Superagonist IL-15-Armed Oncolytic Virus Elicits Potent Antitumor Immunity and Therapy That Are Enhanced with PD-1 Blockade

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Oncolytic immunotherapy is a promising novel therapeutic for cancer, and further preclinical studies may maximize its therapeutic efficacy. In this study, we construct a novel oncolytic vaccinia virus (vvDD-IL15-Rx) expressing a superagonist IL-15, a fusion protein of IL-15 and IL-15Ra. This virus, named vvDD-IL15-Rx, possesses similar replication efficiency as the parental virus vvDD yet leads to significantly more regression of the disease and extends the survival of mice bearing MC38 colon or ID8 ovarian cancer. This novel virus elicits potent adaptive antitumor immunity as shown by ELISPOT assays for interferon-gamma-secreting CD8+ T cells and by the rejection of antitumor immunity as shown by ELISPOT assays for intermediate assays with antibodies showed that this antitumor activity is highly dependent on CD8+ T cells but much less so on CD4+ T cells and NK cells. Finally, the combination of the oncolytic immunotherapy with anti-PD-1 antibody dramatically improves the therapeutic outcome compared to either anti-PD-1 alone or vvDD-IL15-Rx alone. These results demonstrate that the IL-15-IL-15Rx fusion protein-expressing OV elicits potent antitumor immunity, and rational combination with PD-1 blockade leads to dramatic tumor regression and prolongs the survival of mice bearing colon or ovarian cancers.

INTRODUCTION

Cancer immunotherapy has made a breakthrough over the last 5 years. Yet, despite all the efforts in immunotherapy, only a minority of human patients benefit from these novel therapeutic regimens. Studies have shown that systemic potent antitumor immunity is required for significant efficacy of cancer immunotherapy.1 However, the tumor microenvironment (TME) is highly immunosuppressive and limits both innate and adaptive antitumor immunity and promotes tumor growth.2 In addition, the TME impacts the infiltration efficiency of tumor-specific T cells.2,3

Oncolytic virus (OV)-mediated cancer therapeutics are considered to be a novel type of immunotherapy.4,5 The cancer cell death mediated by OVs is considered a form of immunogenic cell death (ICD).6,7 This process of ICD provides multiple immune signals to activate the immune system. First, virally mediated cell death leads to the presentation of potent danger signals (signal 0) and tumor-associated antigens in context with major histocompatibility complex (MHC) class I antigens (signal 1) to dendritic cells (DCs) and other antigen-presenting cells. Then, viruses often induce inflammation and stimulate the production of cytokines and chemokines that activate and attract the immune cells into the TME (signals 1 and 2). These four signals, together with costimulatory signal (signal 2), elicit potent antitumor innate and adaptive immunity.5,8 To enhance the potential efficacy of oncolytic therapy, investigators have designed various strategies to boost antitumor immunity, e.g., expressing genes to enhance tumor immunogenicity,9–12 cytokines to stimulate antitumor immunity,11,13 or in combination with other regimens of immunotherapy.14–16

Interleukin-15 (IL-15) is a cytokine capable of promoting survival, proliferation, and activation of natural killer (NK), NKT cells, and CD8+ T cells,17 as well as CD56+ myeloid DCs.18 In particular, IL-15 can potently and selectively stimulate memory CD8+ T cells.19,20 Despite the fact that it is a cytokine related to IL-2, it possesses contrasting roles in adaptive immune responses.21 A number of studies indicate that IL-15 expressed in the TME may induce rejection of large tumors in a process mediated by T cells.22 One unique property of IL-15 is that its bioactivity in vivo is conferred mainly through a trans-presentation mechanism, in which IL-15 is presented in complex with the α-subunit of soluble IL-15 receptor (IL-15R) to target cells such as NK, NKT, or T cells, rather than interact directly with membrane-bound IL-15R.23,24 With this understanding, investigators have recently generated IL-15 agonists that consist of IL-15 and partial or complete soluble IL-15R to improve
We and others have been developing highly tumor-selective and enhanced antitumor activity in a B16 melanoma model. In addition, an IL-15 superagonist promotes not only immune responses of effector and memory CD8+ T cells, but also antitumor activity of PD-1 blockade.30 Investigators have explored the possibility of using an OV to express IL-15. Indeed, an IL-15-expressing oncolytic vesicular stomatitis virus has been shown to induce strong antitumor immunity in a murine colon cancer model.31 An OV armed with IL-15 and RANTES can enhance the function of CAR T cells.32 Roy and associates33 have shown that an oncolytic myxoma virus expressing this IL-15-IL-15R fusion protein possesses an enhanced antitumor activity in a B16 melanoma model.

