

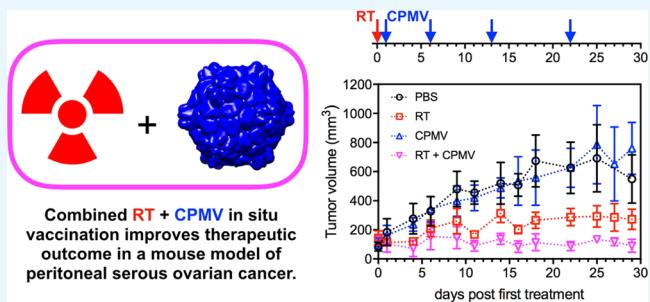
# Radiation Therapy Combined with Cowpea Mosaic Virus Nanoparticle *In Situ* Vaccination Initiates Immune-Mediated Tumor Regression

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**ABSTRACT:** Epithelial ovarian cancer is a deadly gynecologic malignancy because of its late detection, usually after local and distant metastatic spread. These cancers develop resistance to traditional chemotherapeutic agents; therefore, the development of next-generation immunotherapeutic approaches may have a significant promise in improving outcomes. A novel immunotherapeutic approach utilizing combination radiation therapy (RT) with immunostimulatory cowpea mosaic virus (CPMV) was tested in a preclinical syngeneic mouse model of ovarian carcinoma. ID8-Defb29/Vegf tumors were generated in C57BL/6 mice. Compared to placebo-treated control tumors or those treated with a single agent RT or CPMV, the combination treatment resulted in a significantly improved tumor growth delay ( $p < 0.05$ ). Additionally, immunohistochemical profiling of tumor samples after treatment with CPMV demonstrated an increase in tumor infiltrating lymphocytes (TILs). These results suggest that utilizing CPMV particles in combination with RT can turn an immunologically “cold” tumor (with low number of TILs) into an immunologically “hot” tumor. This novel combination treatment approach of RT and CPMV demonstrated the ability to control tumor growth in a preclinical ID8 ovarian cancer model, showing promise as an *in situ* tumor vaccine and warrants further testing.



## 1. INTRODUCTION

Epithelial ovarian cancer (EOC) remains the deadliest gynecologic malignancy, with an estimated 22 400 new cases in 2017 in the US, resulting in 14 000 deaths.<sup>1</sup> Despite progress with newer agents, such as poly (ADP-ribose) polymerase inhibitors and peritoneal chemotherapy, which have been shown to improve progression-free survival and response rates in this disease, the overall survival remains dismal.<sup>1</sup> One of the main difficulties for improving survival in EOC is that while most patients initially respond to traditional platinum-based chemotherapies, resistance inevitably develops. There are few effective alternative agents.

Therefore, newer treatment regimens are needed to improve outcomes in patients with locally advanced ovarian cancer. One strategy that has been investigated in a variety of solid tumors, including EOC, is the use of immune checkpoint inhibitors to potentiate an adaptive host T-cell-based immune response against cancer cells. Therapeutic antibodies targeted to the programmed death receptor one (PD1) or its ligand [programmed death ligand-1 (PDL1)] have produced partial response rates of 20% in early-phase clinical trials in platinum-resistant ovarian cancer.<sup>2,3</sup> However, complete responses that have been seen using these agents in melanoma remain elusive.

A key challenge that the checkpoint therapies fail to address is the recruitment of T cells into an immune-suppressive tumor microenvironment; that is, while checkpoint inhibitors take the “brakes off” T cells, the therapeutic regimen does not promote local immune stimulation and required immune cell infiltration into the tumor.<sup>3–5</sup>

*In situ* vaccination approaches have shown great promise to overcome and relieve tumor-mediated local immune suppression. The idea behind *in situ* vaccination is that an immune-stimulatory agent is introduced into an identified tumor to promote first innate, then adaptive, immune infiltration and activation, essentially turning an immunologically “cold” tumor into a “hot” tumor and thus priming the patient’s own immune system against the tumor. Optimally, this leads to a boost in systemic antitumor immunity to attack untreated tumors of the same type. A number of strategies are being explored, such as a stimulator of interferon (IFN) genes (STING) pathway agonist<sup>6,7</sup> and viral *in situ* vaccination.<sup>8,9</sup> For example, Talimogene laherparepvec (TVEC) is a Food and Drug

