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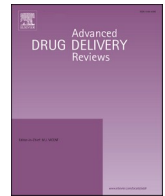
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Cowpea mosaic virus as a candidate for intratumoral immunotherapy: progress, challenges, and future directions[☆]

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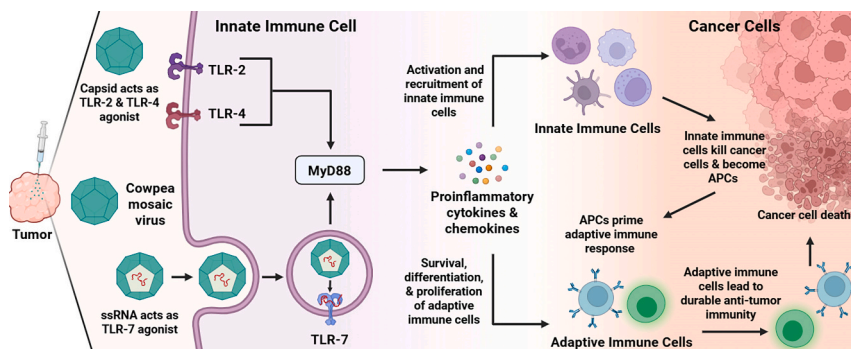
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GRAPHICAL ABSTRACT



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ABSTRACT

Immunotherapy has emerged as a new pillar of cancer therapeutics that utilizes the patients own immune system to target tumor cells, a particular advantage in the context of hard-to-treat cancers. Virus-based immunotherapies have shown great potential as cancer treatments, including oncolytic viruses, viral vectors and virus-like particles. Here, we discuss the development, applications, challenges, and future opportunities of cowpea mosaic virus (CPMV) as a plant virus-based candidate for intratumoral immunotherapy, focusing on the treatment of aggressive, metastatic and refractory solid tumors. Evidence of antitumor efficacy has been gathered from mouse tumor models and veterinary clinical trials involving canine cancer patients. CPMV is promising as a monotherapy and as part of combination therapies, including chemotherapy, radiation, checkpoint inhibitors, and cytokine therapies. The putative mechanism of action is described, highlighting key features such as the capsid

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proteins and RNA acting as Toll-like receptor (TLR) agonists for TLR-2, TLR-4 and TLR-7, as well as the presentation of unique epitopes that prime a Th-1 balanced immune response. The nanoparticle structure of CPMV enhances efficacy by exerting multivalency and avidity effects. The biodistribution, toxicity, and agronomical safety profile of CPMV is also described, especially in relation to tumor retention, hematologic toxicity, allergenicity and adverse events, and viral shedding. This body of work provides a thorough exploration of a cancer immunotherapy candidate in development for more than 10 years, positioning CPMV as a promising intratumoral platform for hard-to-treat cancers.

1. Introduction

Cancer is a leading cause of death worldwide, with an estimated 20 million new cases and ~ 9.7 million cancer-related deaths in 2022 [1]. In the United States of America, cancer is the second leading cause of death, with more than 2 million new cases and more than 600,000 deaths in 2025 [2]. These numbers are predicted to rise due to factors such as a growing and aging global population, improvements in early detection, and risks such as tobacco use, alcohol use, and obesity [1]. Some estimates predict well over 30 million new cancer cases globally and more than 18 million cancer-related deaths in 2050 [1,3,4]. Although outcomes have improved for some malignancies, many solid tumors, including metastatic melanoma, triple-negative breast cancer (TNBC), ovarian cancer, pancreatic cancer, glioma, and recurrent or metastatic head and neck cancers, remain difficult to manage. They are often characterized by poor drug penetration, intrinsic or acquired resistance, and an immunosuppressive tumor microenvironment (TME). There is an urgent need for therapeutic strategies that are both locally effective and capable of reprogramming these hard-to-treat tumors [5].

For decades, cancer treatment could be classified into three ‘pillars’: surgery, chemotherapy, and radiotherapy. More recently, immunotherapy, which utilizes a patient's own immune system to target cancer cells, has emerged as a fourth pillar. Promising immunotherapies that prime the immune system against tumors have been approved by the FDA, including monoclonal antibodies (mAbs), adoptive cell therapies, pathway-specific approaches such as Toll-like receptor (TLR) agonists, and pro-inflammatory cytokines (specifically, IL-2 and IFN- α) [6–9]. Immunotherapy may be used alongside other treatments to prevent immune-evasive cancer progression by targeting specific immune cells or receptors. Some approaches may also induce long-lasting anti-cancer effects that persist beyond the end of treatment [8,10,11]. However, one key challenge is that only a fraction of patients respond, with the immunosuppressive state of aggressive tumors reducing efficacy and tumors developing resistance to the treatment over time [10,12–15]. Another concern is the risk of autoimmune, off-target, and on-target but off-tumor toxicity, which can prevent new therapeutics from moving forward in clinical trials [16–18]. These challenges are particularly relevant in hard-to-treat cancers, where standard systemic regimens often fail to eradicate all tumor cells.

Among the most recent approaches in immunotherapy, oncolytic viruses and mammalian viral vectors have already made progress in the clinic. For example, talimogene laherparepvec (TVEC) is a first-in-class oncolytic virus therapy approved by the FDA for the treatment of advanced melanoma [19]. Several other oncolytic viruses have reached clinical trials [20–22]. However, concerns about the use of mammalian viruses to treat human diseases include the potential for pathogenicity, off-target toxicity, and recombination with wild-type viruses [22–24]. These drawbacks are addressed by intratumoral agents based on plant viruses or bacteriophages. For example, vidutolimod (also known as CMP-001) is a virus-like particle (VLP) based on bacteriophage Q β packaging a TLR9 agonist to prevent its degradation, potentiating the immune response elicited by the VLPs [25]. Vidutolimod has been shown to convert refractory tumors into effective *in situ* vaccines in preclinical models and early clinical studies of PD-1–refractory melanoma [26], and has recently advanced to clinical testing as a monotherapy or in combination with mAb checkpoint inhibitors for the

treatment of difficult forms of melanoma, prostate cancer, and head and neck cancer (NCT02680184, NCT04698187, NCT05445609, NCT04633278). Such agents are particularly attractive for hard-to-treat cancers that do not respond to systemic immunotherapy alone.

Plant viruses offer an interesting platform technology owing to their diversity and versatility. They range from icosahedral structures as little as 17 nm in diameter to rod-like and filamentous forms as long as 2000 nm [27,28]. Their nucleoprotein assemblies form precise nanostructures with a high degree of reproducibility [29]. However, the structures are also dynamic, allowing disassembly and reassembly, as well as morphological rearrangement [30,31]. High yields of plant viruses can be produced by plant molecular farming, which comes with a number of advantages: a production system lacking human pathogens, scalability based on vertical farming, and low-cost, rapid production [32,33].

Several plant viruses have been explored as cancer therapeutics, specifically as carriers of small-molecule drugs, proteins, or nucleic acid therapeutics. Plant virus nanoparticles and corresponding VLPs have also been used preclinically as adjuvants and epitope display platforms for cancer vaccines, and some have been used as immunomodulators for intratumoral immunotherapy candidates. Examples include red clover necrotic mosaic virus as a carrier for the anticancer drug doxorubicin, VLPs based on cowpea chlorotic mottle virus to package and release mRNAs encoding antigens, and VLPs based on potato virus X (PVX) to deliver TRAIL, the tumor necrosis factor-related apoptosis-inducing ligand [34–36]. A VLP based on cucumber mosaic virus displaying a tetanus toxoid epitope has been used alongside a microcrystalline tyrosine adjuvant as a cancer vaccine, and PVX-derived VLPs have also been used to carry an idiopathic tumor antigen to induce antibodies in a cancer model [37,38]. Papaya mosaic virus has been used as an intratumoral agent combined with a PD-1 blockade to synergistically reduce tumor growth [39].

In this focused review article, we discuss the development of cowpea mosaic virus (CPMV) as a drug candidate for intratumoral immunotherapy, particularly in the context of hard-to-treat cancers. CPMV infects cowpeas plants (*Vigna unguiculata*) causing mosaic patterning on leaves as well as chlorosis and necrosis [40]. CPMV as a platform nanotechnology has been developed and repurposed as a foreign epitope display system [41], a carrier of heterologous mRNAs and small-molecule drugs [42,43], and as a potent immunomodulatory effector in cancer immunotherapy for the treatment of solid tumors. Intratumoral CPMV treatment acts as an *in situ* vaccine, engaging multiple pattern recognition receptors (PRRs), recruiting and activating innate immune cells, and driving adaptive responses that control local and distant disease, even in hard-to-treat cancers [44–47]. This includes poorly-immunogenic and metastatic models (e.g., B16F10 melanoma, ovarian cancer, breast cancer, and glioma) as well as spontaneous tumors in canine patients [44,47–51]. CPMV outperforms other plant and bacteriophage VLPs, that our lab has tested, for intratumoral immunotherapy based on comparative efficacy and mechanistic studies [52–54]. The data discussed in this review highlight CPMV as a promising platform to tackle hard-to-treat tumors by reprogramming the immunosuppressive TME and further, synergizing with systemic therapies. This review covers the efficacy of CPMV immunotherapy in challenging preclinical tumor models and canine cancer trials, its mechanism of action, and pharmacology. We also highlight the challenges and future directions of CPMV therapy in the context of hard-to-treat cancers.

2. CPMV genome and structure

CPMV (*Comovirus vignae*) is a non-enveloped plant virus of the family *Secoviridae*. It is primarily transmitted by beetles and has a host range mainly limited to legumes, especially cowpea plants (or black-eyed pea plants), which can serve as molecular farming bioreactors for virus production (Fig. 1a). The structure of CPMV has been characterized at near atomic resolution [55]. The CPMV virion is ~30 nm in diameter and has icosahedral symmetry. Virions assemble from 60 copies of the small (S, 213 amino acids) and the large (L, 374 amino acids) subunit coat proteins (Fig. 1b). The S subunit folds into one jellyroll β -sandwich, whereas the L subunit folds into two jellyroll β -sandwich domains that form an asymmetric unit arranged in a similar surface lattice to $T = 3$ viruses [55]. Because these units have different polypeptide sequences, the particle structure is instead described as pseudo $T = 3$ or $P = 3$ symmetry [55,56]. Interestingly, CPMV is regarded as a plant picornavirus because it shares a taxonomic order, *picornavirales*, with animal picornaviruses. For example, CPMV and poliovirus share similar shapes and homologous genetic arrangement, despite their different hosts [57,58].

CPMV is a bipartite positive-sense ssRNA virus with known nucleotide sequences [60–62]. Both RNAs (known as RNA-1 and RNA-2) are separately encapsidated within isometric particles: specifically the bottom component (B) contains RNA-1, the middle component (M) contains RNA-2, with an RNA-free top component (T), the latter occurs in low quantities (Fig. 1c) [63–65]. Both B and M components (or the RNA isolated from them) are required for infection [64]. Each RNA molecule

is polyadenylated at the 3' end [66] and covalently bound to a VPg (genome-linked viral protein) at its 5' terminus [67,68]. Both RNA-1 and RNA-2 contain one long open reading frame (ORF) each. The RNAs are translated into precursor polypeptides that are cleaved by a virus-encoded proteinase in *cis* and *trans* isomers [69–72]. The larger RNA-1 (5889 nt) encodes the virus replication machinery (the protease cofactor, helicase and CPMV-specific proteinase, the VPg that plays an essential role in initiation of RNA synthesis, and the RNA-dependent RNA polymerase) [70,73]. The smaller RNA-2 (3481 nt) encodes the movement protein and the small (S) and large (L) coat proteins. These coat proteins protect the RNA but also assist in short-range cell-to-cell movement *via* plasmodesmata and long-distance movement *via* the plant vascular system [71,74,75].

3. Efficacy of intratumoral immunotherapy using CPMV in mouse tumor models

CPMV is a potent immunomodulator in the context of intratumoral immunotherapy. In 2015, we discovered that intratumoral CPMV (and its corresponding VLP devoid of the genomic RNA) triggers a robust antitumor immune response that was reproducible in murine models of melanoma as well as colon, ovarian, and breast cancer [44]. Intratumoral CPMV activates innate immune cells leading to a systemic and long-lasting antitumor immune response. Efficacy was shown in murine tumor models and, more importantly, in veterinary clinical trials treating canine cancer patients (companion animals in this and subsequent veterinary clinical studies are pets that spontaneously developed cancer,