We and others have been developing highly tumor-selective and potent oncolytic vaccinia viruses (VVs) for cancer virotherapy.34 The virus vvDD contains two mutated viral genes encoding the thymidine kinase and vaccinia growth factor for improved tumor selectivity.35 This tumor-selective OV was proven to be safe in phase I clinical trials via either intratumoral injection or intravenous infusion. While some clinical responses were identified as a result of viral replication and oncolysis without a therapeutic transgene, an improvement in efficacy is still desired.36,37 To enhance the therapeutic effect, our approaches include arming the virus with genes encoding chemokines, such as CXCL11,31 and combining it with immune checkpoint blockade for synergistic efficacy.15

In this study, we have constructed an oncolytic VV encoding the murine fusion IL-15-IL-15Rz (vvDD-IL15-Rz). We hypothesized that oncolytic VV expressing bioactivity-enhanced IL-15-IL-15Rz complex could not only induce potent oncolytic effects with ICD, but also activate both T and NK cells and modulate the immune TME in favor of antitumor immunity. As a result, it would exhibit a superior antitumor activity and prolong the life of tumor-bearing mice. In this context, it is interesting to note that IL-15 and other γ-chain-sharing cytokines (IL-2, IL-7, and IL-21) induce the expression of programmed death-1 (PD1) and its ligands (PD-L1).38 Thus, the strategy of using PD-1 blockade in combination with this IL-15-armed OV might greatly improve the therapeutic outcome. Our current study has validated the hypothesis, and this new OV provides a superior therapeutic efficacy in both colorectal and ovarian cancer models.

RESULTS
Construction and In Vitro Characterization of the New Oncolytic VV vvDD-IL15-Rz
We constructed a new OV that expresses murine IL-15-IL-15Ra fusion gene in the backbone of a highly tumor-selective oncolytic VV vvDD. This new virus has been named vvDD-IL15-Rz (Figure 1A). First, we would verify the expression of the therapeutic gene IL-15 fusion protein. CV-1 cells were mock infected, infected with vvDD or vvDD-IL15-Rz at an MOI of 1.0, and conditioned media were harvested at 48 hr after infection. The quantity of IL-15-IL15Ra fusion protein was determined by ELISA assay (Figure 1B). As we see, fusion protein reached 747 pg/mL in the media collected from vvDD-IL15-Rz-infected cells, while not detectable in medium from control vvDD-YFP-infected cells. Then we compared replication efficiency of this novel virus with a parental one in cancer cells. We infected MC38-Luc murine colon cancer cells with vvDD-IL15-Rz or parental virus vvDD at MOIs of 0.1 and 1 and then measured the replication efficiency by monitoring viral expansion over time using plaque assays. As shown in Figures 1C and 1D, the two viruses followed almost identical kinetics of progeny virus accumulation in MC38-Luc cancer cells. In addition, their oncolytic
potency in MC38 cancer cells was similar (Figure S1). In summary, these data demonstrated that inclusion of the fusion gene did not affect the functionality of the virus as a replicating OV and the viral construct appropriately secreted the fusion cytokine from infected cells in vitro.

