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Treating Tumors With a Vaccinia Virus Expressing IFNβ Illustrates the Complex Relationships Between Oncolytic Ability and Immunogenicity

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Since previous work using a nonreplicating adenovirus-expressing mouse interferon-β (Ad.mIFNβ) showed promising preclinical activity, we postulated that a vector-expressing IFNβ at high levels that could also replicate would be even more beneficial. Accordingly a replication competent, recombinant vaccinia viral vector-expressing mIFNβ (VV.mIFNβ) was tested. VV.mIFNβ-induced antitumor responses in two syngeneic mouse flank models of lung cancer. Although VV.mIFNβ had equivalent *in vivo* efficacy in both murine tumor models, the mechanisms of tumor killing were completely different. In LKRM2 tumors, viral replication was minimal and the tumor killing mechanism was due to activation of immune responses through induction of a local inflammatory response and production of antitumor CD8 T-cells. In contrast, in TC-1 tumors, the vector replicated well, induced an innate immune response, but antitumor activity was primarily due to a direct oncolytic effect. However, the VV.mIFNβ vector was able to augment the efficacy of an antitumor vaccine in the TC-1 tumor model in association with increased numbers of infiltrating CD8 T-cells. These data show the complex relationships between oncolytic viruses and the immune system which, if understood and harnessed correctly, could potentially be used to enhance the efficacy of immunotherapy.

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INTRODUCTION

Lung cancer is the leading cause of cancer-related death throughout most of the world, with >160,000 cancer deaths per in the US alone. Nonsmall cell lung cancer is the most predominant subtype of lung cancer, which contributes >80% of lung cancer cases. Despite the use of surgery, chemotherapy and radiotherapy, a 5-year survival of about 15% has remained unchanged for decades. New therapeutic strategies are clearly needed.

The use of type I interferons (IFNs) (the IFNα family and IFNβ) as potential antitumor agents was proposed many years ago. IFNs have multiple anticancer mechanisms that include: direct inhibition on tumor cell proliferation and angiogenesis; induction of tumor-specific cytotoxic T-cells; plus other immunoregulatory effects on antibody production, natural killer (NK) cell activation, macrophage function, delayed-type hypersensitivity, and major histocompatibility complex antigen expression.[1,2] Anticancer activity of type I IFNs has been demonstrated in patients with hematological malignancies (*e.g.*, hairy cell leukemia) and solid tumors (*e.g.*, renal cell carcinoma and malignant melanoma),[1] however, the results and overall efficacy have been modest. This may be due to intrinsic resistance to IFN-induced cell death, to the short half-life (~30 minutes) of intravenously or subcutaneously dosed IFN, to dose-limiting systemic toxicities, and/or to the development of neutralizing antibodies against recombinant IFN protein.

One strategy to improve the specificity and efficacy of type I IFNs is to express them using viral vectors. Our group and others have shown that delivery of type I IFNs using replication-incompetent adenoviral vectors (Ad.IFN) in a variety of animal models induces high local levels of IFN and results in strong antitumor immune responses that effectively eliminate tumors, including lung cancer.[3–5] Successful gene transfer using adenoviruses expressing IFNβ and IFNα2b to produce high levels and long-lasting IFN exposure to tumors has also been demonstrated in patients, and this has been associated with antitumor immune responses (such as activation of cytotoxic T-cells and NK cells, as well as generation of humoral responses) and promising clinical antitumor responses.[6–10] However, there are some limitations to the use of Ad.IFN. First, because of almost complete "first pass" uptake in the liver after intravenous injection, Ad vectors can only be given locally, thus limiting the range of applicable tumors to diseases such as malignant mesothelioma, brain tumors, and bladder tumors. Second, the vector can only be administered once (or twice within a very short three day window) because rapid generation of anti-Ad neutralizing antibodies rapidly inactivate the vector and prevent generation of IFNβ after repeated dosing.[6–10]