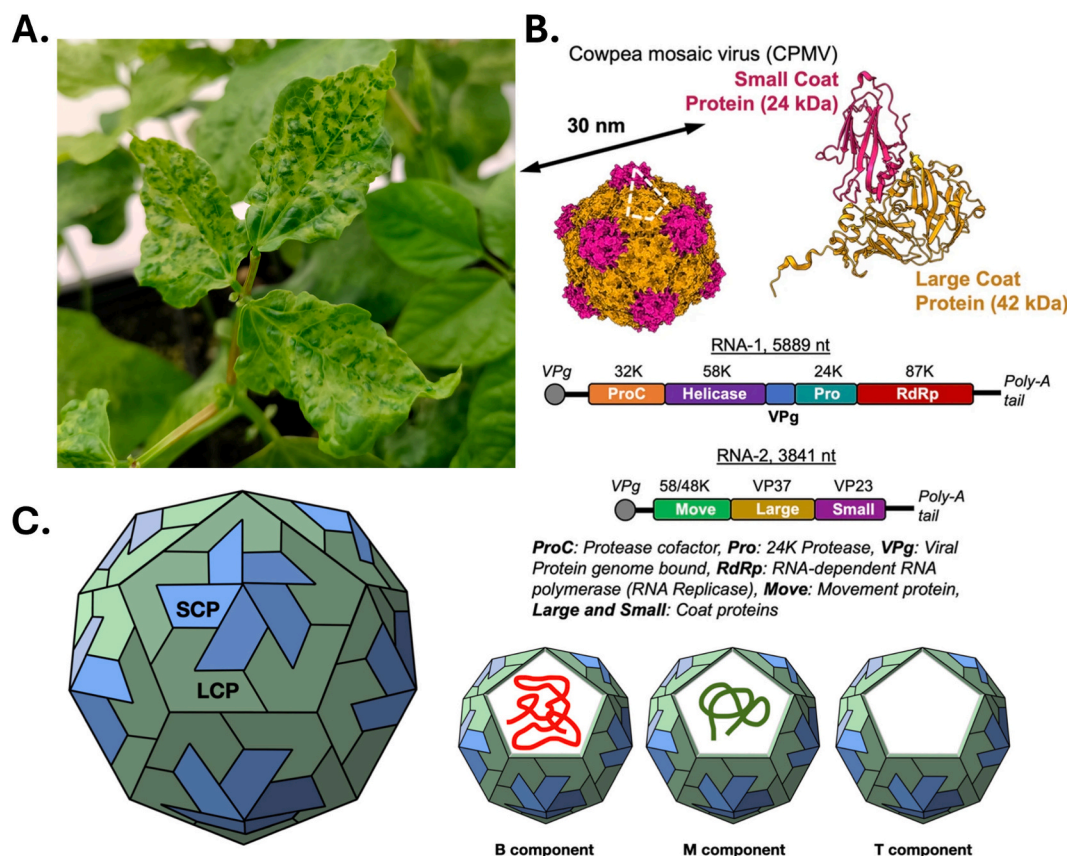


Fig. 1. CPMV structure (based on [55,59]). A) Cowpea plant infected with CPMV. B) Cowpea mosaic virus (CPMV) has a bipartite ssRNA genome. RNA-1 and RNA-2 are separately encapsulated into 30-nm icosahedral capsids assembled from small (S) and large (L) coat proteins. CPMV RNA-1 encodes the 32K protease cofactor, 58K helicase, VPg, 24K protease, and 87K RNA-dependent RNA polymerase. RNA-2 encodes the movement protein (MP) and the L and S coat proteins. PDB: 1NY7, CPMV structure generated with UCSF's ChimeraX. Reproduced with permission from [46], CC BY 4.0. C) The B, M and T components. The B component packages RNA-1, the M component packages RNA-2, and the T component is devoid of RNA. The S and L coat protein (SCP and LCP) are colored in blue and green. Capsid structures drawn by Justin McCaskill (UC San Diego). Adapted with permission from [52], © 2022 American Chemical Society. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

as opposed to being induced with cancer, which are further described in [section 3](#)) [44,45,49,54,76–81]. Mechanistically, intratumoral immunomodulatory CPMV overcomes immunosuppression within the TME, priming innate immune activation and the recruitment of immune cells, ultimately launching both local and systemic adaptive antitumor immunity (mediated by tumor-specific adaptive antitumor immunity). Systemic antitumor immunity mediated by intratumoral CPMV suppresses both local and distant metastases, while inducing immune memory that prevents recurrence.

3.1. Intratumoral CPMV demonstrates efficacy against multiple solid tumors in murine models

The antitumor response induced by intratumoral CPMV immunotherapy is tissue and tumor agnostic; models demonstrate potent efficacy against a range of tumor types with a high degree of reproducibility ([Table 1](#), [section 6](#)). Potency was observed in C57BL/6 and BALB/c mice and in male and female mice [82,83]. C57BL/6 mice are known for a Th1-biased immune response favoring cell-mediated immunity while BALB/c mice favor a Th2-biased humoral response, which demonstrates the broader and agnostic efficacy of CPMV [84,85]. Efficacy in both male and female mice is also encouraging, as human sex-based differences in immune response due to genetics and hormones have been described and could be a concern when developing a novel immunotherapy [86–88].

CPMV immunotherapy has been extensively studied using an intradermal B16F10 melanoma model, achieving systemic and durable efficacy as indicated by the abscopal effect and protection from re-challenge ([section 2.3](#) and [2.4](#)) [52,54,77,78,90]. Similarly, intratumoral CPMV immunotherapy shows efficacy in disseminated intraperitoneal tumors

based on the analysis of ID8-Defb29/Vegf-A tumors as a model of ovarian cancer [54,77,79,92]. Here, CPMV was dosed intraperitoneally achieving control and reduction of tumor growth rate resulting in increased survival. The potent efficacy of CPMV was also demonstrated in an intraperitoneal CT26 colon tumor mouse model [54,77]. Excitingly, intracranial CPMV injections resulted in tumor reduction and complete regression in an intracranial GL261 glioma mouse model, a common model for the hard-to-treat glioblastoma [93]. CPMV efficacy is not limited to the injected tumor – rather intratumoral CPMV modulates the TME resulting in immune-mediated tumor cell killing and antigen processing, ultimately launching systemic antitumor immunity, with reduced tumor growth also observed in distant (non-injected) tumors in multiple cancer models ([section 3.3](#)) [45]. Beyond mouse models, intratumoral CPMV immunotherapy improved outcomes for canine cancer patients with mammary cancer, melanoma, and sarcoma ([section 3](#)); systemic efficacy was also observed in these canine cancer trials [49,80]. Efficacy in multiple disease states and immune profiles is encouraging for the development of a broadly applicable treatment.

3.2. CPMV immunotherapy induces immunological memory to prevent cancer recurrence

Intratumoral CPMV immunotherapy has been shown to induce long-lasting antitumor efficacy and immunological memory to prevent cancer recurrence after rechallenge in tumor mouse models ([Fig. 2](#)). After an initial successful treatment of B16F10 melanoma tumors using CPMV particles, surviving mice underwent a secondary tumor re-challenge after a period of 28 days.

These mice exhibited resistance to re-challenge, with the majority failing to develop new tumors, indicating that CPMV can induce durable

Table 1

Relevant works related to pharmacology, pharmacodynamics, and toxicology.

Study Title	Design	Findings	Route	Ref.
Pharmacology				
Efficacy of IT CPMV in mouse solid tumor models	Two-tumor models (B16F10, 4 T1, CT26, MC38). IT CPMV, 100 µg × 2 weekly doses.	Efficacy in injected & distant tumors (B16F10, MC38, CT26); variable in 4 T1.	IT	[45]
IT CPMV + Cyclophosphamide in 4 T1 breast cancer	BALB/c mice with 4 T1 tumors. IT CPMV, 100 µg weekly × 3 + IP cyclophosphamide.	Reduced tumor burden (abscopal effect in 2-tumor model), prolonged survival, suppressed lung metastases.	IT	[48]
CPMV + checkpoint blockade in mouse tumors	ID8 ovarian (IP CPMV, 100 µg weekly × 6 + 50 µg α), CT26 (IP CPMV, 50 µg weekly × 2 + 50 µg α), B16F10 (ID, 100 µg CPMV × 2 + 100 µg α).	Combination with α-PD-1 or OX40 agonist reduced tumor burden, extended survival, induced immune memory.	IT	[77]
IT CPMV in canine mammary tumors (neoadjuvant)	11 dogs. IT CPMV, 0.2 mg × 2 (D0, D6–9), followed by surgery (D12–17).	Tumor regression (5.8–63%); no recurrence or metastasis.	IT	[81]
IT CPMV in canine oral melanoma & STS	9 dogs. IT CPMV 0.5–2.5 mg weekly × 4.	Stable disease/remission.	IT	[89]
IT CPMV + anti-PD-1 in canine mammary tumors	6 dogs. IT CPMV, 0.2–0.4 mg + 0.125–1 mg α-PD-1 weekly × 4–11.	Tumor shrinkage in injected & distant tumors; regression of lung metastases (abscopal effect).	IT	[50]
Pharmacodynamics				
Dose escalation in ovarian tumor model	ID8 ovarian tumors. IP CPMV 30, 100, or 500 µg weekly × 4.	Delayed tumor progression; all doses prolonged survival; 500 µg nearly doubled survival.	IP	[79]
Biodistribution (IT)	B16F10 tumors. IT CPMV, 100 µg weekly × 3.	Majority retained in tumor; clearance via liver, spleen, kidneys; no shedding.	IT	[90]
Toxicology				
Toxicology (IP, mice)	Healthy mice. IP CPMV, 100 µg single or weekly × 6.	Normal histology in all CPMV+ organs (liver, spleen, kidney, lung, stomach).	IP	[47]
Toxicology (SC/IV, rats)	Female Sprague Dawley rats. SC or IV ~20 mg/kg weekly × 3.	Mild immune activation (↑WBC, neutrophils, monocytes); no MTD/NOAEL defined.	SC/IV	[91]
IT CPMV in canine mammary tumors	11 dogs. IT CPMV, 0.2 mg × 2 (D0, D6–9) prior to surgery.	No blood/chemistry abnormalities; transient neutrophilia & cytokine shifts; no hypersensitivity.	IT	[81]
IT CPMV in canine melanoma & STS	9 dogs. IT CPMV, 0.5–2.5 mg weekly × 4.	Well tolerated; no systemic toxicity; 1 unrelated renal failure (chemo/RT).	IT	[89]
IT CPMV + anti-PD-1 in canine mammary tumors	6 dogs. IT CPMV, 0.2–0.4 mg + 0.125–1 mg α-PD-1 weekly × 4–11.	Safe and well tolerated; no irAEs; mild reversible blood changes.	IT	[50]
NCL Cascade Assays (ex vivo human blood)	CPMV tested <i>in vitro/ex vivo</i> across NCL safety panels.	No hemolysis, thrombosis, complement activation, or leukocyte dysfunction.	ex vivo	[46]

IP = Intraperitoneal, IT = Intratumoral, IV = Intravenous, SC = Subcutaneous, α = antibody.

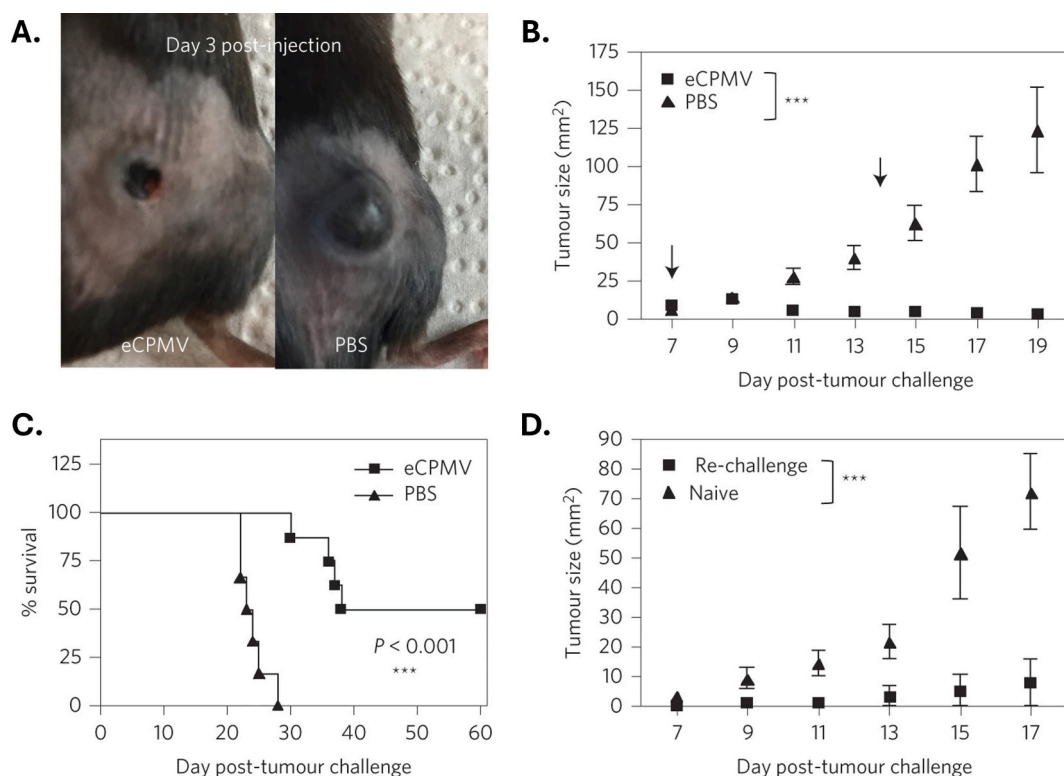


Fig. 2. CPMV treatment generates immune memory. A-B) Mice bearing intradermal flank B16F10 melanomas directly injected with eCPMV showed remarkable arrest of tumor progression (eCPMV = empty cowpea mosaic virus; arrows = treatment days). C) 50% of eCPMV-treated mice experienced complete elimination of tumors. D) Mice cured of primary tumors by eCPMV treatment and re-challenged on the opposite flank 4 weeks later failed to develop new tumors. Reproduced with permission from [44], © 2016 Macmillan Publishers Limited.

protective immunity (Fig. 2d) [44]. B16F10 tumors treated with CPMV see the significant recruitment of CD4⁺ and CD8⁺ cells as well as effector memory cells, which play a key role in establishing immune memory to protect mice from re-challenge [44,83]. Systemic antitumor immunity has been reproducibly observed in multiple tumor mouse models and canine cancer patients. For example, in a treatment study, mice with localized A20 lymphoma were treated with CPMV; the treated and

surviving mice did not show signs of tumorigenesis upon re-challenge 10 weeks later [82]. Another study, using intraperitoneal ID8 ovarian tumors, found that CPMV improved effector and memory CD4⁺ and CD8⁺ T cell responses, with no tumorigenesis upon re-challenge (99 days after tumor regression) [79]. Taken together, these studies highlight the long-lasting antitumor immune response induced by CPMV treatment. Although not a perfect model for cancer relapse, these results

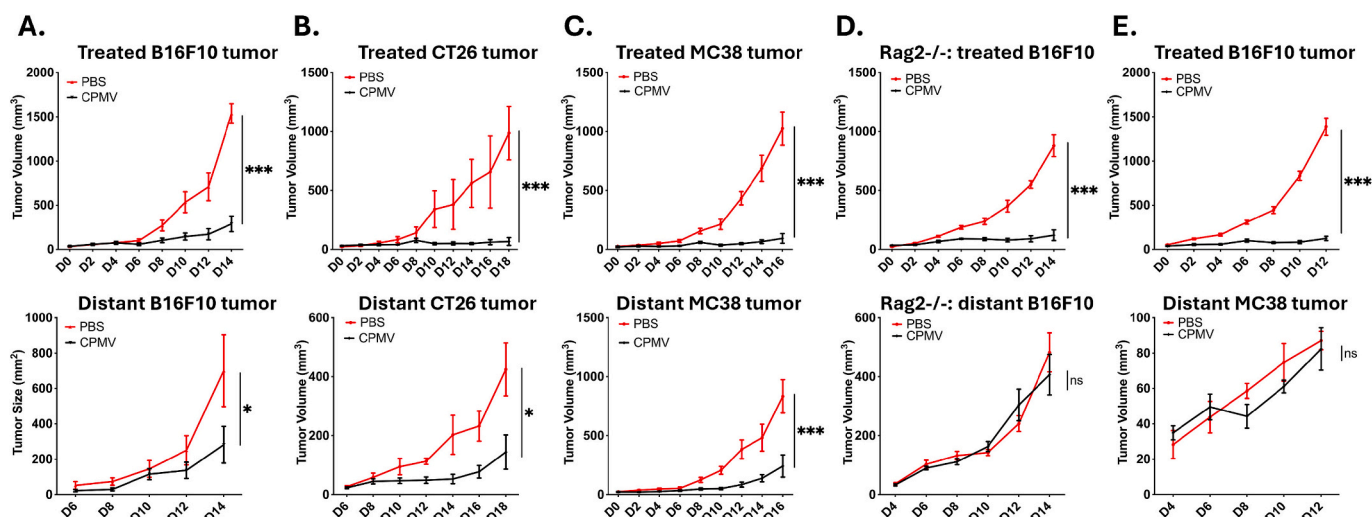


Fig. 3. Abscopal effect following CPMV treatment. A-C) Tumor growth curves for both treated and distant tumors in three different tumor models: B16F10 melanoma, CT26 colon carcinoma, and MC38 colon adenocarcinoma. All cancer cells were inoculated intradermally in C57BL/6 J mice. D) Tumor growth curves for CPMV-treated and PBS-treated B16F10 in Rag2^{-/-} knockout mice, both treated and distant. B16F10 cells were inoculated intradermally. E) Unmatched two-tumor-bearing mice with B16F10 (treated) and MC38 (distant) tumors. Cancer cells were inoculated intradermally in C57BL/6 J mice. Reproduced from [45] with permission from BMJ Publishing Group Ltd.

are promising for the prevention of cancer recurrence.