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Administration (FDA)-approved oncolytic and immune-stimulatory (through expression of granulocyte-macrophage colony-stimulating factor) therapy approved as *in situ* vaccine for the treatment of melanoma.<sup>10</sup>

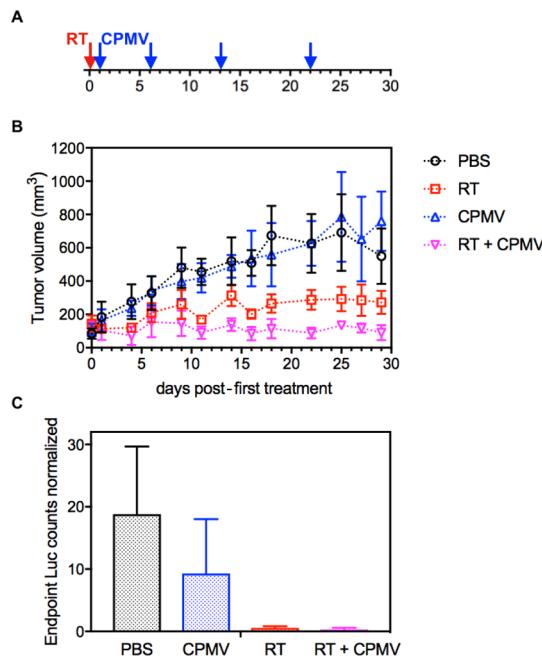
Our group has developed an *in situ* vaccination approach using virus-like nanoparticles derived from the plant virus cowpea mosaic virus (CPMV).<sup>11</sup> In previous work, we demonstrated efficacy across various mouse tumor models; our data indicate that the plant viral *in situ* vaccine acts in part to activate intratumoral neutrophils which in turn lead to immune-mediated, systemic and memory responses against tumors, including melanoma, colon cancer, breast cancer, and ovarian cancer. Specifically, weekly intraperitoneal (IP) treatment with CPMV has been shown to significantly improve survival after IP tumor challenge with a hyperaggressive clone of ID8 epithelial mouse ovarian cancer cells compared to phosphate-buffered saline (PBS)-treated controls.<sup>11</sup>

Nevertheless, although these studies highlight the potential of plant viral nanoparticles as *in situ* vaccines, immunotherapy as monotherapy is generally effective only against small tumors, and most patients do not respond to single-approach immunotherapy.<sup>5</sup> Combining multiple treatment regimes, however, could form the basis for success. Therefore, in the present work, we examined the combination of CPMV *in situ* vaccination with radiation therapy (RT). We hypothesized that the combination of RT with our CPMV *in situ* vaccine would enhance the antitumor effect. Specifically, the hypothesis is that RT would debulk the tumor to provide a burst of tumor antigens in the context of immunogenic cell death that fosters specific immune recognition and response to those antigens;<sup>12</sup> in turn, CPMV-mediated immune stimulation would further augment antitumor immunity to protect from outgrowth of metastases and recurrence of the disease. Here, we report a test of this hypothesis using a mouse model of serous ovarian cancer.

## 2. RESULTS

**2.1. Tumor Growth Delay.** Subcutaneous ID8-Defb29/Vegf-A-Luc tumors in C57BL/6 female mice were treated with RT, *in situ* vaccine (CPMV), both RT + CPMV, or PBS (placebo group); treatment was begun when tumors reached a volume of 100–150 mm<sup>3</sup>. RT (10 Gy) was given in a single session on day 0, whereas the CPMV treatment was repeated 5 times in weekly intervals starting on day 1 (Figure 1A). As evidenced by tumor volume measurements, the tumor growth was delayed after day 5 in both RT arms compared to the arms receiving CPMV alone or PBS ( $p < 0.05$ ) (Figure 1B). From day 18 post-RT through the end of the study, the combination of RT + CPMV produced significantly greater tumor growth delay than did RT alone ( $p < 0.05$ ). Tumor volumes of animals treated with RT + CPMV combination were on average 2–3× smaller than those measured for animals receiving the RT alone; and this difference was apparent 14 days post-treatment (Figure 1B).