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One potential solution would be to develop alternative vectors. A vaccinia virus (VV) vector-expressing IFNβ was of particular interest for us, as the antitumor efficacy and safety of modified VV vectors have been demonstrated in other tumor models and recombinant VV vectors are being developed for clinical trials.[11–15] The *mouse IFNβ* gene was originally inserted into VV to enhance safety since many tumor cells are resistant to the antiviral effects of type I IFNs, whereas viral replication in normal tissue is limited.[11,12] Thus, production of IFNβ would theoretically not impede viral replication in the tumor, but would effectively prevent replication in normal tissues that have intact IFN-dependent antiviral defenses. However, to prevent VV-mediated neutralization of the IFNβ being produced, the vaccinia *B18R* gene (which binds and inactivates IFN) was deleted and the mouse *IFNβ* transgene was cloned into and replaced the *thymidine kinase* gene.[11] The resulting virus displayed highly selective replication in the tumor with very little gene expression in any other organs or tissues, including the liver. Moreover, several strategies have been reported that can allow repeat delivery of vaccinia to the tumor even in the face of an antiviral immune response. These include cell-based delivery within cytokine-induced killer cell carrier vehicles[16,17] and the incorporation of mutations that enhance production of the extracellular enveloped virus.[18] The extracellular enveloped virus form of the virus is shrouded in a host cell-derived membrane containing host cell complement control proteins, and so is relatively protected from complement or antibody-mediated neutralization.[12,18,19]

We therefore hypothesized that this oncolytic VV mutant (*TK⁻/B18R⁻/IFNβ⁺*) would be a better therapeutic than Ad.IFNβ for treating lung cancer, due to its oncolytic effect plus IFNβ-mediated anticancer activity.

RESULTS

Local and systemic administration of VV.mIFNβ induces therapeutic response in two mouse lung cancer models

We first determined whether vaccinia viral vector-expressing mIFNβ (VV.mIFNβ) could induce therapeutic responses in two lung cancer models. TC-1 and LKRM2, two mouse lung cancer cell lines, were inoculated subcutaneously into the right flanks of syngeneic immunocompetent mice. When tumor sizes reached 200 mm^3, a sublethal dose of VV.mIFNβ [10^8 plaque-forming units (pfu)][11] was given intratumorally and tumor measurements obtained. VV.mIFNβ was able to significantly ($P < 0.05$) slow tumor growth by ~40% in both mouse models (**Figure 1a,b**).

We then compared similar doses of VV.mIFNβ with our adenovirus-expressing mouse IFNβ (Ad.mIFNβ) vector in which antitumor efficacy in mouse lung cancer has already been demonstrated by our group.[3,4] A single dose of either IFNβ vector was given intratumorally to mice with large (200 mm^3) TC-1 and LKRM2 tumors. Consistent with the previous study,[4] Ad.mIFNβ significantly inhibited LKRM2 flank tumor growth (**Figure 1d**), however, at the dose used in this experiment (10^8 pfu), it had no antitumor activity against TC-1 flank tumor (**Figure 1c**). Since LKRM2 flank tumors spontaneously metastasize to the lung (**Supplementary Figure S1**),[20] we also compared the ability of each vector to inhibit lung metastases and found that intratumoral VV.mIFNβ (versus Ad.mIFNβ) was superior (**Supplementary Figure S2**). Given that the viruses made similar amounts of IFNβ (see below), these data suggested that the additional oncolytic effect of VV.mIFNβ might be active in the TC-1 model.

Another possible advantage of VV.mIFNβ over an adenoviral vector is that it could potentially show activity when given intravenously.[11] To test this, VV.mIFNβ was delivered either intratumorally or intravenously (via tail vein) to immunocompetent mice bearing large (200 mm^3), subcutaneous TC-1 or LKRM2 tumors. The results showed that intravenous administration was equally effective as intratumoral injection on inhibition of subcutaneous tumor growth in both models (**Figure 1e,f**). Both routes of VV administration were equally effective in inhibiting growth of lung metastases in the LKRM2 model (**Supplementary Figure S3**).

To summarize, the recombinant VV-expressing mouse IFNβ was effective against both our mouse lung cancer models when given either locally or systemically. Moreover, VV.mIFNβ was as effective as, or better than Ad.mIFNβ at the same pfu in the TC-1 model.