3.3. CPMV immunotherapy induces the abscopal effect

CPMV's effect is not only seen in the injected tumor – the CPMV-stimulated systemic antitumor immunity controls distant tumors as well. In one study, mice groups were inoculated with B16F10 melanoma, CT26 colon cancer, MC38 colon cancer on both flanks, then received CPMV treatment in only one tumor while the distant tumor was left untreated [45]. According to the mechanism of action (section 5) and priming of adaptive antitumor immunity, the distant tumor's growth rate was also reduced [45]. This result is tumor-specific and highly reproducible in the different tumor types tested (Fig. 3a-c). Mechanistically, CPMV immunotherapy recruits and activates innate immune cells such as M1 macrophages, N1 neutrophils, dendritic cells (DCs) and natural killer (NK) cells, thereby modulating the TME and initiating a cascade of tumor cell killing mechanisms [44]. This results in the presentation of both tumor-associated antigens and neoantigens. Additionally, functionally active adaptive antitumor immunity is generated via tumor antigen-specific CD4⁺ and CD8⁺ effector and memory T cells [65,76]. Therefore, the CPMV-stimulated immune-mediated antitumor

response is not only localized against the injected tumor, but also against non-injected (untreated) distant sites, a phenomenon known as the abscopal effect. When *Rag2* knockout mice, which lack B and T cells, were inoculated with B16F10 melanoma and treated with CPMV, the abscopal efficacy of CPMV was completely abolished in the untreated tumor while retaining the typical response of tumor reduction in the locally treated tumor (Fig. 3d) [45]. CD8⁺ but not CD4⁺ cells are required for CPMV to elicit systemic efficacy, as shown in T cell depletion studies [77]. This is consistent with the tumor antigen-specific nature of CPMV systemic efficacy: CPMV does not elicit abscopal effects against an unmatched syngeneic tumor (Fig. 3e) [45].

4. Efficacy of intratumoral immunotherapy using CPMV in canine cancer patients

Translation of the CPMV immunotherapy approach to canine cancer patients with melanoma, breast cancer, or sarcoma in veterinary clinical trials has demonstrated potent antitumor efficacy [80,94,95]. In early studies, CPMV was used in combination with therapeutic hyperthermia and/or hypofractionated radiation, and efficacy (as well as the abscopal effect) was demonstrated in spontaneous canine tumors (Fig. 4) [80]. As

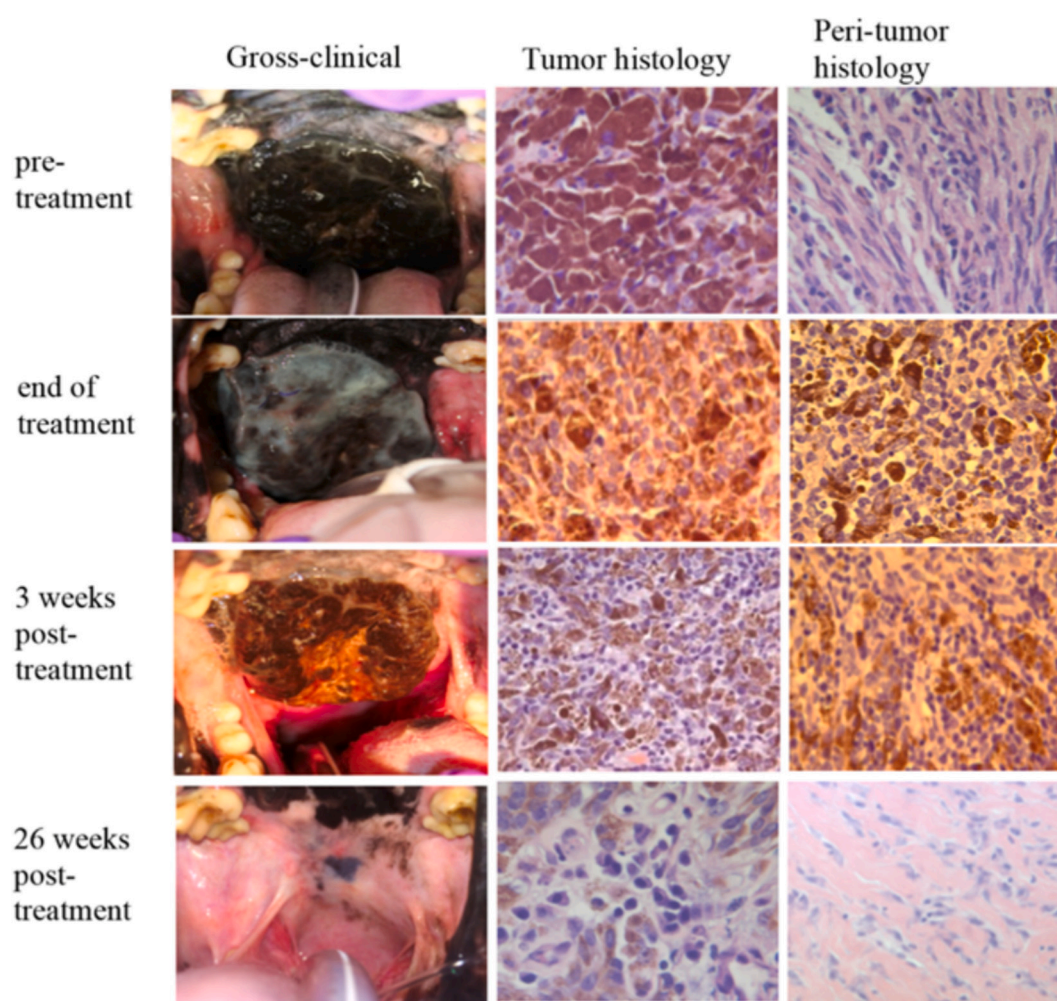


Fig. 4. Efficacy of CPMV plus radiation in a canine cancer patient. The images are from a 12-year-old female beagle patient after treatment with CPMV and radiation. In addition to complete tumor resolution that was durable at 20 months, there was a dramatic inflammatory/immune response in the weeks following treatment. The images compare the gross clinical response and the level of immune cell infiltration in the tumor and peri-tumor tissue at selected time points. The response is largely mononuclear cell (macrophage/lymphocyte, small blue cells with high nucleus/cytoplasm ratio) but pockets of neutrophils are also seen in some areas. As shown in the final two histology photomicrographs, there is no residual tumor but some ongoing active fibroplasia, although most of the response at this point is mature fibrosis. Reprinted with permission from [80], © 2018 American Chemical Society. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

preliminary studies, the treatment groups had small sample sizes and no CPMV monotherapy group for comparison. However, the CPMV combination treatment showed improvement beyond the expected outcome and remission, and patients experienced prolonged survival. These trials were therefore not only an opportunity to de-risk the technology by testing in outbred animals with spontaneous tumors, but importantly also a chance to treat and save someone's beloved pet.

Intratumoral CPMV was also provided to canine mammary cancer (CMC) patients as a neoadjuvant prior to surgery followed by adjuvant chemotherapy [81]. These studies demonstrated potent antitumor efficacy, reflected by the shrinkage of injected and non-injected distant tumors, thus indicting the priming of systemic antitumor immunity (Fig. 5A–D) [81]. Dogs receiving CPMV neoadjuvant therapy showed increased survival compared to standard of care and recurrence was not

observed, highlighting the durable efficacy induced by intratumoral CPMV immunotherapy [49,81]. Therapeutic success was also evident from histology of biopsies showing the infiltration of neutrophils, T and B cells into CMC tumors (Fig. 5E).

The efficacy of CPMV combined with a canine anti-PD-1 antibody was also shown, specifically confirming reduction in tumor size in both injected and non-injected tumors and, importantly, showing efficacy against lung metastases, which indicates a systemic immune response and abscopal effect [50]. Potent efficacy was also demonstrated using intratumoral CPMV to treat canine patients with oral melanoma or soft-tissue sarcoma (STS) [89], achieving stable disease and partial remission. CPMV increased immune cell activity in both blood and tissue samples. The number of circulating CD4⁺ and CD8⁺ T cells increased, and both the circulating cells and those found in tumor biopsies

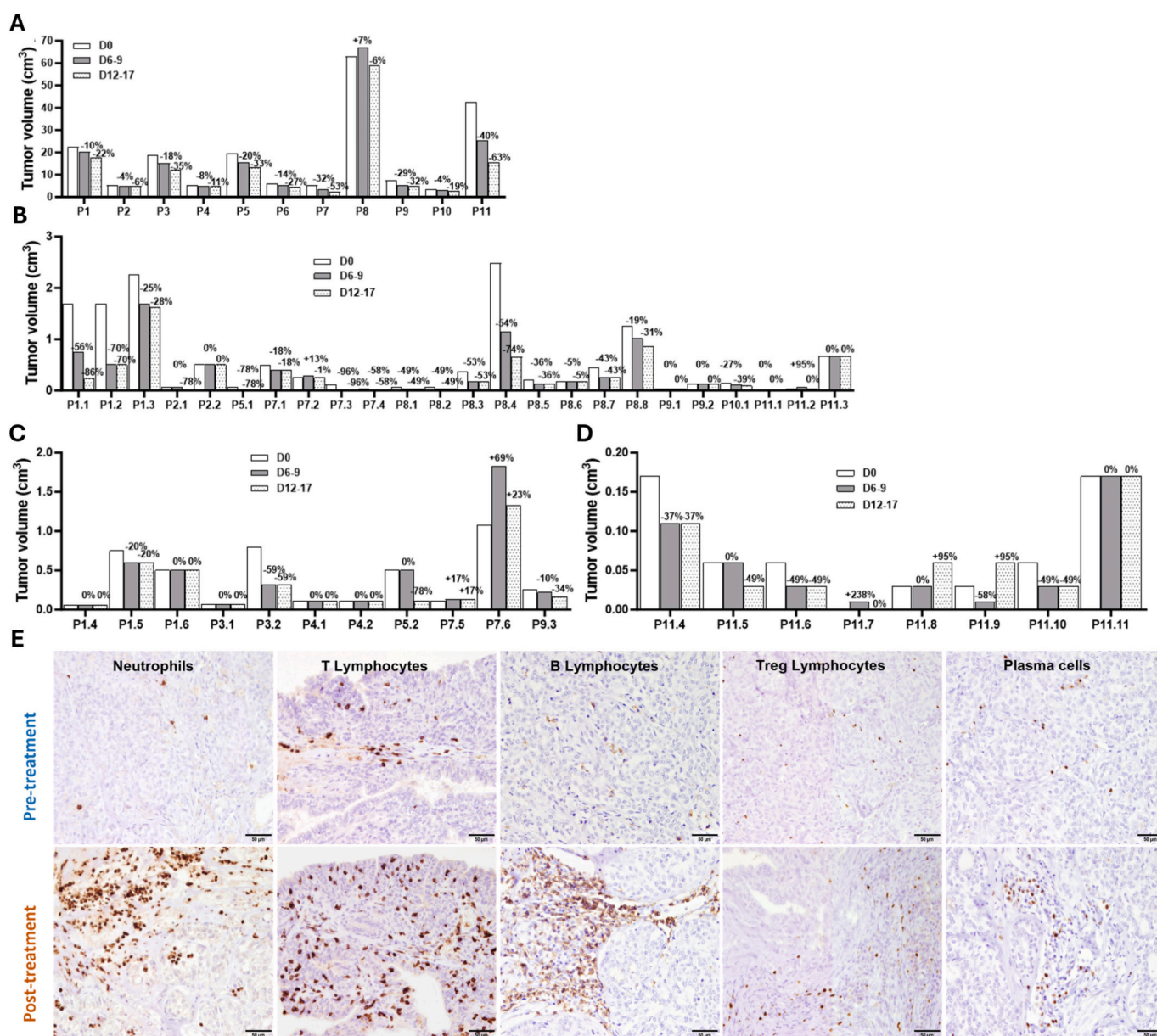


Fig. 5. Tumor volume changes and immune cell infiltration in canine cancer patients treated with CPMV. Canine mammary cancer (CMC) patients received the first eCPMV injection in the target tumor at D0 and the second injection at D6–9, followed by surgery at D12–17. (A–D) Tumor reduction was observed in the injected tumor (A) and noninjected tumors in both ipsilateral (B) and contralateral (C, D) mammary chains. The percentage of tumor growth inhibition (%TGI) relative to D0 is indicated at different times. E) Representative immunostaining of pre-treatment (D0) and post-treatment (D12–17) canine mammary tumor tissues. When compared with pre-treatment tumor biopsies, post-treatment tumor biopsies have significant increases in intratumor infiltration with neutrophils (MPO⁺), T cells (CD3⁺), B cells (CD20⁺), T regulatory cells (FoxP3⁺), and plasma cells (MUM1⁺). Reproduced with permission from [81], CC BY 4.0.

expressed more IFN- γ . The greater abundance of circulating CD4⁺ and CD8⁺ T cells correlated with STS tumor regression.