**vvDD-IL15-Rα Infects MC38 Colon Cancer Tissue, Expresses the Superagonist, and Induces Immune Cell Infiltration in Vivo**

MC38-Luc tumors were established in C57BL/6 (B6) mice by injecting 5.0e5 MC38-Luc cells into the subcutaneous tissue of the right flank. Once tumors reached 5 mm in diameter, mice were treated intravenously with 1.0e8 plaque-forming units (PFU) of vvDD, vvDD-IL15-Rα, or just saline PBS. Tumor tissues were harvested 2, 4, and 6 days after treatment. Analysis by qRT-PCR of tumor homogenates showed viral infection of tumor tissues at all time points for vvDD and vvDD-IL15-Rα as evidenced by expression of A34R, a vaccinia coat protein gene (Figure 2A). Clearly, there was more accumulation of vvDD than vvDD-IL15-Rα (p < 0.05), suggesting that innate immunity activated by IL15-IL15Rα may facilitate the clearance of the virus. More importantly, the mRNA encoding the IL-15 fusion protein was expressed within tumor tissues of mice treated with vvDD-IL15-Rα at all examined time points (Figure 2B). These *in vivo* studies verified that both vvDD and vvDD-IL15-Rα were able to infect tumor implants *in vivo* and that the novel virus vvDD-IL15-Rα expressed the IL-15 fusion protein *in vivo*.

MC38-Luc tumor implants from mice treated with intravenous oncolytic VV were assessed for infiltration of immune cells after viral treatment with qPCR analysis. Both vvDD and vvDD-IL15-Rα induced expression of CD8+T cells (Figure 2C). vvDD-IL15-Rα notably increased expression of activating NK cell receptors NKp46 (Figure 2D) and NKG2D (Figure 2E) at day 4 and day 6, suggesting increased activated NK cells in the TME.

**vvDD-IL15-Rα Exhibits High Therapeutic Potency in Syngeneic MC38 Colon and ID8 Ovarian Cancer Models**

We then tested the therapeutic potency of this new virus in murine tumor models. The peritoneal MC38 tumor model mimics a significant population of colorectal cancer patients with peritoneal metastases. 5.0e5 MC38-Luc cancer cells were injected into the peritoneal cavity of B6 mice (day 0). When the tumors were well established (day 5), randomized groups of mice were treated with PBS, vvDD, or vvDD-IL15-Rα at 2.0e8 PFU/mouse, intraperitoneally. Again, tumor growth was monitored by bioluminescence imaging, and animal health was closely monitored by observations.
and body weight measurements. Animal survival was recorded (Figure 3).

On day 4 (1 day before treatment), the tumor size, indicated by bioluminescence, was uniform in all groups. On day 9 (4 days after treatment), the mice in the control group had significant tumor mass on imaging. The parental virus vvDD displayed potent tumor growth inhibition; however, vvDD-IL-15Ra treatment resulted in a significant decrease in tumor mass (Figure 3A). vvDD initially inhibited tumor growth, yet tumor progressed at later time points (days 23 and 30). However, vvDD-IL15-Ra exhibited potent and long-term effects, with little tumor growth throughout 30 days of imaging (Figure 3B). When the animal survival was presented as a Kaplan-Meier survival curve, vvDD had a significant therapeutic effect, yet vvDD-IL15-Ra displayed far improved therapeutic effects, including 80% long-term cured mice (Figure 3C).

We performed a similar experiment with a syngeneic ID8 ovarian peritoneal cancer model (Figure 4). Three and a half million ID8-luc cells were injected into B6 mice, and tumor growth was monitored by bioluminescent imaging. On day 11, three randomized groups of mice were treated with PBS, vvDD, or vvDD-IL15-Ra. As demonstrated in Figure 4A, vvDD treatment resulted in significant growth inhibition, and vvDD-IL15-Ra treatment significantly decreased tumor mass compared to vvDD on day 25. In Figure 4B, it is apparent that vvDD inhibited tumor growth compared to control, but vvDD-IL15-Ra significantly reduced tumor mass compared to vvDD. The Kaplan-Meier survival curve demonstrates a similar pattern of potencies for the two OVs in this ovarian cancer model as observed in the previous colon cancer model (Figure 4C). Overall from both tumor models, it is clear that vvDD-IL15-Ra demonstrates marked anti-cancer therapeutic efficacy.