Differential replication of VV.mIFNβ in the two mouse lung cancer models: the role of the oncolytic effect

Given that VV.mIFNβ generated significant clinical responses in both mouse lung cancer models, we next investigated the antitumor mechanisms in each model. First, we examined how efficiently the virus replicated and killed tumor cells in culture, *i.e.*, the oncolytic activity. TC-1 and LKRM2 cells were infected with either a recombinant VV-expressing luciferase (VV.Luc; control vector with the same *TK⁻/B18R⁻* background) or VV.mIFNβ (test vector) at an multiplicity of infection (MOI) 1, and the cell viability was determined 48 hours postinfection. Interestingly, despite the similar *in vivo* antitumor activity of VV.mIFNβ in the two mouse tumor models, TC-1 cells were killed much more efficiently by both control and test vectors compared with LKRM2 cells (**Figure 2a**). There was slightly less killing in both cell lines with the VV.mIFNβ vector compared to the VV.Luc vector, which likely reflects that these two tumor cell lines are partially "IFN-responsive," as determined by their diminished susceptibilities to VSV-mediated killing in the presence of IFN (data not shown).

A likely explanation for the differences in VV-induced cell death is that VV's replicate more efficiently in TC-1 than in LKRM2 cells. Both lung cancer lines were thus infected with MOI 1 of VV.mIFNβ, and the infected cells were harvested over 72-hour period to check for viral titers with plaque assays, as well as viral gene expression by real-time PCR. As expected, VV.mIFNβ replicated efficiently in TC-1 cells, with the viral titer increasing by 4–5 logs over the first 48 hours (**Figure 2b**). Concurrently, the amount of VV *K1L* gene expression present in the infected cells following infection using real-time PCR increased linearly with time in TC-1 cells and reached the peak level 48 hours postinfection (**Figure 2c**). In contrast, the replication of VV.mIFNβ was limited in LKRM2 cells, with only approximately a one to two log increase over the first 48 hours (**Figure 2b**). Consistent with the viral titer

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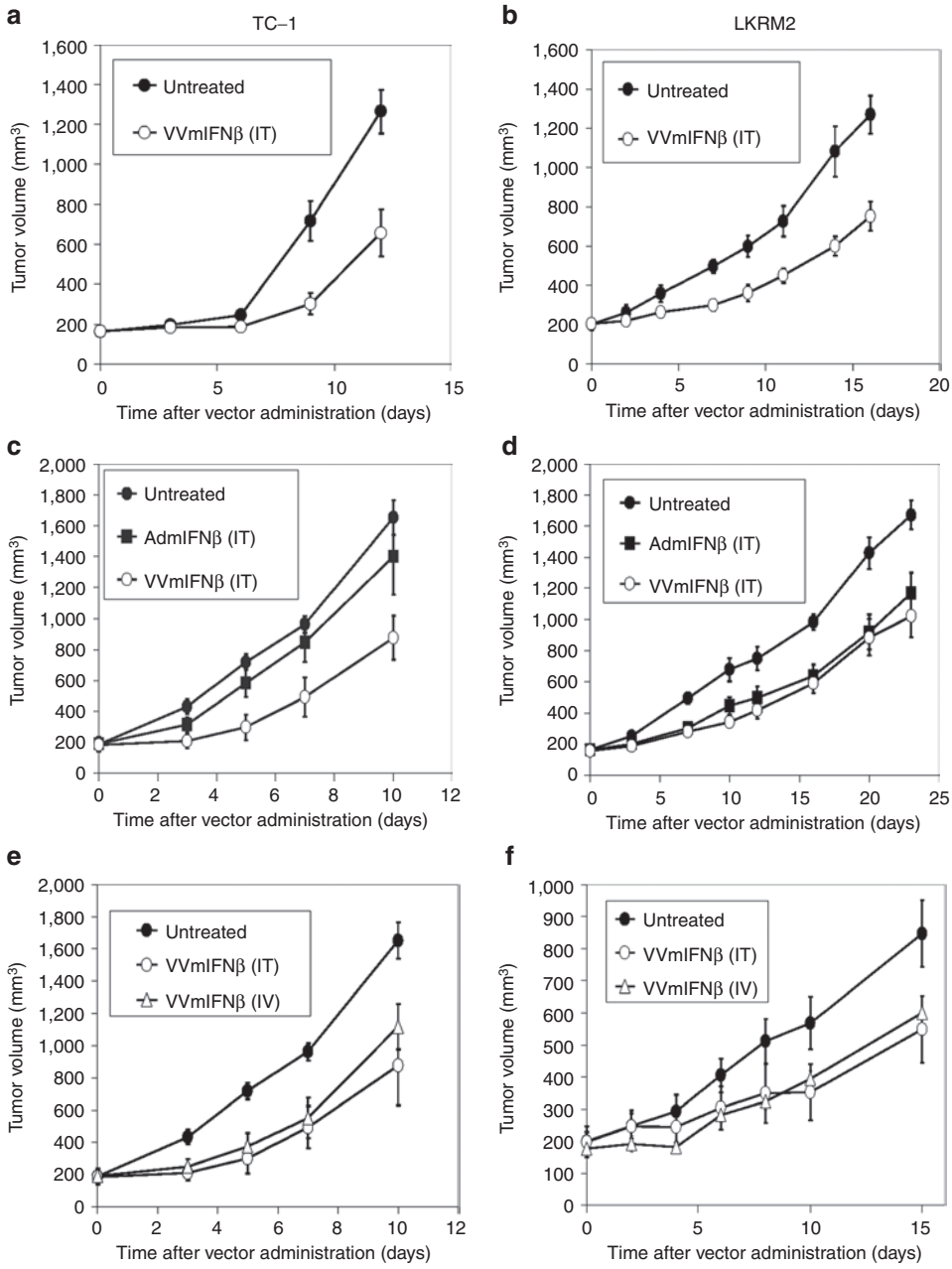