Tumor growth as assessed by bioluminescence showed similar results, with the RT and RT + CPMV arms having significantly reduced ( $p < 0.05$ ) tumor burden compared to the arms receiving CPMV alone or PBS (Figure 1C). There was also a trend toward a reduction in the luminescence counts in the combination RT + CPMV arm compared to the RT alone arm ( $p = 0.06$ ). Although we previously observed a potent efficacy of CPMV *in situ* as solo therapy in early-stage IP disseminated ID8-Defb29/Vegf-A in C57BL/6 mice,<sup>11</sup> in the



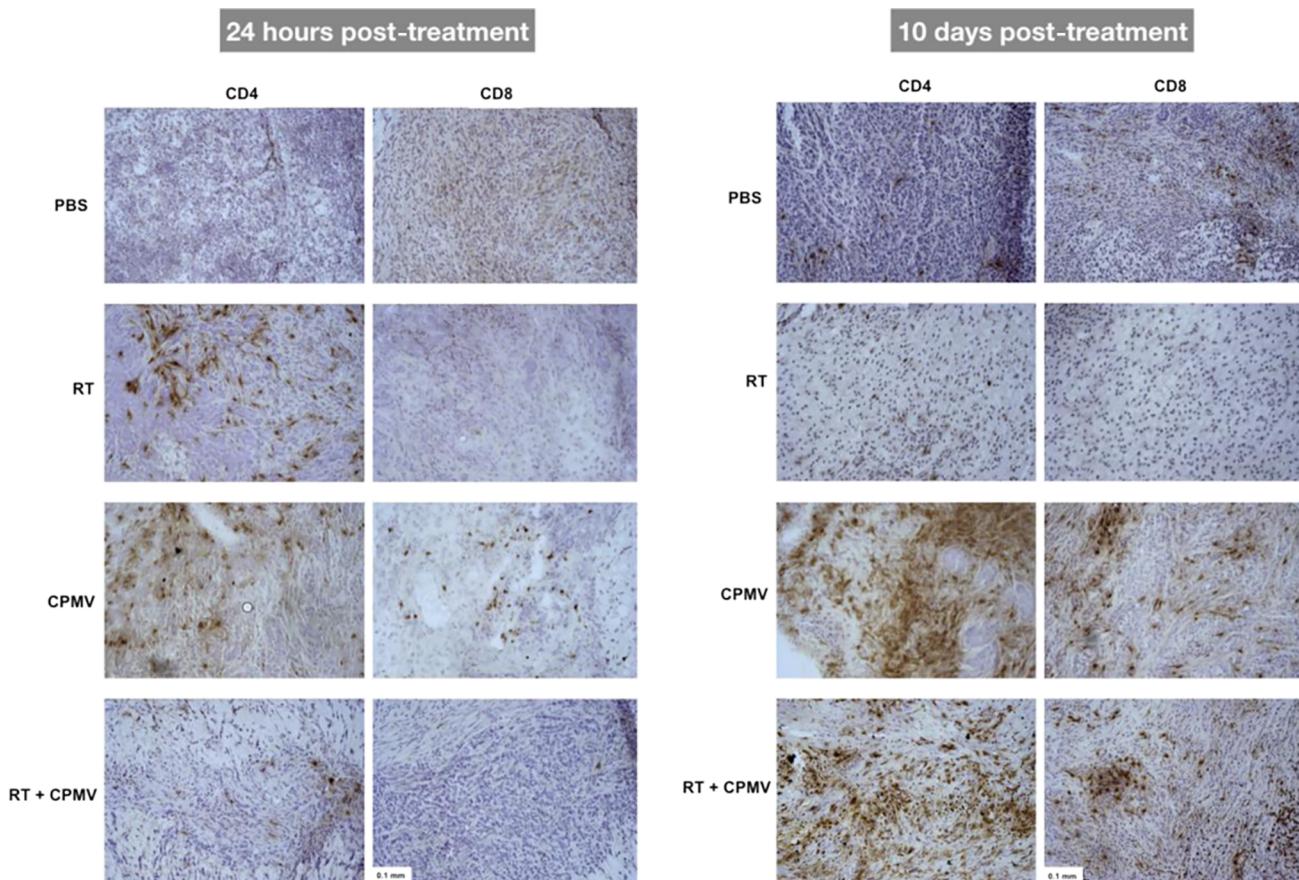
**Figure 1.** Tumor growth was monitored by caliper measurement and by bioluminescence. (A) Schedule for treatment of tumors with RT and CPMV *in situ* vaccine injections. (B) Tumor volume as a function of time is plotted for each treatment group ( $n = 4$ ). (C) Total luciferase counts on day 29 (endpoint) normalized to their starting baseline values is shown for each treatment group ( $n = 4$ ).