Studies involving canine cancer patients are an important step toward human clinical trials of CPMV. Given the variability of cancer type, tumor location, disease progression, and patient history, which differs greatly from canine to canine, CPMV can be better measured in terms of robustness in a manner that cannot be replicated in highly controlled laboratory mouse models. Taken together, these studies highlight the efficacy and safety of CPMV in canine cancer patients. The pharmacology and toxicology findings from canine cancer trials are described in section 7.

5. Mechanism of action

CPMV has remarkable potency compared to other plant viruses or small-molecule adjuvants (TLR, STING agonists) in terms of immune activation mechanisms and antitumor efficacy [44,52,54,96], reflecting its unique mechanism of action (Fig. 6). CPMV is taken up by myeloid cells such as macrophages and neutrophils. The nucleoprotein assembly activates TLR-2 and TLR-4 (stimulated by the protein component) and TLR-7 (stimulated by the RNA component) [78,97]. Activation of innate immune cells within the tumor leads to the release of cytokines and chemokines, primarily those related to interferon signaling [98], recruiting and activating additional innate immune cells, including M1-type macrophages, N1-type neutrophils, NK cells, and dendritic cells. These innate immune cells induce tumor cell killing and lead to the processing and presentation of tumor-associated and neo-antigens, priming CD4⁺ and CD8⁺ T cells that lead to systemic antitumor efficacy [44,45,79,99]. CPMV's potency reflects its status as a multi-TLR agonist, thus activating innate immune cells through multiple pathways. The nanoparticle's multidentate structure leads to tumor retention and avidity effects, but immunomodulation is also achieved through unique and potent T-helper epitopes priming a Th1-balanced response and the robust recruitment and activation of myeloid effector cells.

5.1. CPMV is a multi-TLR agonist

Using mouse knockout models, we showed that CPMV signals through MyD88 and acts as a TLR agonist targeting TLR-2, TLR-4 and TLR-7 (Fig. 7) [78]. Briefly, CPMV's repetitive, multivalent protein

assemblies are recognized as pathogen-associated molecular patterns (PAMPs) that activate the innate immune system. CPMV's capsid proteins are recognized by TLR2 and TLR4, which activate the innate immune response as well as bridging the innate and adaptive immune responses [100]. RNA-containing native CPMV particles additionally signal through TLR-7, which boosts the production of type I interferon [78,97] (Fig. 7d). This is one of the reasons CPMV is more potent than RNA-free CPMV (also known as eCPMV - empty CPMV) (Fig. 7a).

5.2. Beyond TLR agonism – multifaceted immunomodulation by CPMV

It is important to note that CPMV is more than a triple-TLR agonist. Immunomodulation is also achieved through unique and potent T-helper epitopes, priming a Th1-balanced response [101]. This Th1 response results in the production of pro-inflammatory cytokines such as type I interferons (IFNs), which can then go on to activate other immune cells [98]. The nanoparticle structure of CPMV ensures prolonged tumor retention, and its multivalency leads to avidity effects that boost overall efficacy, an advantage to small-molecule PRR agonists (STING and TLR) that act through a single pathway, thus limiting efficacy [90]. Although these small-molecule agents have shown promise in preclinical studies, clinical translation is also hampered by poor pharmacokinetic and physiochemical properties, and rapid wash-out effects after intratumoral administration. However, CPMV shows good tumor penetration and extended tumor retention (~ 5 days) [90]. The plant virus does not target or lyse cancer cells, but recruits and activates myeloid effector cells such as macrophages, neutrophils, DCs and NK cells (Fig. 8a). These not only lyse cancer cells directly, but also present antigens to the adaptive arm. Therefore, whereas CPMV acts on innate cells, it primes durable adaptive immunity (CD4⁺ and CD8⁺ effector and memory T cells) (Fig. 8b) [44,45,79,99]. Intratumoral CPMV immunotherapy leads to the robust recruitment and activation of M1 macrophages and tumor-infiltrating neutrophils (TINs) or N1 neutrophils [44,79,93,97]. M1 macrophages and N1 neutrophils, both strongly associated with a pro-inflammatory response and tumor resistance, are activated by IFN- γ and TNF- α [102,103]. A recent publication highlighted the role of neutrophils in successful immunotherapy and showed that therapy-elicited neutrophil responses in patients are associated with better outcomes [104].

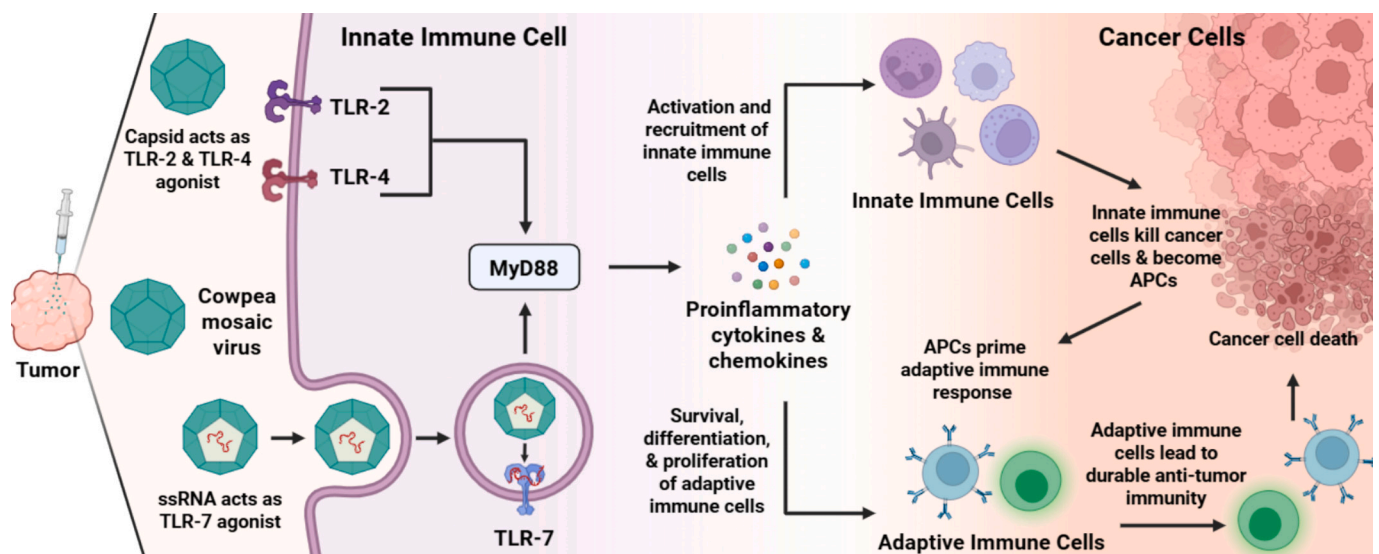


Fig. 6. Mechanism of action of intratumoral CPMV. CPMV activates the innate immune system through TLRs 2, 4 and 7. CPMV activates and recruits innate immune cells through key cytokines (type I interferons and inflammatory cytokines such as TNF- α , IL-1 α , IL-1 β , and CXCL10). Innate immune cells (M1 macrophages, N1 neutrophils, NK cells) kill tumor cells, releasing tumor-associated antigens and neo-antigens. Innate immune cells become APCs, which help generate an adaptive immune response. Potent and durable antitumor immunity is mediated by CD8⁺ T cells and memory cells. Figure prepared using BioRender.com.

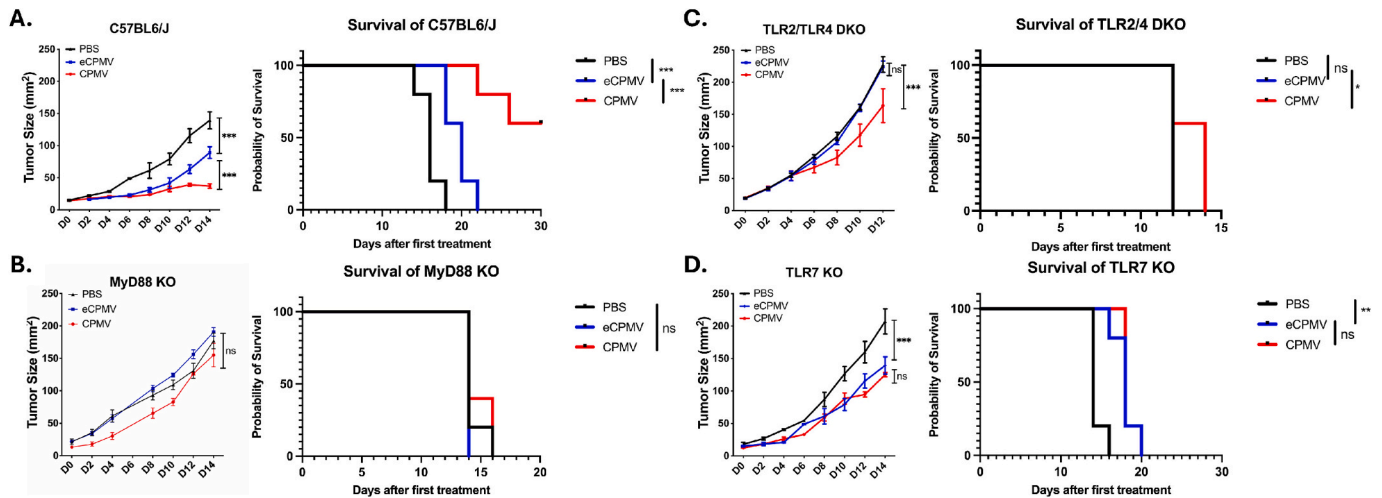


Fig. 7. CPMV signaling pathway involves TLRs 2, 4 and 7. Mice were inoculated intradermally with B16F10 melanoma cells before treatment with PBS, eCPMV (empty CPMV) or CPMV. A) Baseline tumor growth and survival in a B16F10 melanoma model using C57BL/6/J mice. B) Tumor growth and survival using MyD88^{-/-} mice, highlighting MyD88 requirement for CPMV and eCPMV-elicited antitumor efficacy. C) Tumor growth and survival using TLR2/4^{-/-} dual knockout mice. Knocking out TLR2 or TLR4 alone was not sufficient to fully eliminate antitumor efficacy. D) Tumor growth and survival using TLR-7 knockout mice, which eliminated the antitumor efficacy of eCPMV and CPMV (KO = knockout; DKO = dual knockout; D0-D14 = days after initial treatment; eCPMV = empty CPMV lacking RNA). Reproduced from [78] © 2021, with permission from Elsevier.

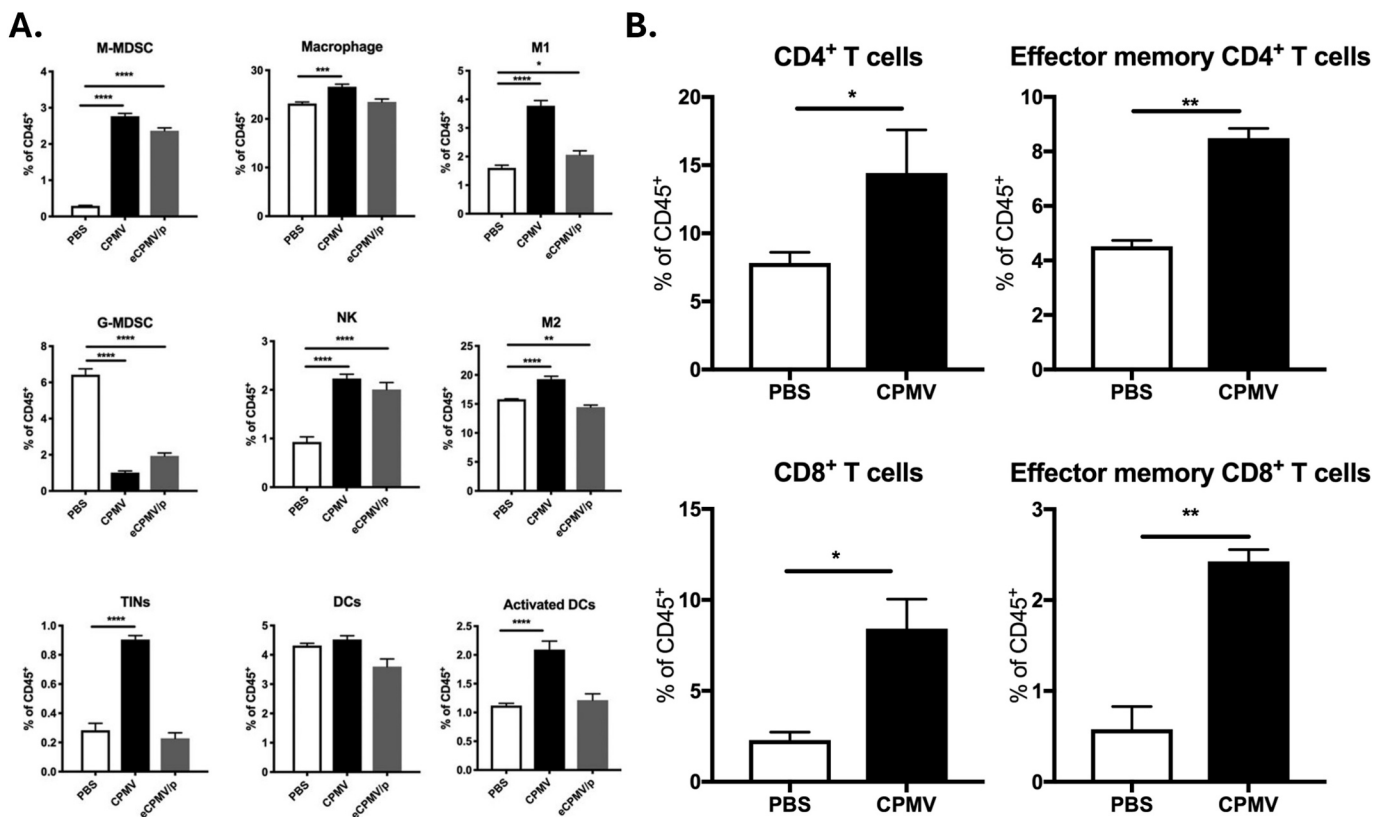


Fig. 8. CPMV recruits and activates key innate and adaptive immune cells. A) Peritoneal cavity lavage cells of tumor-bearing mice were collected 24 h after intraperitoneal treatment with CPMV and eCPMV/p (plant-derived eCPMV) for analysis by flow cytometry. Various immune cells were measured: M-MDSC = monocytic myeloid-derived suppressive cell; M1 = type-1 tumor-associated macrophage; G-MDSC = granulocytic myeloid-derived suppressive cell; NK = natural killer cell; M2 = type-2 tumor-associated macrophage; TiN = tumor-infiltrating neutrophil; DC = dendritic cell. B) Tumor-bearing mice were dosed five times with CPMV or PBS. Six hours after the final treatment, peritoneal lavage fluids were collected and analyzed by flow cytometry. The percentage of CD4⁺ T cells, CD44⁺ CD62L⁻ CD4⁺ effector memory T cells, CD8⁺ T cells, CD44⁺ CD62L⁻ CD8⁺ effector memory T cells, and infiltration of CD45⁺ cells in the peritoneal washes is shown. Reproduced with permission from A) [97], © 2019 American Society for Microbiology; B) [79], © 2019 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