**vvDD-IL15-Ra Induces Potent Adaptive Anti-tumor Immunity**

After demonstrating potent antitumor efficacy of this novel virus, we explored the mechanisms of action. First, we assessed the number of CD8+ T cells and their anti-tumor activity, collected from peritoneal cavity lavage 10 days after viral treatment (Figure 5). We observed that about 10% of the total i.p. cells were CD8+ T cells in the PBS-treated mice. However, in mice treated with either virus, the
percentage significantly increased to about 30% (Figure 5A). This may indicate that the inflammation caused by the virus results in a significant infiltration of CD8+ T cells into tumor tissue and vicinity. We examined the anti-tumor activity of these CD8+ T cells by interferon (IFN)-γ ELISPOT assay, in the presence or absence of irradiated MC38-Luc cells (Figure 5B). The CD8+ T cells harvested from PBS control group showed very few IFN-γ + spots, indicating few tumor-specific activated CD8+ T cells. The vvDD virus-treated mice did show increased yet very limited numbers of IFN-γ + spots. On the other hand, vvDD-IL15-Rx-treated mice showed a dramatically enhanced number of IFN-γ + spots, indicating significantly more tumor-specific activated CD8+ T cells (Figures 5B and 5C). The vvDD-IL15-Rx-treated mice which had been cured with a single treatment (n = 4) were re-challenged with parental MC38-Luc cells. The mice were completely resistant to re-challenge (Figure 5D). These results demonstrate that this superagonist protein in the context of an OV potently stimulates anti-tumor adaptive immunity.

To determine which major types of immune cells are needed for the virus-induced anti-tumor activity, we then repeated the murine experiments by depleting NK, CD4+, and CD8+ cells using antibodies (Figure 5E). The dosing and timing of the antibody administration were done under the conditions where the efficiency of cell depletion reached in previous studies.13 In addition, we have done monitoring of efficacy of cell depletion in small groups of mice to show the depletions of immune cell types worked in the current study. For example, we showed that CD3+CD8+ T cells had been reduced 80%-90% at 27 days post-first-injection of the anti-murine CD8 antibody.

Under these conditions, tumors rapidly progressed as expected in control-treated mice, while mice treated with vvDD-IL15Rz had delayed progression. When CD8+ cells were depleted in the mice treated with the OV, tumors progressed more rapidly, especially when compared to mice with depletion of NK and CD4+ T cells (Figure 5F). Correspondingly, the control mice had a median survival of 18 days, while the mice treated with vvDD-IL15-Rx all had a significant survival benefit. The depletion of CD8+ cells led to significantly reduced survival compared to other viral treatments (median survival 47 days), however, the depletion of either CD4+ cells or NK cells had minimal effects, with median survival greater than 150 days (data not shown). These results demonstrated that CD8+ T cells play a critical role for the therapeutic efficacy mediated by this IL-15 superagonist-expressing OV.

**Rational Combination of the OV with PD-1 Blockade Dramatically Improved the Therapeutic Efficacy**

During the molecular analysis of tumor immune microenvironment, we found that PD-1 is in the tumor tissue were significantly increased on days 4 and 6 after treatment with vvDD-IL15-Rx (Figure 6A; Figure S2A). It is interesting to note that PD-L1 was also significantly upregulated on day 2 (Figure S2B). In our previous study, we found that the combination of vvDD with PD-L1 blockade could work synergistically to improve therapeutic efficacy in both MC38 colon and ID8 ovarian cancer models.12 Here, we chose to explore whether the improved results with vvDD-IL15-Rx could be further enhanced with anti-PD1 therapy in MC38 tumor model. Again, both OVs had significant therapeutic effects, with greater results from vvDD-IL15-Rx (Figure 6B). The treatment with anti-PD-1 antibody also improved survival, and combination of vvDD with anti-PD-1 further improved the anti-tumor efficacy. When all groups are plotted together, the combination of vvDD-IL15-Rx plus anti-PD-1 antibody had the most dramatic effect, extending the survival of mice to over 200 days (p ≤ 0.001 compared to any other group). This results showed greatly improved survival when compared to the combination of vvDD-CXCL11 and anti-PD-1 antibody used in the tumor model in our previous study.13 In summary, the combination of
vvDD-IL15-Rα and anti-PD1 dramatically improved the therapeutic efficacy and survival of the mice bearing MC38 colon tumor.