present study, we did not observe the same efficacy using the same dose of CPMV administered directly into the subcutaneous tumors.

**2.2. Tumor Immune Cell Infiltration.** Tumor immune cell infiltration was determined by immunohistochemistry (IHC) on samples of tumors recovered on day 2 and 11, following treatment with RT, CPMV, RT + CPMV, or PBS (Figure 2). Early-stage PBS-treated tumors (volumes of 100–150 mm<sup>3</sup>) did not reveal significant levels of CD4- or CD8-positive cells. By day 11, the untreated later-stage tumors (volumes of ~500 mm<sup>3</sup>) showed some degree of CD4- or CD8-positive cells; however, compared to other treatment groups, this level of T cell infiltration was minimal. By day 2, 24 h after the start of CPMV treatment, a modest CD4-positive infiltrate was seen in tumors treated with RT or CPMV alone or in combination, with no significant CD8-positive effector immune cell infiltrate in any group at that time. In contrast, 10 days following initiation of CPMV treatment, both the CPMV alone group and the RT + CPMV group showed significant infiltration of CD8-positive effector immune cells, in addition to the CD4-positive immune infiltrate. The lymphocyte infiltration was observed only in groups exposed to CPMV or RT + CPMV and not in those given RT alone or PBS. While quantitative studies were not carried out, there appears to be a trend toward a greater lymphocyte infiltration into RT + CPMV-treated tumors than into tumors treated with CPMV alone (Figure 2).

## 3. DISCUSSION

Ovarian cancer remains difficult to treat and a deadly malignancy even with the advent of new therapeutic approaches and novel drug agents. Therefore, the initial promise of newer immunotherapy paradigms is of significant interest. In our



**Figure 2.** Tumor sections stained for CD4 and CD8 lymphocytes on day 2 and 11 corresponding to 24 h and 10 days post CPMV or PBS placebo treatment.

study, we examined a novel immunotherapeutic viral nanoparticle, CPMV, which has previously been shown to be an effective *in situ* vaccination strategy in a syngeneic mouse ID8 ovarian cancer model.<sup>11</sup> Although we previously observed high potency of the CPMV *in situ* vaccine in early-stage metastatic disseminated disease,<sup>11</sup> the CPMV monotherapy had no apparent effect on the growth of subcutaneous tumors derived from the same cell line (ID8-Defb29/Vegf-A) (Figure 1). This difference may be explained by the fact that in our previous study early-stage disease was treated, that is, the CPMV *in situ* vaccine was given as early as 3 days post-IP ID8-Defb29/Vegf-A cell injection. In contrast, in the present study, mice were treated ~10–14 days post-ID8-Defb29/Vegf-A cell injection when subcutaneous tumors reached a volume of 100–150 mm<sup>3</sup>. Differences in tumor burden at the time of treatment and the different anatomical locations may both explain the difference in response to CPMV monotherapy.