5.3. Human PBMC response to CPMV *in vitro*

Studies with human PBMCs indicate that these cells recognize CPMV and respond by producing cytokines similarly to mouse immune cells [78]. Importantly, large increases in the abundance of cytokines like IL-6, commonly observed during a cytokine storm, were not detected following CPMV treatment of human immune cells. Instead, increases in type I, II and III interferons, known for their potent anti-proliferative and immunomodulatory activity, were detected [105,106]. These interferons provide direct, specific, rapid, and sustained antitumor activity while minimizing inflammation-mediated damage to surrounding healthy tissues. They also display indirect antitumor effects through the inhibition of neoangiogenesis. In collaboration with the Nanotechnology Characterization Laboratory (NCL), we recently completed multiplex ELISA and single-cell sequencing analysis of human PBMCs exposed to CPMV (Fig. 9) [53]. The key conclusions were: functional patterns of gene expression are consistent between all donors; activation of all cell populations was observed, although monocytes and DCs responded most robustly (consistent with CPMV's known mechanism of action); and pathway analysis showed that CPMV induced acute inflammation, antigen presentation, lymphocyte homing, and adaptive and trained immunity. All data were in agreement with functional assays conducted by NCL in that many genes and pathways demonstrated the activation of type I interferons, consistent with the activation of myeloid

and lymphoid cells. The data also indicated the upregulation of tumor-suppressor genes and the downregulation of proto-oncogenes, metabolic changes in the TME, and changes in expression of long non-coding RNAs. More analysis is required because this study involved healthy blood cells; plans have been established to repeat these assays on patient-derived tumors and blood samples.

6. Combination therapies

CPMV is useful both as a solo treatment and as part of a treatment regimen in murine models. CPMV, as part of a combination therapy with radiation, delayed tumor growth and showed an increase in tumor-infiltrating lymphocytes in a model of ovarian cancer [107]. Another study showed that CPMV enhanced the effects of hypofractionated radiation in a model of melanoma [108]. CPMV has also been studied in combination with current chemotherapy medications. A combination of CPMV and cyclophosphamide demonstrated an increase in survival, attenuated primary tumor growth, suppressed distant tumor growth, and reduced incidence of lung metastases in a breast cancer model [48]. Co-administration with the chemotherapeutic agent oxaliplatin improved efficacy compared to the treatments alone, with extended survival and reduced tumor burden in models of ovarian cancer and melanoma [109]. Recently, CPMV was combined with IL-12 plasmid electroporation therapy (IL-12 plasmid electroporation is being assessed

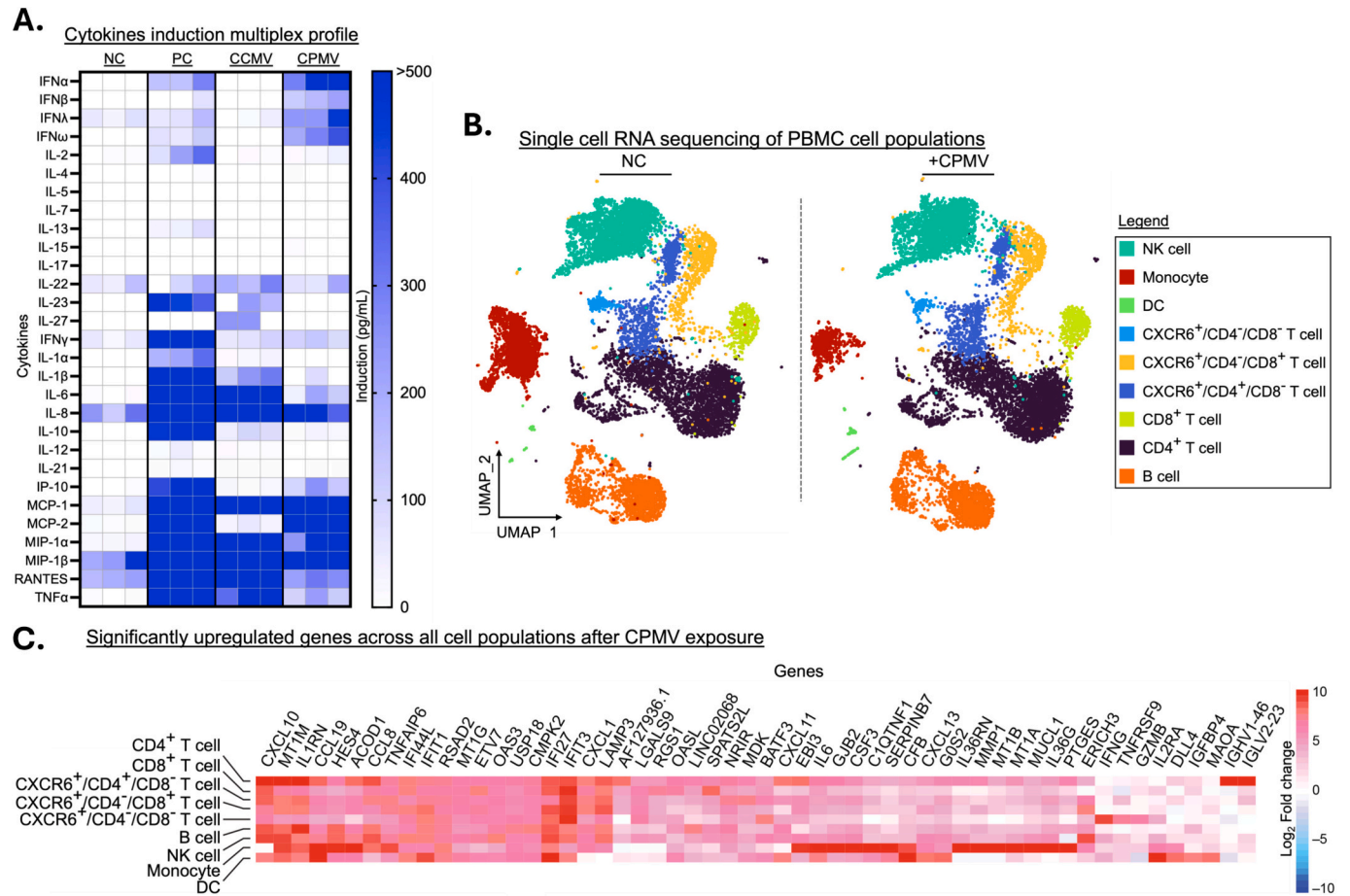


Fig. 9. Human PBMCs respond to CPMV. A) Multiplex cytokine assay from supernatant of human PBMCs grown in culture, showing the differential cytokine profile comparing CPMV, CCMV (control: does not induce antitumor immunity), negative control (NC: PBS), and positive control (PC: ODN2216, LPS, and PHA-M). CPMV is more potent at inducing type I and type III interferons and IL-2 (pDC and T cell activation). B) UMAP plots of PBMC single-cell RNA-Seq after CPMV exposure ($n = 3$). NK = natural killer cell; DC = dendritic cell; CXCR6⁺/CD4⁻/CD8⁻ probably represents NK T cells; CXCR6⁺/CD4⁺/CD8⁻ = memory cytotoxic T cells; CXCR6⁺/CD4⁺/CD8⁺ = memory Th cells; CD8⁺ T cells = cytotoxic T cells; CD4⁺ T cells = Th cells. C) Heat map showing significantly upregulated genes across all cell populations in human PBMCs. Reproduced with permission from [53], this article was published in Cell Biomaterials, vol 1, Omole et al., Comparative analyses for plant virus-based cancer immunotherapy drug development, page 100095, © Elsevier (2025).

in phase II clinical trials [110]). The CPMV plus IL-12 plasmid electroporation regimen led to clearance of local tumors and suppression of non-injected, distant tumors in mouse models of melanoma and colorectal cancer. Finally, when CPMV is co-administered with checkpoint blocking antibodies, such as anti-PD-1 or agonistic OX40-specific antibodies, it also improved the efficacy of systemic immunotherapy by expanding the pool of antitumor T cells that respond to the checkpoint blockade and establishing tumor-specific immunological memory (Fig. 10) [45,77]. In mouse tumor models, CPMV immunotherapy has been shown to upregulate immune checkpoints such as PD-1, OX40, and Lag-3, helping to overcome resistance to checkpoint therapy [45,77,111].

7. Biodistribution, safety and dosing

A number of studies have been conducted which, together, describe the safety profile of CPMV (Table 1). This includes dose escalation, biodistribution, and various measures of toxicity in murine, canine, and human samples.

7.1. Biodistribution

A 2007 study reported on the “biodistribution, toxicology and pathology of CPMV in mice after intravenous (IV) administration” [112]. This paper showed that intravenous CPMV particles are cleared rapidly from the blood plasma compartment with a half-life of 4–7 min. By 30 min, CPMV is distributed largely to the liver and, to a lesser extent, the spleen. No toxicity was seen at doses of up to 100 mg/kg body weight and hematology was essentially normal. The authors also studied the histopathology of the spleen and other relevant tissues following intravenous administration of CPMV. In the spleen, they found cellular infiltration attributed to elevated B cells on the first day after administration. Evaluation of various tissues did not show any signs of tissue degeneration or necrosis. CPMV biodistribution has also been evaluated after repeated intratumoral treatment of tumor-bearing (B16F10 melanoma) C57BL/6 mice [90]. Minimal leaching from the tumor was observed, consistent with the favorable tumor retention properties of CPMV, but any leached particles were cleared through liver and spleen, which is consistent with the earlier reported study (Fig. 11a) [112]. These data indicated that CPMV is cleared *via* the reticuloendothelial system (RES) followed by biliary excretion. Longitudinal western blot tests did not show any evidence of CPMV coat protein in urine or stool. RT-PCR did indicate RNA shedding in some stool and urine samples but there was no evidence that the RNA was infectious. This work was recently repeated after intraperitoneal dosing using a mouse model of intraperitoneal disseminated ovarian cancer (ID8-Defb29/Vegf-A).

CPMV was administered six times weekly starting a week after tumor inoculation. CPMV delayed tumor progression compared to the PBS group as observed by the lower body weight and circumference [47]. After treatment, organs were harvested and analyzed for the presence of CPMV RNA and coat protein, which was detected in multiple organs to varying degrees, but no toxicity was observed after hematoxylin and eosin staining (Fig. 11b & c) [47].

7.2. Toxicology and pharmacology

7.2.1. *In vivo* toxicology

A toxicology study was completed in collaboration with the Nanotechnology Characterization Laboratory (NCL) where CPMV was found to be non-toxic *in vivo*. Repeat-dose toxicity was assessed in rats using a fixed dose (~20 mg/kg) and three weekly subcutaneous injections to mimic the intended route vs intravenous injections, mimicking the worst-case scenario of leaching from tumor and systemic exposure. Analysis by necropsy, clinical chemistry, hematology and histopathology was carried out 24 h (main group) or 14 days post-administration (recovery group). The study concluded that CPMV treatment did not result in abnormal clinical signs; immune activation markers support the mechanism of action; other findings were mild, within historical ranges, or not biologically significant; and no maximum tolerated dose (MTD) nor no observed adverse effect level (NOAEL) was established during the repeat-dose toxicity study [91].

7.2.2. Hematologic toxicity of CPMV

We completed the NCL assay cascade (Fig. 12) [46]. Immunological and physicochemical characterization of CPMV did not identify adverse reactions and otherwise were consistent with the intended mechanism of action [46]. Hemocompatibility was assessed using human whole blood and its derivatives. CPMV was tested at concentrations up to 10-fold higher than the maximum CPMV concentration at the intended therapeutic dose. Under *in vitro* conditions, CPMV did not induce hemolysis, platelet aggregation, or complement activation. It also did not affect collagen-induced platelet aggregation or normal plasma coagulation time at all tested concentrations (Fig. 12 a-d). CPMV did not cause toxicity in immune function tests. The normal phagocytic function of myeloid cells and influenza-antigen specific proliferation of T-cells were unaffected, and chemotaxis and leukocyte procoagulant activity was not induced (Fig. 12 e-h). These data show that, even at high concentrations, CPMV does not affect erythrocytes, has a negligible risk of complement activation-related pseudo-allergy (CARPA), does not promote thrombosis, and has no effect on normal human leukocyte functions. The hematological assays used in this NCL study show good *in vitro*–*in vivo* correlation [113]. Collectively, these results indicate that the risk of

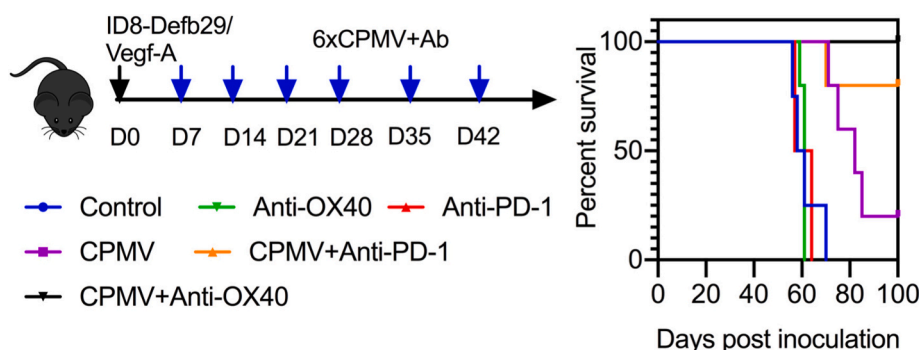


Fig. 10. CPMV shows efficacy as part of combination therapy. Synergistic effects of CPMV and PD-1 antagonist or OX40 agonist antibody therapy in a model of disseminated peritoneal ovarian cancer. C57BL/6 J mice were inoculated intraperitoneally with ID8-Defb29/Vegf-A cancer cells. Mice were given weekly intraperitoneal injections of CPMV, PD-1 antagonist or OX40 agonist solo or as part of a combination therapy. Mechanistic data show that CPMV therapy upregulates PD-1 and OX40 expression, therefore sensitizing the tumors to checkpoint therapy. Reproduced with permission from [77], © 2020 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim.