Figure 1 Therapeutic responses induced by vaccinia viral vector-expressing mIFNβ (VV.mIFNβ) and adenovirus-expressing mouse interferon-β (Ad.mIFNβ) in two mouse lung cancer models, TC-1 (left panel) and LKRM2 (right panel). Tumor cells were inoculated subcutaneously into the right flanks of syngeneic mice. When tumor reached 200 mm^3, a sublethal dose of VV.mIFNβ [10^8 plaque-forming units (pfu)] was given to mice either (**a**, **b**) intratumorally or (**e**, **f**) intravenously via tail vein. Antitumor efficacy of Ad.mIFNβ at the same dose, 10^8 pfu, was also compared with VV.mIFNβ in these (**c**, **d**) two mouse lung cancer models. The values are expressed as the mean + SEM (n = 6/group).

data, VV *K1L* gene expression detected in LKRM2 cells remained relatively constant (**Figure 2c**).

Given that the VV vector expresses the *IFNβ* gene, one would hypothesize that the IFNβ secretion would increase while the virus was replicating. We infected both TC-1 and LKRM2 cells with VV.mIFNβ and measured IFNβ protein level over time using enzyme-linked immunosorbent assay. The result is consistent with the viral replication data (**Figure 2d**). IFNβ protein level was maintained at high level in TC-1 cell culture over 72 hours, whereas IFNβ levels reached their peak at 6 hour and dramatically decreased in LKRM2 within the first 24 hours. We also compared the amount of IFNβ (area under the curve) induced by either Ad.mIFNβ or VV.mIFNβ in an IFN-nonresponsive mesothelioma cell line, and found that they produced similar amounts of IFNβ protein in culture (**Supplementary Figure S4**).

Taken together, these results indicate that in the *in vitro* system, the differential susceptibility of tumor cells to VV-mediated oncolysis is dependant on how well the virus replicates in those cells. The amount of IFNβ transgene expression also correlates well with the amount of viral replication in culture.