Another difference between our earlier work and the present study is the fact that we used a nucleic-acid-free, viruslike particle of CPMV, termed “empty” CPMV (eCPMV), for the treatment of early-stage, IP tumors,<sup>11</sup> whereas the present study used nucleic-acid-containing CPMV particles to treat later-stage subcutaneous tumors. CPMV contains a bipartite RNA genome, with RNA-1 and RNA-2 encapsulated separately into virions of identical protein composition. The RNA cargo may affect the type of immune response induced; RNA is an immune-stimulatory danger-associated molecular pattern known to stimulate innate immune responses through Toll-like receptor signaling. Although others reported the

encapsulated RNA in the plant viruslike nanoparticle derived from papaya mosaic virus to be beneficial in triggering an antitumor immune response,<sup>33</sup> the underlying mechanism of immune stimulation comparing different plant virus-based nanoparticles of different geometry and molecular composition remains to be elucidated. It cannot be ruled out that RNA-containing CPMV and nucleic-acid-free eCPMV have distinct potencies based on differences in immune activation. Our laboratory is investigating this question, but this is beyond the present study.

Nevertheless, even though the potency of CPMV monotherapy was less profound than in our previous animal model treated with eCPMV, luminescence measurements indicated reduced disease burden for CPMV versus PBS control groups at the conclusion of the study (Figure 1C). The reduced disease burden at the endpoint and the observation of immune cell infiltration into the tumor tissue upon CPMV *in situ* vaccination indicate that animals with the subcutaneous ID8 tumors indeed responded to the treatment, although dosing may be suboptimal. It is clear that dosing and administration schedules need to be optimized for each disease model; however, this was beyond the scope of the present study. In fact, the suboptimal dosing was advantageous to allow testing of RT + CPMV combination therapy. ID8 ovarian tumors are known to be radiosensitive;<sup>16</sup> thus, it was expected that RT alone would produce a tumor growth delay. Nevertheless, the combination treatment, RT + CPMV *in situ* vaccine, resulted in significantly reduced tumor growth compared to RT or CPMV

treatment alone. These findings were also confirmed by our bioluminescence studies (Figure 1B,C).

There are several mechanisms by which RT can improve the efficacy of immunotherapeutic agents, including inducing immunogenic cell death,<sup>12</sup> enhancing release of neoantigens,<sup>17</sup> altering the tumor cell phenotype,<sup>18–20</sup> and stimulating the immune system via the STING pathway and IFN signaling.<sup>21,22</sup> The mechanism of cell death traditionally attributed to radiation is the mitotic catastrophe or p53-mediated intrinsic pathway apoptosis.<sup>23</sup> However, radiation can also upregulate CD95/Fas, tumor necrosis factor (TNF)-related apoptosis-inducing ligand, and TNF alpha death receptor pathways involved in the extrinsic pathway of apoptosis.<sup>24,25</sup> Upregulation of CD95/Fas on tumor cells has been shown to improve tumor cell kill by CD8<sup>+</sup> T lymphocytes.<sup>26</sup> In addition to enhancing immune-mediated cell death, radiation has also been shown to alter tumor cell phenotype. Tumor cells often downregulate major histocompatibility complex I (MHC-I) to evade the immune system. However, radiation exposure can also elicit a TNF and IFN response that results in MHC-I upregulation on tumor cells, as well as increased ICAM-1 expression, to improve the binding and interaction of T cells with MHC-I in multiple patient-derived human EOC cell lines.<sup>20</sup> Moreover, tumor cells exposed to radiation have been shown to have a greater diversity of antigens presented on their surface MHC-I molecules.<sup>27</sup> Finally, radiation itself can induce an inflammatory response via the Cgas/STING pathway either via cytosolic DNA or via DNA double-strand breaks within micronuclei.<sup>21,28</sup> Although activation of this pathway may initially lead to a cytotoxic T-cell-mediated antitumor response, IFN-mediated immune-suppressive pathways are also activated with recruitment of myeloid-derived suppressor cells,<sup>29</sup> regulatory T cells (Tregs),<sup>30</sup> and induction of tumor cell PDL1 expression.<sup>31</sup>