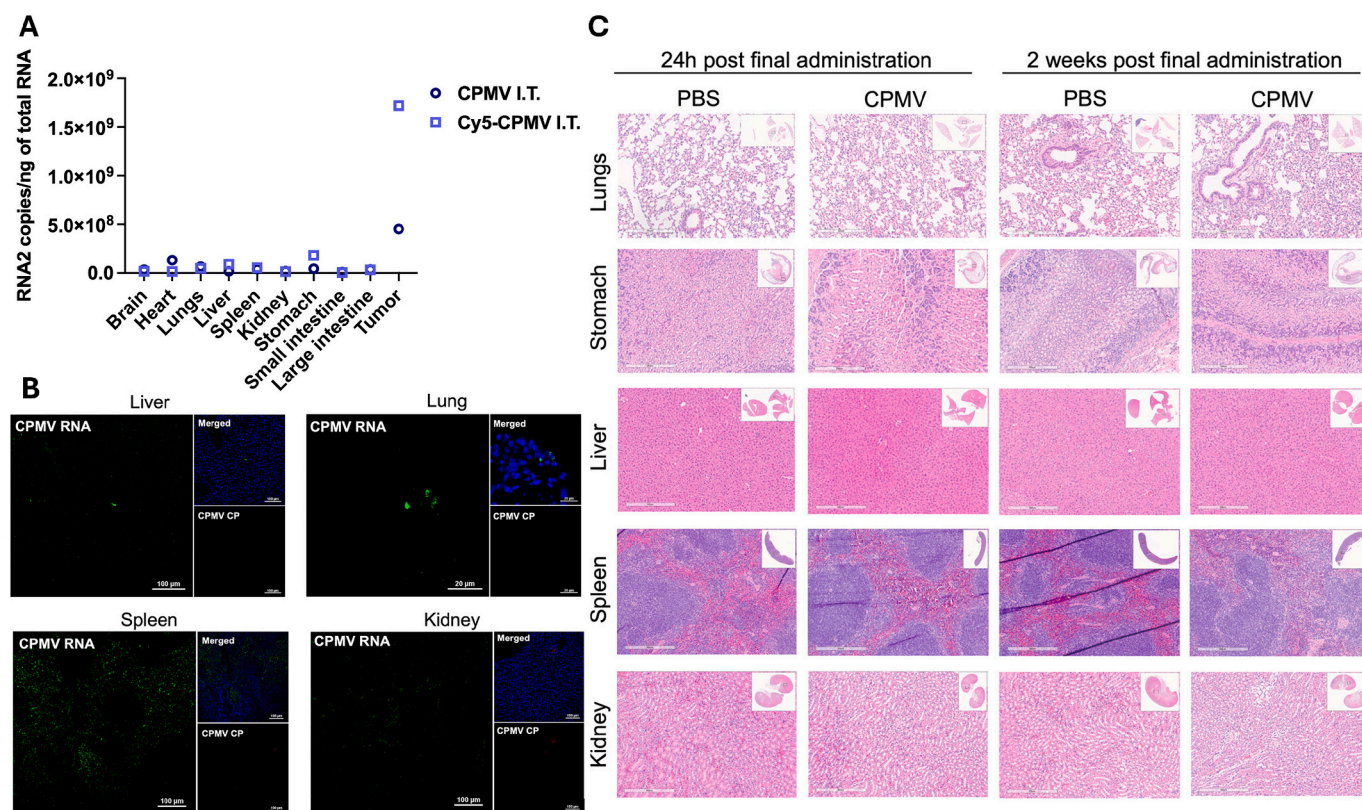


Fig. 11. A) CPMV retention in B16F10 tumors following IT administration. RT-qPCR was carried out after 24 h post 3 weekly IT administrations; CPMV is mostly retained in the tumor and cleared by the RES organs followed by biliary excretion. B) CPMV RNA (but not coat protein) persistence 2 weeks after final administration. Multiplexed detection of CPMV RNA and coat protein in tissue slices. Green: CPMV RNA, red: CPMV CP, blue: DAPI. Images taken on confocal with a 20× objective. C) Histopathology of organs treated with CPMV. H&E staining of organ sections from mice treated with CPMV and PBS 24 h and 2 weeks after final dose administration. Scale bar: 300 μ m. Fig. 11a was adapted with permission from [90], © 2022 American Chemical Society, Fig. 11b & c were adapted with permission from [47], © 2024 American Chemical Society. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

CPMV-mediated hematological toxicity *in vivo* is low.

7.2.3. Allergenicity

Antibody responses, and adverse effects. No allergic/anaphylactic reactions have been reported following repeated intratumoral injections in mouse tumor models and canine cancer patients. However, the mammalian immune system does react to the presence of CPMV, which results in cell and antibody-mediated responses [79,92]. We have not observed CPMV-specific immunoglobulin E (IgE) responses, which play an important role in type I hypersensitivity (true allergy). However, CPMV-specific IgM and IgG have been observed in mice and dogs, but these host antibodies are not neutralizing. Instead, they enhance immunity through innate immune cell Fc receptors [92]. Although the presence of CPMV-specific IgM and IgG has the potential to trigger complement activation and complement-mediated anaphylactoid reactions, this has not been observed in the many canine cancer patients treated with CPMV thus far. Complement activation by nanoparticles in dogs is greater than in humans, with a strong predictive value for human complement [114]. The lack of anaphylactoid responses in the canine studies provide additional reassurance that the risk to humans is likely to be low. Moreover, our collaborative studies with NCL showed no complement activation *in vitro* following exposure of human plasma to CPMV at concentrations relevant to the intended clinical dose (Fig. 12c). One study indicated the presence of pre-existing anti-CPMV antibodies in human plasma, with ~50% of human patient samples testing positive for CPMV antibodies in a 50-patient pool [90]. Taken together, these results suggest that the risk of anaphylactoid reactions and true allergy to CPMV adjuvant in humans is low.

In canine cancer patients (pets), hematological, biochemical, and

blood plasma analysis showed no abnormalities among the indicators used to track canine adverse events per the Veterinary Cooperative Oncology Group-Common Terminology Criteria for Adverse Events, and quality of life was not reduced in the treated dogs [81]. Regarding systemic immune changes, RNA-Seq analysis of blood from CPMV-treated canine patients revealed that CPMV induced a significant (but not pathological) increase in mature and immature blood neutrophils in a majority of the dogs. These numbers returned to basal levels by day 45 [81]. Further, decreases in IL-6, IL-7, and monocyte chemoattractant protein-1 (MCP-1) levels were observed after CPMV treatment ($p < 0.04$ for all), returning to basal plasma levels by 7 days post-treatment. These data collectively indicate that intratumoral CPMV immunotherapy does not induce large systemic release of cytokines (Fig. 13) [81], hence there is no indication of hypersensitivity reactions.

7.3. Dosing

In most mouse studies, the standard dose for tumors is 100 μ g CPMV per treatment, with the typical tumor area being ~20 mm², or 5 μ g CPMV/mm² tumor. Canine patients received doses of 200 μ g and up to 1 mg CPMV per treatment, with tumor area being anywhere from ~3–15 cm², or ~0.5 μ g CPMV/mm² tumor. Given the good safety profile of CPMV [91,112] and the difficulty with injecting large volumes intratumorally, it is possible and expected that a MTD cannot be achieved by intratumoral dosing alone. For example, 5 mg of CPMV dosed in the 3 cm² tumor of a 70 kg patient equates to a ~0.07 mg/kg dose. Dosing beyond 30 mg per tumor (~0.4 mg/kg in a 70 kg patient) is probably not feasible due to limits in injection volumes, which are one third of the size of the tumor, and upper CPMV solubility limits in PBS (50 mg/mL). This

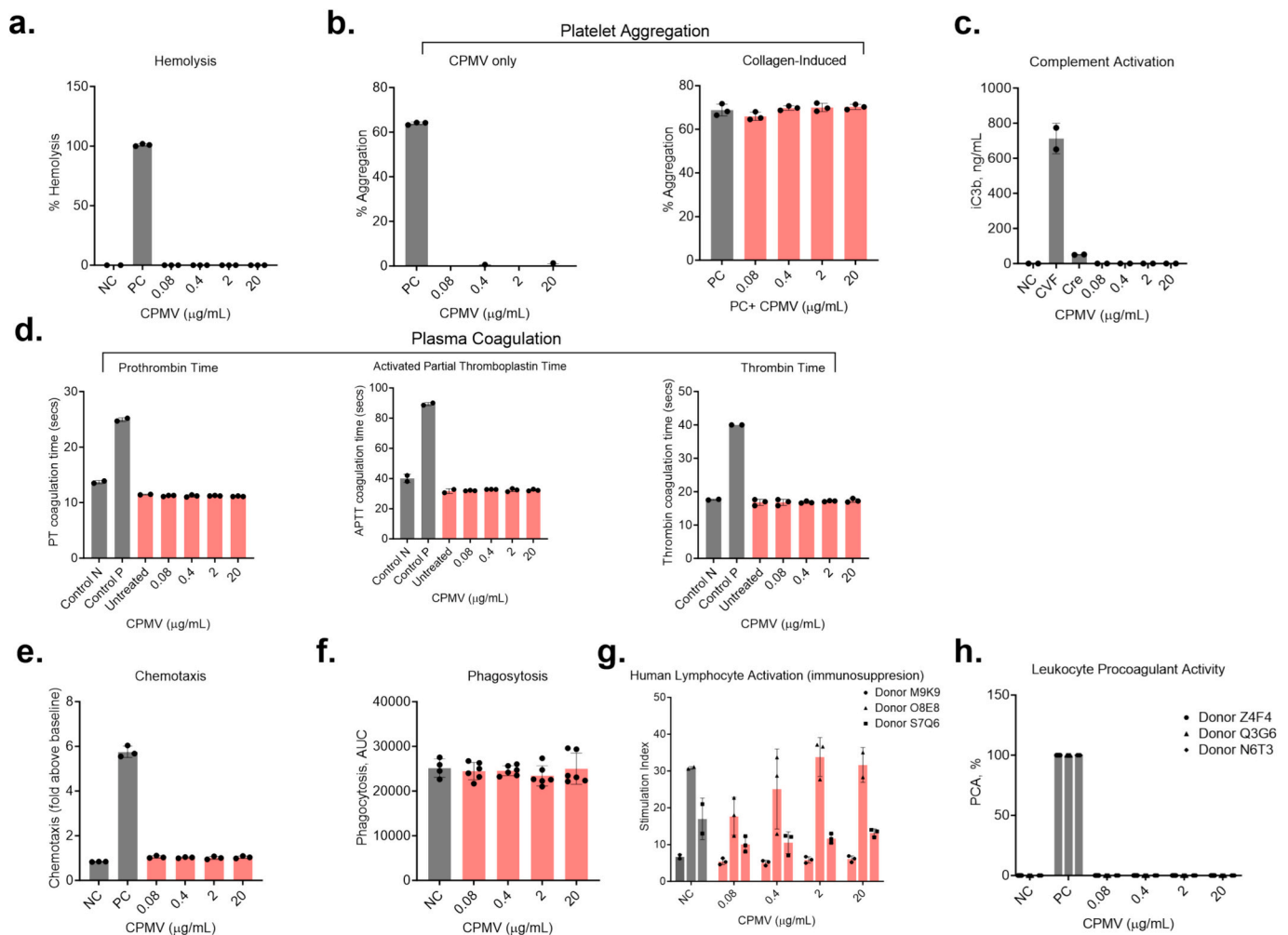


Fig. 12. CPMV demonstrates hemocompatibility in healthy human blood and has no negative effect on the function of human immune cells *in vitro*. A) Hemolysis assay using human whole blood. Negative control (NC) is PBS. Positive control (PC) = Triton X-100. B) Platelet aggregation. PC = collagen. C) Complement activation was not detected in human plasma in the presence of CPMV. PC = cobra venom factor. Cremophor-EL (Cre) at the same concentration as in the clinical formulation Taxol was used as additional nanoparticle-relevant PC. D) Plasma coagulation in the presence of CPMV using prothrombin time (left graph), activated partial thromboplastin time (middle graph) and thrombin time (right graph) tests. E) Chemotaxis of human HL-60 myeloid cells was studied toward PBS (NC), serum (PC) or CPMV. F) CPMV does not affect phagocytosis of Zymosan A by HL-60 myeloid cells. G) Influenza antigen-specific proliferation of human primary T-cells isolated from three healthy donors (M9K9, O8E8 and S7Q6) was not affected by the presence of CPMV. H) Leukocyte procoagulant activity (PCA) was not induced by CPMV at all tested concentrations in peripheral blood mononuclear cells isolated from three healthy donors (Z4F4, Q3G6 and N6T3). NC = PBS; PC = a combination of *Escherichia coli* K12 LPS and calcium ionophore. Reproduced with permission from [46], CC BY 4.0.

indicates that a dose of <1 mg/kg is unlikely to be toxic.