**DISCUSSION**

The IL-15 superagonist, a fusion protein of IL-15 and IL-15Rα, has been previously shown to be superior to IL-15 in promoting adaptive antitumor immunity. Our highly tumor-selective OV, vvDD, has undergone two clinical trials and proved to possess a high safety profile, with some evidence of therapeutic efficacy in a subgroup of human cancer patients.36,37 To further improve its efficacy, we incorporated this IL-15-IL-15Rα fusion gene into vvDD, hoping to achieve the best combination of two classes of therapeutic agents. We have confirmed that this armed OV expressed the fusion protein from the virus-infected cells by ELISA assay and then in MC38 tumor tissues by RT-PCR.

We then moved to test its gene expression kinetics, potential safety, and efficacy in colon and ovarian tumor models in syngeneic mice.

We first examined the expression kinetics of the superagonist by qRT-PCR. In this model of MC38 colon cancer, the expression of IL-15 fusion protein (or mRNA) peaked on day 4 and declined to low levels by day 6. We observed increased expression of markers for NK and T cells, suggesting superagonist IL-15 functions to enhance the infiltration and/or activation of NK and T cells in the tumor tissues, when compared to the parental virus vvDD. The kinetics of the inflammatory response between vvDD-IL15-Rα and vvDD seem to be different. vvDD-IL15-Rα induces CD8 T cells rapidly, leading to rapid clearance of the virus and tumor cells. The parental virus (vvDD) activates the immune system with slower kinetics, probably reaching a plateau by day 6.

Then we examined the efficacy of the armed OV. In the intraperitoneal (i.p.) model of MC38 colon cancer, vvDD-IL15-Rα exhibited more potent antitumor activity and thus displayed better survival of mice initially bearing MC38-Luc tumors. Similar pattern of antitumor activity was also observed in ID8-Luc ovarian cancer in syngeneic
mice. Our study strongly suggests that IL-15 superagonist and OV together elicited highly potent antitumor CD8+ T cells, as shown by ELISPOT assay and parental tumor cell re-challenge (MC38 tumor) in the cured mice. Cell depletion experiments demonstrated that CD8+ T cells, but not CD4+ T cells or NK cells, are important in mediating antitumor activity under these conditions. In summary, this superagonist IL-15-expressing OV acts through CD8+ T cell-dependent adaptive antitumor immunity.

It is intriguing that this antitumor activity was independent of NK cells as one of the major functions of IL-15 is to activate NK cells in addition to T cells. There are several explanations for the result. One is that the timing of anti-Asialo GM1 antibody treatment (5 and 9 days after viral treatment) may not have been appropriate to have an effect on the rapidly activated NK cells that function with an early kinetics. The other possibility is that VV can inhibit NK cell functions in the TME through direct infection. The third possibility is that the virus encodes multiple genes whose products are to inhibit NK cells as well as other immune cells via inhibition of the nuclear factor κB (NF-κB) pathway. In this regard, it is tempting to explore the removal of these NF-κB inhibitors from this OV, as it may greatly enhance the therapeutic response.