In contrast to RT, which has been exhaustively studied, CPMV is a novel immunotherapeutic agent and to date, we have limited data surrounding its mechanism of immune activation. Our IHC results demonstrate that CPMV with or without RT can increase both CD4<sup>+</sup> and CD8<sup>+</sup> tumor-infiltrating lymphocytes. In our study, we targeted relatively “cold”, well-established tumors; although CPMV monotherapy did not show apparent efficacy over placebo-treated control groups, IHC data indicate that CPMV alone recruits large numbers of T cells, particularly CD4<sup>+</sup> T cells. Although CPMV clearly initiates an immune response, the inflammatory response that it generates in this model likely recruits both effector and immunosuppressive cells. It is possible that a significant number of recruited CD4<sup>+</sup> T cells are CD4<sup>+</sup> T regulatory cells that are strongly immunosuppressive through multiple mechanisms. If this is the case, it likely explains why CPMV alone had minimal effect on tumor growth in this model, in that both effector and suppressive T cells are being recruited, and the regulatory T cells can limit effector T cell antitumor responses. Exposure to RT prior to CPMV in situ vaccination may help shift the balance toward a more cytotoxic immune response. Detailed time-dependent phenotyping of recruited T cells would better elaborate how and when the T cell subsets are recruited to these tumors in response to various treatments. An alternative possibility that was not explored is that the innate cell responses which can also be immunosuppressive or immunostimulatory were tilted toward immunosuppressive with CPMV alone but were altered toward immunostimulation in combination with RT.

Although our results demonstrated significantly improved tumor growth delay, as measured both by tumor volume and by bioluminescence, we were unable to achieve complete tumor regression, even in the RT + CPMV arm. Future optimization of dosing and administration schedule is expected to further increase efficacy. Another possible approach to improve our treatment paradigm would be to add a checkpoint inhibitor to eradicate suppressive regulatory T cells prior to priming with RT and initiating an effector immune response with CPMV. Anti-CTLA4 treatments have previously been shown to eradicate regulatory T cells and help illicit a robust immune response in syngeneic mouse breast cancer models.<sup>32</sup> Therefore, anti-CTLA4 checkpoint blockade may also eradicate suppressive regulatory T cells in our ovarian cancer model and hence remove suppressive immune cell barriers prior to RT and CPMV. Furthermore, the checkpoint blockade has been shown to synergize with viral immunotherapy.<sup>33</sup> It is thought that the viral in situ vaccination approach augments other immunotherapies by expanding antigen-specific T cells.

#### 4. CONCLUSIONS

We have demonstrated the potent efficacy of RT + CPMV in a mouse model of serous ovarian cancer. Data indicate that the combination of RT + CPMV enhances efficacy over RT alone, and that this may be attributed to expansion of T cells within the tumors. Although it is clear that more studies are needed on the mechanism(s) of immune activation by CPMV and other viral nanotechnologies, our data indicate this to be a promising immunotherapy. In situ vaccination with plant-derived viral nanotechnologies has advantages over mammalian vectors or systemically administered checkpoint blockade: plant virus-based nanotechnologies are not infectious toward mammals; the localized treatment is safer than systemic administration of immunotherapeutic reagents; and there is no requirement to identify antigens in the tumor or relate those antigens to the patient's human leukocyte antigen. The combination of the viral in situ vaccine with RT may be a particularly powerful strategy because RT debulks tumors, providing a burst of tumor antigens in the context of immunogenic cell death, therefore, synergizing with the CPMV in situ immune stimulation that further augments antitumor immunity.

#### 5. EXPERIMENTAL SECTION

**5.1. Preparation of CPMV Nanoparticles.** CPMV was propagated in black-eyed pea plants (*Vigna unguiculata*) and isolated using previously reported protocols.<sup>13</sup>

**5.2. Cells.** The highly aggressive murine ovarian cancer cell line ID8-Defb29/Vegf-A<sup>14</sup> was maintained in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, and 100 U penicillin/streptomycin. The cells were stably transfected with luciferase to enable in vivo tracking. Luciferase was expressed using vesicular stomatitis virus-glycoprotein (VSVG) retroviral transduction of pBabe, and luciferase-expressing cells were selected with puromycin. In brief, plasmids VSVG and pBabe-puro-Luc (10 µg each) were added to GP2-293 packaging cells at 40% confluence in a 75 cm<sup>2</sup> flask (maintained in 7 mL of complete Dulbecco's modified Eagle's medium) following a 30 min incubation with 60 µL of TransIT (Mirus). GP2-293 cells and plasmids were a generous gift from Dr. William Schiemann, Case Western Reserve University. Following a 48 h incubation,