7.4. Agronomical safety

One concern over the use of CPMV is the potential infectivity of post-treatment excrement in contact with plants. To investigate the infectivity of excrement, mice treated intratumorally or by oral gavage with CPMV were placed in a metabolic cage and urine and stool samples were collected. The samples that tested positive for CPMV RNA by RT-PCR were pooled and used for attempted mechanical inoculation of cowpea plants. There was no evidence of shedding of infectious CPMV particles. When plants were challenged with urine and stool extracts, the infection of plants was not apparent, indicating agronomical safety [90]. This study was also repeated after intraperitoneal administration using a mouse model of intraperitoneal disseminated ovarian cancer, and again shedding of infectious CPMV was not apparent [47]. We also confirmed there is no evidence of replication or translation of CPMV in mammalian cells [46]. Although CPMV is infectious in plants, there are no reports indicating infectivity or pathogenicity of CPMV in mammals.

8. Manufacturing and quality control of CPMV

Efforts have been made to identify a CPMV-derived lead candidate for immunotherapy, and to improve CPMV extraction, purification yield, and quality control. This will streamline production when scaling up CPMV manufacturing for larger studies and, ultimately, human treatment. Process optimization begins with the upstream production of CPMV-infected plant materials, followed by optimization of downstream purification and storage – we are beginning to establish protocols that are in compliance with FDA and EMA guidelines (guided by an FDA INTERACT meeting held in Q1 2026).

8.1. Identification of a lead CPMV-based candidate

Earlier studies focused on the application of empty CPMV particles (eCPMV), a VLP that does not package any RNA. This approach was selected because eCPMV is non-infectious in plants and has scalable manufacturing by agroinfiltration—the state of the art for plant molecular farming [115]. The VLPs were highly effective as an

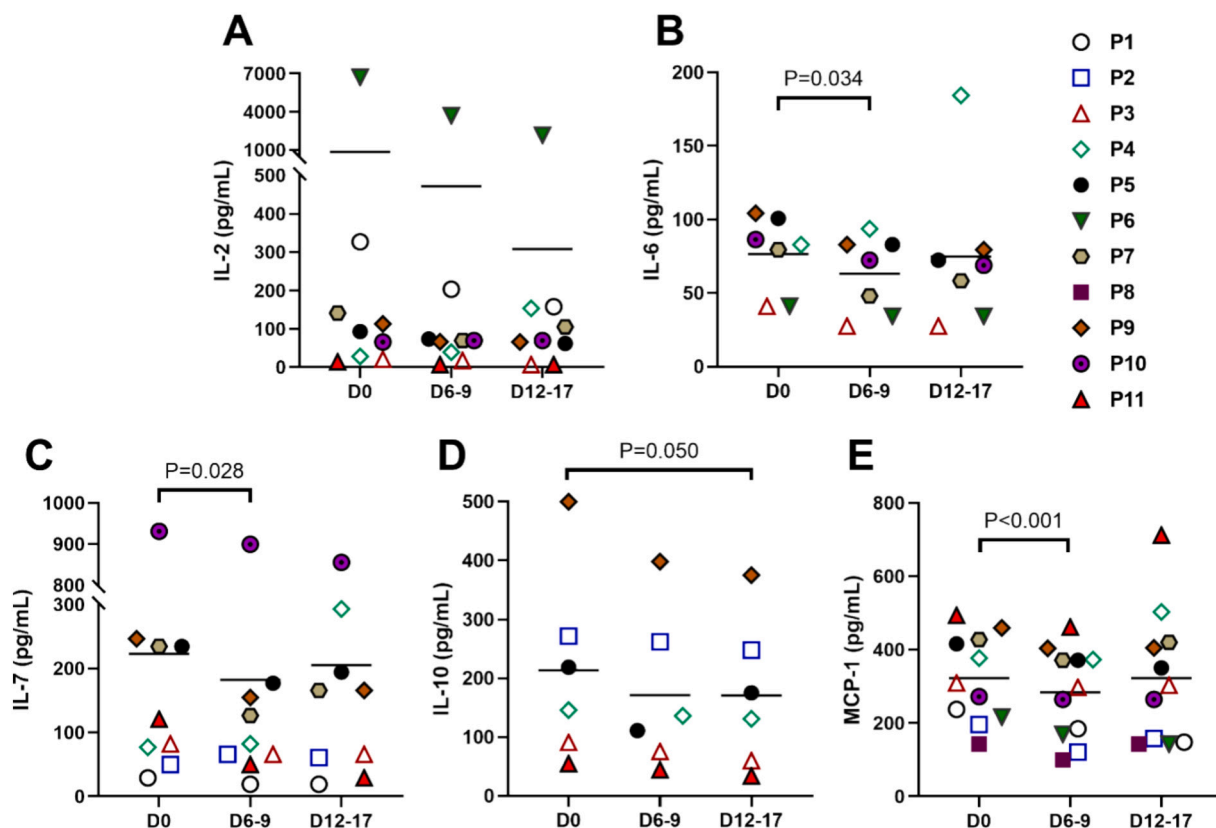


Fig. 13. CPMV immunotherapy induced modest and transient changes in plasma cytokines in CPMV-treated dogs. Changes induced by empty CPMV (eCPMV) injections in A) IL-2, B) IL-6, C) IL-7, D) MCP-1 and E) IL-10 plasma levels. Each companion dog is represented by a colored individual shape as indicated on the right side of B). The x-axis indicates the day when measurements were taken. The y-axis shows percentage changes in the plasma concentration of different cytokines. Reproduced with permission from [81], CC BY 4.0.

immunotherapy against different murine cancer models (Fig. 14a–14c) and in canine cancer studies [44,78,80,97]. Nevertheless, side-by-side comparisons between CPMV and eCPMV showed that CPMV is more potent (Fig. 14d & 14e) [78,97,116]. This increased potency of CPMV vs eCPMV is explained by additional signaling through TLR-7, resulting in a type-I interferon response (IFN- α/β) (section 4) [78,97]. The efficacy of native CPMV was also compared against chemically or UV-inactivated particles, which are non-infectious in plants, but the inactivation/crosslinking of the RNA/protein reduces the potency of CPMV (Fig. 14e) [116,117]. Therefore, the lead candidate has been defined as RNA-laden CPMV particles.

8.2. Manufacturing of upstream CPMV-infected plant material

For future production of clinical material, plant growth and CPMV propagation must be performed with temporal and spatial separation of the pre-infection and post-infection growth areas and precautions must be taken to avoid potential transfer of CPMV to plants outside of the facility. Currently, cowpea seeds are planted in pots filled with soil and allowed to grow for roughly 7 days in a growth chamber maintained at specific conditions (25/22 °C, 60% relative humidity, and ~100,000lx 16h photo period) [116]. At this point, infection is carried out by mechanical inoculation using ~4 μ g CPMV per leaf, with carborundum used as abrasive. Infected plants are placed back in the growth chamber and grown for an additional 10–14 days. Primary and tri-foliolate leaves with symptoms of infection (mosaic and mottling spots) are harvested and plant biomass is stored frozen. The growth chamber and laboratory are maintained under a USDA PPQ526 permit.

8.3. Downstream CPMV purification process

We recently established a scalable CPMV purification process that omits the use of ultracentrifugation (commonly used in the laboratory setting for virus purification) through the use of ultrafiltration/diafiltration (UF/DF) (Fig. 15a). This process has three main steps as previously described and are briefly summarized below [118]:

Extraction and pH treatment: Frozen leaves are homogenized in a blender [119]. Extracts are treated with MES buffer to achieve a pH of 4–5. This allows ~80% of plant host cell proteins to be removed during acidic extraction. Extracts are then filtered through two layers of Miracloth (MilliporeSigma) and clarified by centrifugation.

Ultrafiltration: A Minimate bench-top tangential flow filtration system (Cytiva) is currently being used for all UF/UD, with a transmembrane pressure of 0.5 bar and a tangential flow rate of 40 mL/min. Hydrophilic polyethersulfone filtration membranes with a MWCO of 1000 kDa and 300 kDa are fed the plant extract and are used to remove impurities that are larger and smaller than CPMV. Through this process, bulk water is removed and CPMV is concentrated, which reduces sample volumes and equipment footprint. Notably, UF membranes can be regenerated overnight, which may reduce operating expenditures if scaled up.

Ion exchange chromatography (polishing step): After purification by ultrafiltration, CPMV preparations are further polished by ion exchange chromatography (AEX). This step can remove any remaining impurities. To remove endotoxins, the non-ionic detergent Triton-X114 is added to UF/UD-purified samples prior to polishing using AEX.

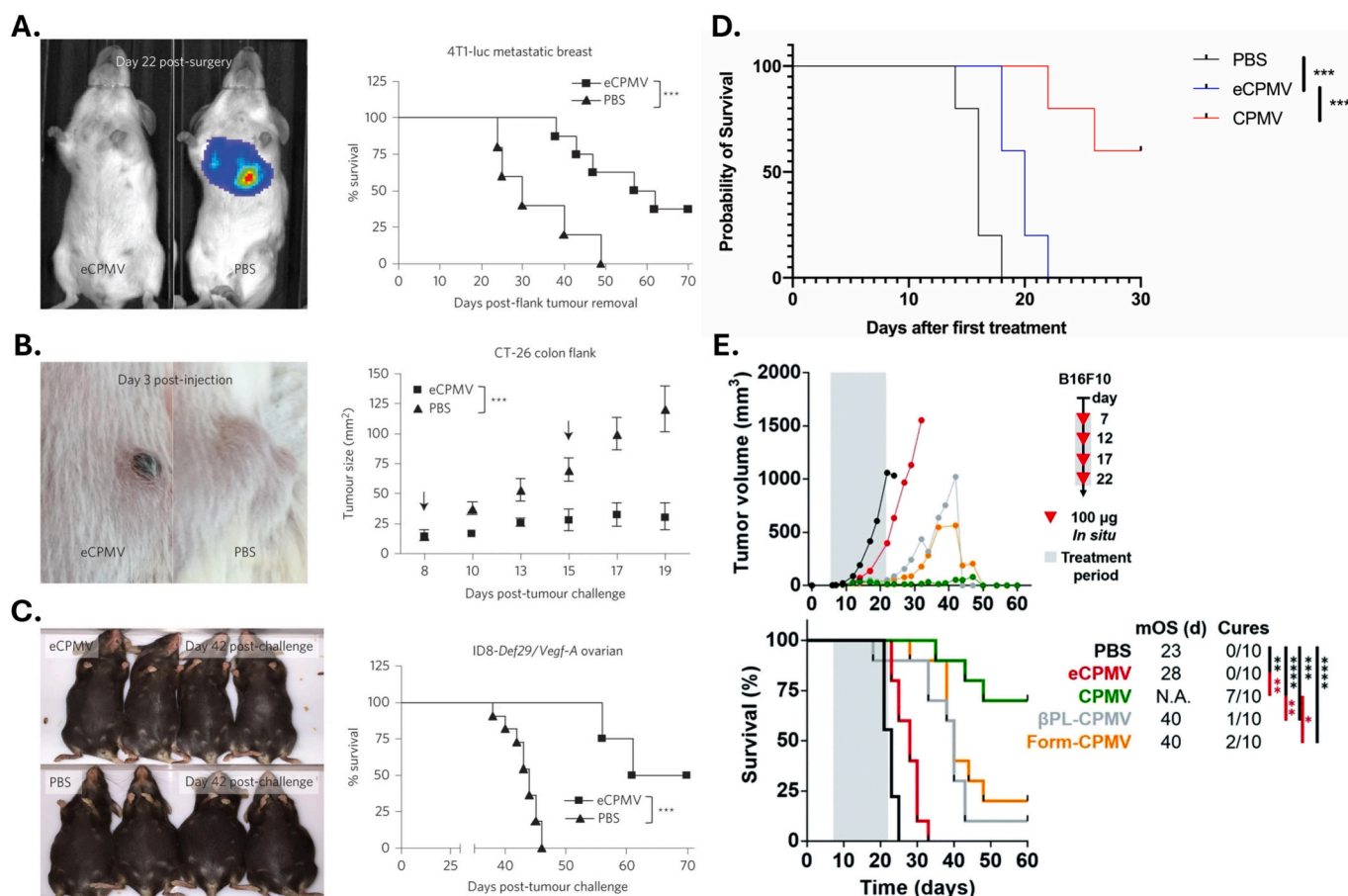


Fig. 14. CPMV is efficacious in tumor-bearing mice. CPMV can treat different tumor types in several anatomical sites (eCPMV is empty CPMV, an RNA-free version of CPMV). A) 4 T1 was grown in the breast and surgically removed on day 15 at which time CPMV was inhaled once. Survival curve reflects death from spontaneous lung metastases. B) CT26-colon cancer on the flank was injected once with CPMV on day 8 and growth was followed for both eCPMV and PBS-treated tumors. C) Hyperaggressive, disseminated, intraperitoneal ovarian cancer model (using ID8-Defb29/Vegf-A cells) was injected with eCPMV VLP intraperitoneally once weekly for 4 weeks, starting on day 3. Survival was followed over 70 days. D) Survival curve of C57BL6/J mice bearing intradermal B16F10 melanoma tumors treated with PBS, eCPMV, or native CPMV. E) Average tumor volume and survival in C57BL6/J mice bearing intradermal B16F10 melanoma tumors treated with PBS, eCPMV, CPMV, βPL-CPMV, or Form-CPMV (βPL-CPMV = CPMV inactivated with β-propiolactone; Form-CPMV = CPMV inactivated with formalin; mOS = median overall survival). Reproduced with permission from (A-C) [44], © 2016 Macmillan Publishers Limited, (D) reprinted from [78], © 2021 with permission from Elsevier, and (E) [116], CC BY-NC 3.0.

8.4. Characterization of CPMV

We established a UC San Diego CPMV Research bank and verified for CPMV identity, biophysical parameters, and purity. The CPMV Research Bank will serve as starting material to establish a CPMV Master Bank for future production of clinical material. To confirm purity, the presence of 24-kDa small and 42-kDa large coat proteins as well as the absence of protein contaminants is confirmed by SDS-PAGE; the presence of encapsidated RNA and absence of free RNA is confirmed by native agarose gel electrophoresis (Fig. 15b & 15c). UV/Vis Spectroscopy also indicates purity with the CPMV characteristic A260/280 ratio of ~1.7 (Fig. 15d); UV/Vis spectroscopy and the CPMV-specific extension coefficient is used to determine its concentration. To confirm structural integrity and sizing of CPMV, a combination of dynamic light scattering (DLS), size exclusion chromatography (SEC), and transmission electron microscopy (TEM) imaging is used (Fig. 15e-g). Particle sizing by DLS indicates monodispersity of the sample and should show a particle of roughly 30 nm in size. SEC is used to confirm purity and the absence of aggregation, degradation, or impurities. The characteristic A260:280 ratio can also be observed via SEC. Finally, TEM is used to show a uniform distribution of nanoparticles and the absence of broken particles.