OVs have demonstrated promising results in the treatment of cancer as showcased by the FDA approval of first-in-class drug talimogene laherparepvec (T-VEC). The combinatorial strategy for cancer therapy has been a well-recognized consensus for highly efficacious means among leading investigators. We and others have explored the combination of virotherapy with immune checkpoint blockade. In our previous study, we showed that the viral infection of cancer tissue caused inflammation and induction of PD-L1 and possibly PD1, and thus it was logical to combine an OV and anti-PD-L1 antibody for synergistic therapeutic effects in multiple tumor models. It has been shown that PD1 and PD-L1 could be induced by IL-15 and other common γ-chain-sharing cytokines. Indeed, we showed here that after treatment with vvDD-IL15-Rx, PD-1, and PD-L1 as well were upregulated in tumor tissues. It is not clear which cell types in the TME account for the increase in PD-L1, and further studies delineating the origin may be indicated. IL-15 is known to increase PD-L1, and this may account for the differences noted. When this OV was combined with anti-PD-1 blockade, we observed our most striking therapeutic efficacy ever, with all tested mice surviving for the duration of the experiment, over 200 days (Figure 6). To the best of our knowledge, this is the best therapeutic efficacy in this syngeneic tumor model among any treatment strategies.

One rationale for the combination with immune checkpoint blockade is that an OV often induces inflammation and upregulates PD-1 and/or PD-L1 in the tumor tissues. It is interesting to note this combination has been tested in human cancer patients, with two clinical studies combining an OV (GM-CSF-armed oncolytic herpes simplex virus) and one of the immune checkpoint blockers. In the first, a phase I study in patients with metastatic melanoma, the authors reported that the injection of T-VEC intratumorally in combination with systemic anti-PD-1 treatment resulted in enhanced T cell infiltration in the OV-injected tumor lesions and 62% response rate. In the second, randomized, open-label phase II study, ipilimumab, an anti-CTLA4 antibody, was used alone or in combination with T-VEC to treat advanced melanoma patients. The results showed that 39% in the combination arm and 18% in the ipilimumab alone arm had an objective response. Interestingly, responses were not limited to injected lesions. These studies demonstrate that the combination regimens are better than monotherapy in achieving higher objective responses in melanoma patients.
In summary, our current study has revealed that an OV armed with an activity enhanced superagonist IL-15 can elicit potent adaptive antitumor immunity and significant antitumor activity in two tumor models in mice, and the combination of this OV with anti-PD1 blockade exhibits striking therapeutic efficacy in a murine colon cancer model. While this data is based on a murine version of IL-15-IL-15Rz, the homologous human version can be substituted for easy translation into patients. Our results warrant clinical applications with such combination regimen of vvDD-IL-15Rz in cancer patients.

MATERIALS AND METHODS

**Mice and Cell Lines**

Female C57BL/6j mice (B6) were purchased from the Jackson Laboratory (Bar Harbor, ME) and were housed in the University of Pittsburgh animal facility under pathogen-free conditions. The Institutional Animal Care and Use Committee approved all animal studies and procedures performed. A murine colon cancer cell line transduced with a lentivirus expressing firefly luciferase gene, MC38-Luc, was previously used in the laboratory, and a murine ovarian cancer cells labeled with firefly luciferase, ID8-Luc, were obtained from Dr. Natasa Obermajer (University of Pittsburgh). HeLa and CV-1 cells, for viral amplification and titering, were originally obtained from ATCC (Manassas, VA). All cells were grown in DMEM supplemented with 1× penicillin and streptomycin (Invitrogen, Carlsbad, CA), 2 mM L-glutamine, and 10% fetal bovine serum (FBS) in an incubator at 37°C with 5% CO₂.

**Generation of vvDD-IL15-Rz**

The DNA fragment containing murine IL-15Ra sushi domain-IL-15 fusion gene was amplified via PCR from plasmid pBS-IL-15RLtdTomato, using primer set SalI-Sushi forward primer 5'-TACGCTAGCTATATAGTGGAGACAGACACACT-3' and Ascl-mIL-15 reverse primer 5'-TAGCCCGGCTTATCAGCTGGTGTTGATGAACATC-3'. Then the restriction enzyme (SalI and AscI)-digested DNA fragment was cloned into the respected sites in pCMS-IRES, a derivative of pCMS1 plasmid. This results in a new shuttle vector, pCMS-IL15-RMP1-14, BioXCell) was utilized to inhibit PD-1/PD-L1 interaction and was injected intraperitoneally (200 μg/injection) every other day for four doses total.