the medium was collected, filtered to remove cellular debris, and added to ID8-Defb29/Vegf cells in the presence of polybrene (8 µg/mL, Santa Cruz Biotechnology (SCBT)) in a ratio of 50:50 with modified RPMI 1640 medium typically used with this cell line. ID8-Defb29/Vegf cells were incubated with retroviral particles for 24 h and allowed to recover in fresh medium for an additional 24 h; cells expressing the plasmid were selected with puromycin for 5 days (5 µg/mL medium, SCBT). Expression of luciferase was confirmed by IVIS Spectrum BLI (PerkinElmer) imaging of cells in a 96-well plate in the presence of 15 µg of luciferin/well. Measured luminescence was found to be linearly correlated with cell number in the range of 5000–500 000 cells per well. The resultant cell line is referred to as ID8-Defb29/Vegf-A-Luc.

**5.3. Tumor Model.** Animal studies were carried out using IACUC-approved protocols. Female C57BL/6 mice were obtained from Jackson Labs and used in these experiments at 8–10 weeks of age. Mice were shaved, and 5 million ID8-Defb29/Vegf-A-Luc cells in a 50:50 mixture of PBS cell suspension and matrigel were injected subcutaneously in the right flank. Once tumors were approximately 100–150 mm<sup>3</sup>, mice were randomized into four treatment groups ( $n = 4$ ): radiation (RT) alone, RT + CPMV, CPMV alone, and PBS placebo injections. RT was delivered on day 0 via a Cs-137 irradiator (Shepard Mark I; dose rate ~2.75 Gy/min) to a total dose of 10 Gy with a source aligned with the tumor and the rest of the animal shielded with lead blocks. On day 1 and then once weekly for a total of five treatments, 100 µg of CPMV in  $\alpha$  µL of PBS or the same volume of PBS were injected intratumorally. Tumor growth was monitored using caliper measurements as well as by luminescence imaging of the luciferase-expressing cancer cells. For the latter, mice were injected intraperitoneally with luciferin (15 mg/mL, 150 µL) and imaged 5 min post-injection with a 3 min exposure time using a PerkinElmer IVIS Spectrum in vivo imaging system. Total luminescence was determined using Living Image software, and total counts per mouse were graphed.

**5.4. Immunohistochemistry.** Primary antibody for CD4 was purchased from TONBO Biosciences, purified antimouse CD4 (clone GK1.5) and CD8 from eBioscience, and purified antimouse CD8a (clone 53-6.7). IHC was performed on tumor sections from the mice of each treatment group to characterize and quantify tumor immune cell infiltrate on day 1 and 10, following the initiation of treatment using a previously established protocol.<sup>15</sup> Briefly, tumors were surgically dissected and immediately cryopreserved in optimal cutting temperature compound. Tissue sections were microsectioned and plated on slides. Frozen sections on slides were fixed with acetone and washed with PBS, and then antigen blocking was performed with 5% (v/v) rabbit serum. Next, primary antibodies were added to coat the tissue sample and allowed to incubate at 4 °C overnight. Slides were then washed with PBS and stained with immPRESS HRP anti-rat IgG peroxidase polymer detection kit (Vector Laboratories) and 3,3'-diaminobenzidine tetrahydrochloride solution. Sections were also stained with hematoxylin and washed with PBS, dehydrated with ethanol, cleaned with xylene, and finally permanently mounted. Slides were imaged using a Zeiss Axio Imager.Z1 inverted high-resolution microscope with motorized stage.

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### Author Contributions

R.P. and A.E.C. contributed equally. N.F.S., N.L.O., and R.P. conceived and designed the experiments; A.E.C. and R.P. performed the experiments; A.E.C., R.P., and N.F.S. analyzed the data; S.F. contributed analysis and writing; R.P. and A.E.C. wrote the paper with editing from N.F.S., N.L.O., and S.F.

### Notes

The authors declare no competing financial interest.

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