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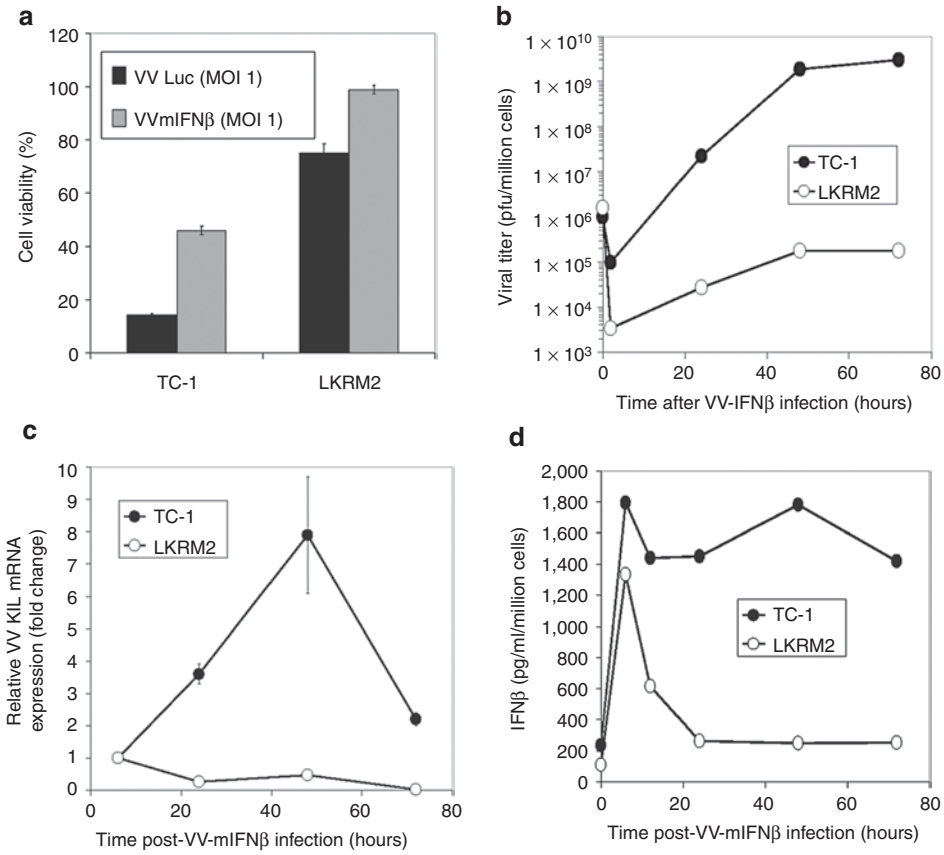

Figure 2 The ability of our vaccinia virus (VV) vectors to (a) lyse tumor cells, (b, c) replicates and (d) induce interferon-β (IFNβ) in culture. TC-1 and LKRM2 tumor cells were infected with either VV.Luc or vaccinia viral vector-expressing mIFNβ (VV.mIFNβ) at multiplicity of infection (MOI) of 1. The cell viability was then determined with MTT assays 24 hours following infection (**a**). The viral replication was determined using (**b**) plaque assay and (**c**) real-time PCR. One million of TC-1 or LKRM2 cells were infected with either VV.Luc or VV.mIFNβ vector at MOI of 1, and cells were harvested at various timepoints to determine the amount of the infectious virus. At the same time, the culture supernatants were collected and analyzed with enzyme-linked immunosorbent assay (ELISA) to measure IFNβ protein level (**d**).

Induction of IFNβ by VV.mIFNβ does not correlate with its ability to replicate in mice

We next conducted studies to evaluate virus replication and IFNβ secretion in immunocompetent mice bearing established TC-1 or LKRM2 tumors. VV.mIFNβ was injected intratumorally into TC-1 or LKRM2 tumors when the tumor size reached ~300 mm^3. Tumors were harvested 6 and 72 hours after injection, homogenized, and assayed for viral titer using plaque assays. Consistent with the *in vitro* data, the amount of virus recovered in TC-1 tumors increased by three logs within the first 72 hours (**Figure 3a**), while a log decrease in viral titer was seen in LKRM2 tumors (**Figure 3b**). We also conducted experiments to evaluate the role of the immune system in limiting viral replication. TC-1 and LKRM2 cells were inoculated into NOD/SCID/γ2 knockout (NSG) mice and VV.mIFNβ was injected into tumors when the size reached 250 mm^3. Overall, we did not see any significant changes in the amount of viral replication in either tumor cell line when comparing immunocompetent to immunodeficient mice (**Figure 3a,b**).

We also measured the amount of IFNβ production induced by VV.mIFNβ within infected tumors. We found that, in general, the IFNβ levels correlated well with viral titers in the tumors (**Figure 3c**), with high and prolonged levels seen in the TC-1 tumors. VV.mIFNβ induced significant early levels of IFNβ in LKRM2 tumors (which peaked at 6 hours), then subsequent lower levels that decayed relatively quickly (**Figure 3d**).