Long-term survivors of intraperitoneal MC38 tumor inoculation and vvDD-IL-15-Rz treatment were re-challenged with MC38-Luc tumor implants (1.0e5 cells/mouse) injected subcutaneously on the right flank 132 days after initial intraperitoneal tumor inoculation. Tumor growth was monitored via digital caliper volume measurement and compared to naïve B6 mice inoculated with MC38-Luc tumor implants at the same time. Tumor volume was calculated as: $V_{tumor} (mm^3) = 0.5 (L \times W^2)$.  

**Live Animal Bioluminescence Imaging**

Implanted tumor cells were originally transduced with lentivirus expressing firefly luciferase, allowing for in vivo live animal bioluminescence imaging with a Xenogen IVIS 200 Optical In Vivo Imaging System (Caliper Life Sciences, Hopkinton, MA), using well-established standard procedures. In brief, mice were injected peritoneally with 0.1 mL of 0.1 M D-luciferin, potassium salt (GoldBio, St Louis, MO), for 5–10 min before they were anesthetized and aligned in the compartment of the instrument for bioluminescence imaging (BLI). BLI was performed prior to virus or mock treatments to ensure that tumor implants were present and that groups had comparable in 1 mL of 2% FBS-containing DMEM media for 2 hr. The cells infected with virus at MOI 0.1 were then harvested 6, 12, 24, and 36 hr later, while the cells infected with virus at MOI 1 were harvested after 24, 36, 48, and 72 hr. Following harvest, the cell pellets were homogenized to release intracellular virions using Precellys 24 Tissue Homogenizer (Bertin Instruments, Rockville, MD). The viral load of cell lysates was then determined by viral plaque assay in CV-1 cells.
tumor burden. IVIS live animal BLI was then performed periodically following treatment to monitor tumor progression.

Assessment of Animal Health and Survival
Animal health status and survival was monitored closely. Abdominal girth of mice bearing intraperitoneal tumor implants was monitored with caliper measurement, and mice were sacrificed when girth exceeded 1.5× original measurements. Mice either succumbed to their disease or were sacrificed when abdominal girth exceeded allowable measurements as above. Mice with subcutaneous tumor implants were sacrificed when tumors reached maximum diameter of 2 cm, became ulcerated, and/or interfered with murine activity.

Tissue Homogenizer (Bertin Instruments, Rockville, MD).

RT-qPCR
RNA was isolated from tumor homogenates of subcutaneous MC38-Luc tumor implants using RNeasy kit (QIAGEN, Germantown, MD). cDNA was then created using from 2 μg of RNA using qScript cDNA SuperMix (Quanta Biosciences, Gaithersburg, MD) and Dyad Peltier Thermal Cycler (Bio-Rad, Hercules, CA). qPCR was then performed using TaqMan analysis with PerfeCTa qPCR SuperMix (Quanta Biosciences, Gaithersburg, MD) and Dyad Peltier Thermal Cycler (Bio-Rad, Hercules, CA). RT-qPCR

Statistical Analysis
GraphPad Prism version 7 (GraphPad Software, San Diego, CA) was used in creating graphs and analyzing data from both in vitro and in vivo studies. The data analysis was performed using non-parametric Student’s t test or two-way ANOVA. Survival of animals was assessed using Kaplan-Meier survival curves and was analyzed using log rank (Mantel-Cox) test. A p value of < 0.05 was considered statistically significant. The symbols used in figures were standard ones: *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001; and NS, not significant.

SUPPLEMENTAL INFORMATION
Supplemental Information includes two figures and two tables and can be found with this article online at https://doi.org/10.1016/j.ymte.2018.07.013.

AUTHOR CONTRIBUTIONS
S.J.K., Z.S.G., and D.L.B. conceived and designed the experiments and interpreted the data. S.J.K., Z.L., M.F., S.E.B., C.M., R.R., and E.D. performed the experiments. E.J.R. provided essential reagents. S.J.K., Z.S.G., and D.L.B. wrote the paper.

CONFLICTS OF INTEREST
D.L.B. has financial interests with SillaJen Biotherapeutics. The other authors declare no conflict of interest.

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