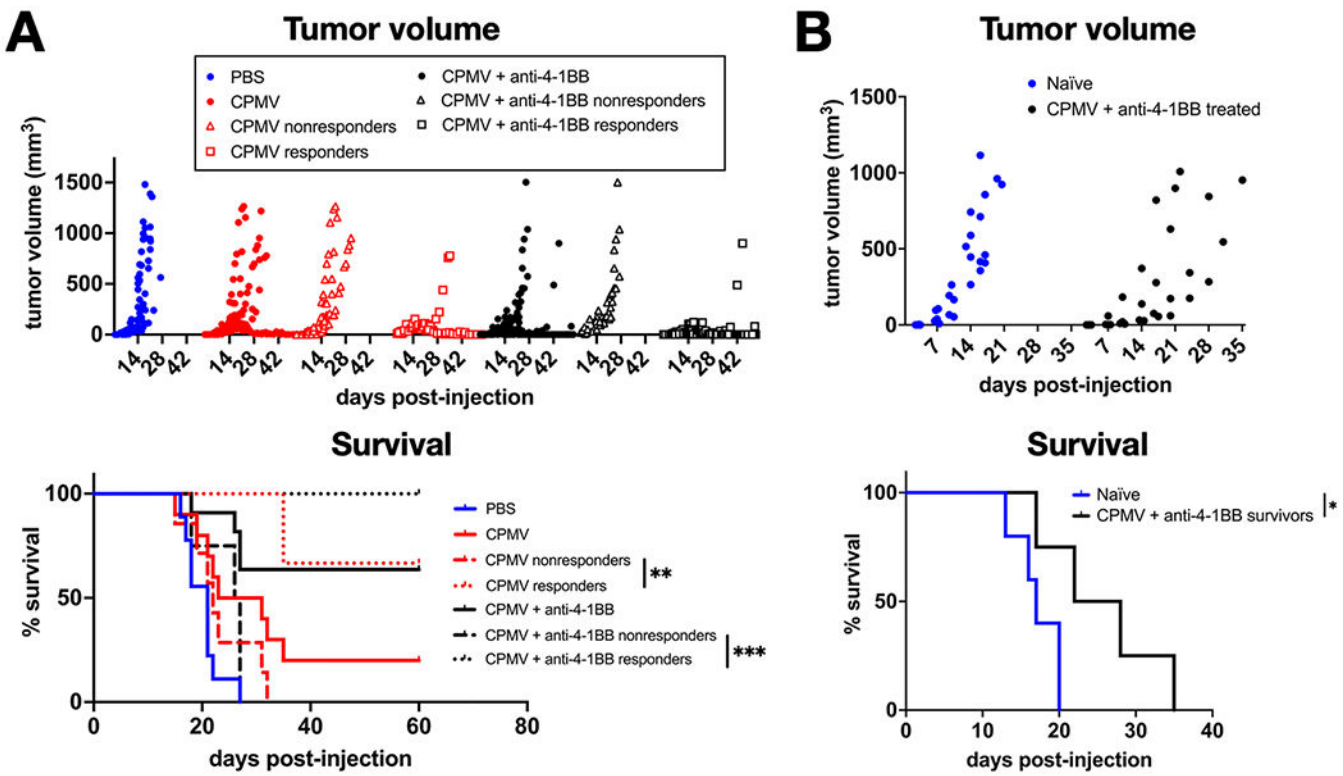


Figure 5.

CPMV monotherapy and CPMV + anti-4-1BB dual therapy groups demonstrate distinct subgroups of responders and nonresponders to therapy in a B16F10 model of dermal melanoma. Survival of treatment groups was plotted and statistical analysis was performed using a Mantel–Cox test (**$p < 0.01$, ***<0.005). In the CPMV monotherapy group, out of 7 mice there were 3 responders and 4 nonresponders. There was a median survival of 21 days for PBS, 27 days for CPMV, and indeterminate for CPMV + anti-4-1BB treated groups. For the CPMV subgroups, there was a median survival of 22 days for CPMV nonresponders and indeterminate for CPMV responder subgroups. For the dual therapy CPMV + anti-4-1BB subgroups, there was a median survival of 26.5 days for CPMV + anti-4-1BB responders and indeterminate for CPMV + anti-4-1BB responders. Data are combined from two independent experimental repeats. (B) Tumor rechallenge in CPMV + anti-4-1BB treated survivors and naïve C57Bl/6J mice with median survival of 17 days (naïve C57B1/6J, $n = 5$)) vs 21 days (CPMV + anti-4-1BB survivors, $n = 4$). Data are combined from two experimental repeats. Statistical analysis was performed using a Mantel–Cox test (*$p < 0.05$).