Extraction of CPMV from plants is performed according to an established protocol intended to avoid bacterial contamination and

reduce endotoxin concentrations. Endotoxin analysis is performed following purification and characterization using turbidity and chromogenic Limulus Amoebocyte Lysate (LAL) assays according to the United States Pharmacopeia Bacterial Endotoxins Test <85> (USP BET85), which aims to detect endotoxins in pharmaceutical products [120]. The purified CPMV particles should have low levels of endotoxins below the endotoxin limit (EL), calculated as described in USP BET85 using the following formula: $EL = K/M$, where K is the threshold pyrogen dose for all routes of administration except the intrathecal route (5 EU/kg) and M is the maximum dose intended for intratumoral administration (30 mg). With a maximum intratumoral dose of 30 mg/tumor and assuming a 75 kg patient, the CPMV dose is 0.4 mg/kg. Based on $EL = K/M$, the EL used for non-clinical studies was set to 12.5 EU/mg CPMV [97]. Previous work indicates that the majority of endotoxins are removed during UF/UD [118].

8.5. Stability of CPMV

We have completed a 2-year stability study evaluating storage in phosphate buffer pH 7.0 and stored at -80 °C, -20 °C, 4 °C, room temperature, and 37 °C, as well as in liquid nitrogen. Samples were taken and analyzed after 1 week, 1, 3, 6, 9 months, and after 2 years of storage. Data showed that samples stored at -20 °C and colder were

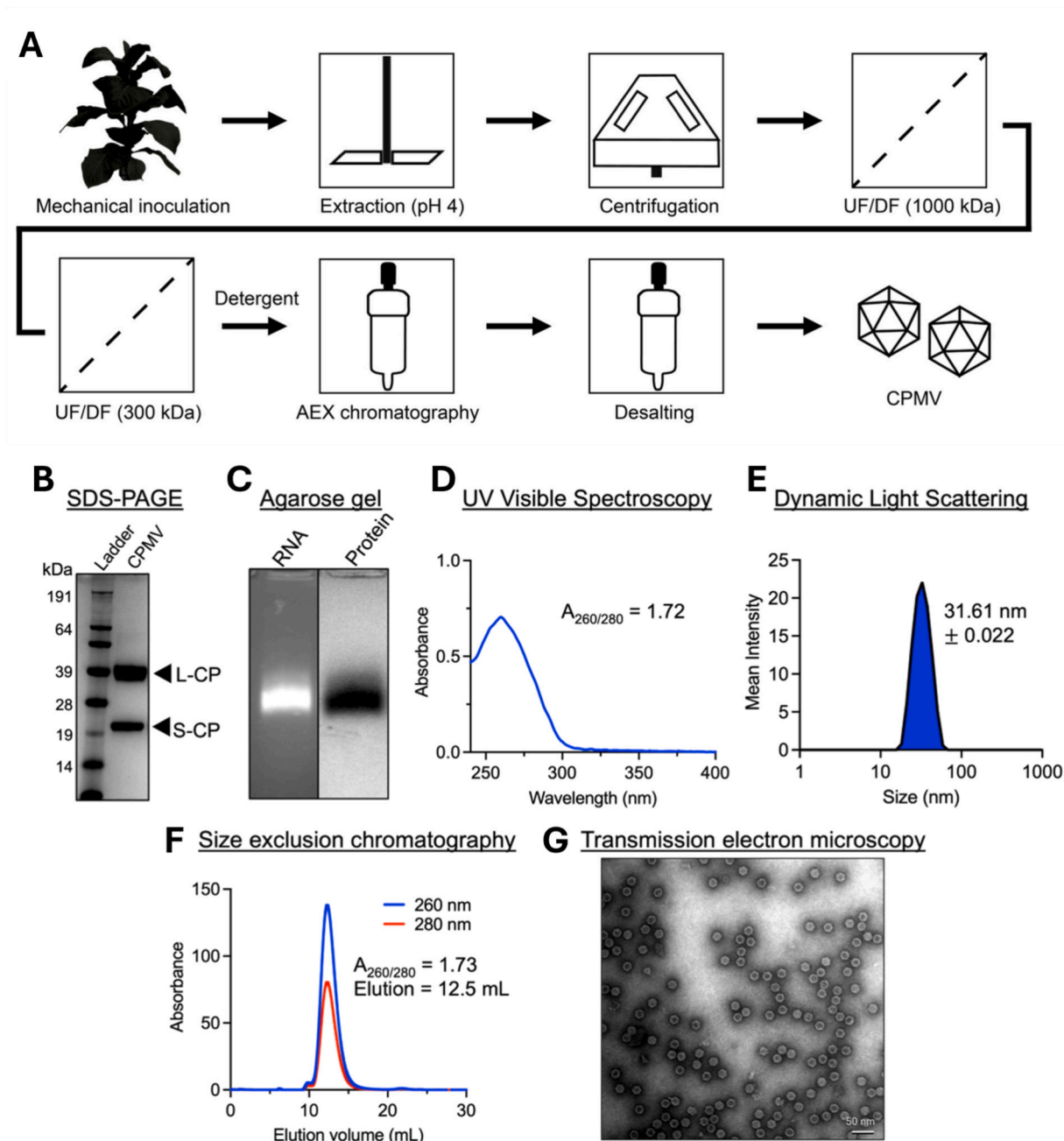


Fig. 15. A) Process flow diagram for CPMV manufacturing. Scalable CPMV purification with the ultrafiltration/diafiltration (UF/DF) process. Ultrafiltration is used in place of ultracentrifugation. A 1000kDa MWCO membrane is used to remove impurities that are larger than CPMV and a 300kDa membrane is used to remove impurities that are smaller than CPMV. Bulk water is also removed during the process. B-G) Representation figures showing characterization of a CPMV Research Bank sample using SDS-PAGE (B), native gel electrophoresis (C), UV/vis absorbance measurement (D), DLS (E), SEC using a Superose6 Increase column (F), and TEM (G). Fig. 15a was reproduced with permission from [118], CC BY-NC-ND 4.0. and Fig. 15b-g was reproduced with permission from [46], CC BY 4.0.

stable throughout these timepoints and CPMV maintained its structural integrity and biological activity after repeat freeze-thaw cycles [121,122].

9. Challenges and future directions

CPMV has been identified as a lead candidate for translational development. In particular, its ability to reprogram “cold” or immunosuppressive TMEs and induce systemic antitumor immunity in multiple solid tumor models as well as spontaneous canine cancers positions it as a promising intratumoral immunotherapy for hard-to-treat cancers [44–47,49,81]. CPMV has been shown to outperform other plant viruses

from the Secoviridae family; it also outperforms another cowpea blight, cowpea chlorotic mottle virus [52,53]. Several virus-based nanotechnologies are currently being developed from pre-clinical stages to FDA approval stages (Table 2) [123]. CPMV works in a similar fashion to the papaya mosaic virus (PapMV) and bacteriophage therapies by acting as a TLR agonist and priming the immune system, but CPMV is a multi-TLR agonist while PapMV acts on TLR-7 and the bacteriophages primarily act on TLR-9 [25,39,53,124]. The mechanism of action of intratumoral CPMV is well understood, and the large body of efficacy studies in diverse mouse tumor models and canine cancer patients treated at multiple veterinary clinics in the USA, Spain, and Mexico are key steps toward clinical testing in humans. Investigational new drug (IND)

Table 2

Current virus-based nanotechnologies in development and clinical use.

Nanocarrier	Drug	Development Phase	Ref.
Plant virus	CPMV	Pre-clinical	
Plant virus with non-coding RNA	PapMV	Pre-clinical	[39]
Oncolytic virus	TVEC	FDA approved	[19]
Bacteriophage with CpG	Vidutolimod (CMP-001)	Clinical trial: NCT02680184	[25]
Plant virus	PVX	Pre-clinical	[125]
Bacteriophage	M13	Pre-clinical	[126]
Oncolytic virus	Lerapolturev (PVSRIPO)	Clinical trial: NCT0149193	[127]
Oncolytic virus	DNX-2401	Clinical trial: NCT03896568	[128]

PapMV: papaya mosaic virus, TVEC: talimogene laherparepvec, PVX: potato virus X.

enabling chemistry, manufacturing, and controls (CMC) and pharmacology programs must now be completed. Toward this step, our team recently completed an FDA INTERACT meeting to begin the next phase of development.

Given the intended use of CPMV in aggressive and refractory solid tumors, scalable and robust manufacturing will be essential to support multicenter trials of diverse types of cancer. The use of plant viruses as human therapeutics is novel and there is a significant gap in contract development and manufacturing organization (CDMO) capacity for the biomanufacturing of plant-based pharmaceuticals, particularly for virus-based nanoparticles like CPMV produced in cowpea plants. Despite extensive efforts, no CDMO has been identified to support process development or current good manufacturing practices (cGMP)-compliant manufacturing of CPMV in this plant. As a first step, these capabilities must be developed, and we have begun discussions with the regulatory agencies to identify suitable methods for manufacture of CPMV as a drug candidate. Plant-based manufacturing of biologics plant molecular farming has gained commercial interest due to the potential for low cost of production at scale, safety relative to animal-based manufacturing, and sustainability [129,130]. Examples of commercial scale manufacturing facilities for plant-derived therapeutics include a facility able to output over 3500 kg of *Nicotiana benthamiana* biomass per week to create vaccines, and the development of a fully automated facility to produce plant-derived biologics within cGMP standards [131–133]. Despite the gap in CDMO capacity for CPMV production, a scalable product does seem feasible.

Another hurdle to consider when proposing a live plant virus as a drug candidate is that CPMV is a plant pathogen that infects cowpea plants and other legumes via an insect vector [90]. Although there is no evidence of live virus shedding from treated animals [90], the handling of plant infectious material is regulated by the United States Department of Agriculture (USDA) and requires a permit (PPQ 526), which may complicate the CMC program and collaboration with CDMOs, for example for assay development, fill-and-finish, quality control and quality assurance testing. With respect to environmental risk: considering that i) CPMV would be administered subcutaneously or intratumorally in a hospital setting, ii) the nanoparticle character leads to local tumor retention of CPMV, and iii) prior studies have shown no detectable shedding of CPMV (in faeces/urine), we consider the environmental exposure risk from clinical use to be minimal [47,90]. CPMV is a wild-type, non-recombinant plant virus, and these factors may support inclusion of a claim of categorical exclusion (under 21 CFR 25.31(e)) for the proposed IND. If concerns about the use of plant-infectious CPMV present a hurdle, a fall-back strategy may include the separation of the B and M components of CPMV. CPMV-B contains RNA-1 (nonstructural genes) and CPMV-M contains RNA-2 (structural genes). Both components are required for the successful infection of plants but either can achieve potency as an intratumoral agent [134].

With the limitations discussed above, CPMV remains untested in

humans despite the many successful animal studies. A large body of data from rodent testing, trials in large animals (canines) and *in vitro* studies using human cells support the excellent safety profile of CPMV [46]. The first-in-human trials will focus on safety studies and inclusion/exclusion criteria must be established. A potential concern that should be monitored relates to legume allergies. Those with peanut allergies can be sensitized to cowpea proteins, placing patients at risk due to IgE cross-reactivity [135]. For clinical material, we will define the release criteria and host cell protein monitoring strategy [46]. A workaround would be the use of another plant for CPMV production. For example, *Nicotiana benthamiana* is frequently used to produce plant-made pharmaceuticals and is the current industry standard [136]. Finally, with no known MTD, a recommended dose for optimal efficacy must be determined. This could be defined based on biomarkers, such as a certain level of type-I interferons in plasma or biopsy samples, or based on injection volume limitations. For hard-to-treat cancers, early-phase trials could consider rational combinations with immune checkpoint inhibitors, radiotherapy, or chemotherapy, to define where CPMV provides additive or synergistic benefits.

Practical deployment in hard-to-treat cancers will also require strategies for treating deep or multifocal lesions. Image-guided or endoscopic intratumoral administration, repeated dosing schedules, and protocols for injecting multiple lesions in parallel should be developed. Emerging data on the systemic or regional administration of CPMV suggest additional opportunities for reaching non-accessible disease sites, provided the safety and mechanistic advantages of *in situ* vaccination are maintained [47]. CPMV has a uniquely favorable intrinsic immunostimulatory profile relative to related plant virus nanoparticles and other VLPs, supporting its prioritization as a lead for intratumoral immunotherapy development in hard-to-treat cancers [52,53].

10. Conclusions

This review particularly highlights the promise of CPMV as a drug candidate for intratumoral immunotherapy to treat cancer, particularly hard-to-treat solid cancers that do not respond to conventional systemic therapies and current immuno-oncology agents. More than 10 years of work has confirmed the efficacy of intratumoral CPMV immunotherapy in diverse murine cancer models (including poorly immunogenic and metastatic disease), the benefits of using CPMV in combination with other treatment modalities, and promising results from veterinary clinical trials using canine companion animals with spontaneous, clinically challenging tumors. We have explored the various ways that CPMV activates the immune system locally while shrinking distant, non-injected tumors and eliciting long-term immune memory, features that are critical for the control of refractory and disseminated malignancies. Additionally, we have discussed safety, biodistribution, and dosing. CPMV shows good hemocompatibility and no evidence of replication or translation in mammalian cells, and a low-to-negligible allergic/anaphylactic risk, indicating that CPMV is a safe, efficacious immunotherapy. However, paving the way for translational development and clinical testing will require collaborative, creative efforts to address scalability, cost and regulatory concerns, as well as practical considerations around intratumoral accessibility and combination strategies in advanced, treatment-resistant cancers. In conclusion, CPMV as both a cancer monotherapy and combination therapy holds great promise but will require continued research and development to fully explore its safety and efficacy.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. Steinmetz is a co-founder and CEO of and has equity in PlantiosX Inc. Dr. Steinmetz is a co-founder of and has equity in Mosaic Immunoengineering, Inc. Dr. Steinmetz is a co-founder and manager of

Pokometz Scientific LLC, under which she is a paid consultant to Ring Therapeutics, Inc. The other authors declare no potential conflicts of interest.

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Data availability

No data was used for the research described in the article.

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