Evaluation of oncolytic versus immunological effects *in vivo*

Since we found that the antitumor activity induced by VV.mIFNβ in the replication-resistant LKRM2 model was not due to its oncolytic effect, we tested if host immunity was required. TC-1 and LKRM2 tumor cells were inoculated subcutaneously to immunodeficient NSG mice. Once tumors reached 200 mm^3, VV vectors expressing Luciferase or mIFNβ were injected intratumorally. Both VV.Luc and VV.mIFNβ vectors were still very effective in slowing TC-1 tumor growth in immunodeficient mice (**Figure 4a**). Consistent with its enhanced ability to replicate (see **Figure 2a**), the VV.Luc vector was even more effective than the VV.mIFNβ vector (**Figure 4a**). In contrast, in the LKRM2 model, both VV.Luc and VV.mIFNβ lost all of their antitumor efficacy in immunodeficient mice, indicating that intact host acquired immunity is required for antitumor efficacy in this model (**Figure 4b**).

To more precisely define the immunologic effector cells in VV.mIFNβ-mediated therapeutic activity, we selectively depleted CD8 T-cells by administering an anti-CD8 antibody to

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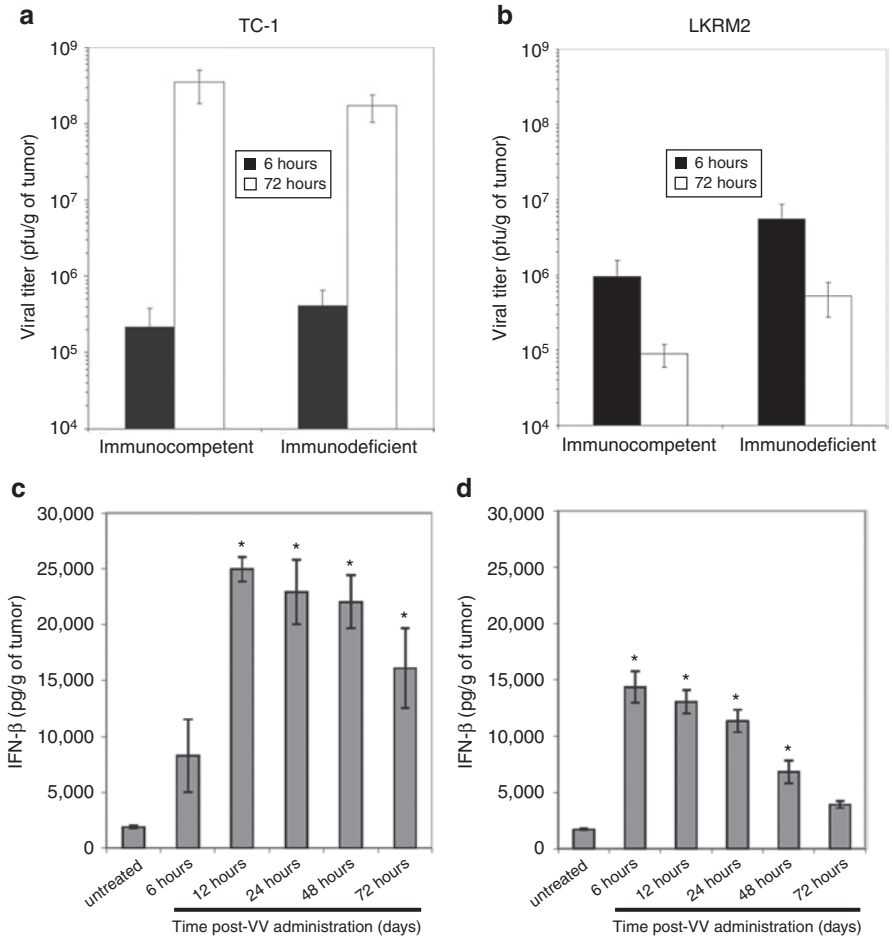

Figure 3 The ability of our vaccinia virus (VV) vectors to (a, b) replicate and induce (c, d) interferon-β (IFNβ) in flank tumors. (**a**, **c**) TC-1 and (**b**, **d**) LKRM2 tumor cells were inoculated into the right flanks of syngeneic immunocompetent C57Bl6 or hybrid mice, respectively. The NOD/SCID/γ2 knockout immunodeficient mice were used to study the role of immunological effect. When the tumors reached 300 mm^3, 100 million plaque-forming units (pfu) of vaccinia viral vector-expressing mIFNβ (VV.mIFNβ) were injected intratumorally. At various timepoints, tumor tissues were then harvested and assayed for viral replication with plaque assay (**a**, **b**) or IFNβ production with enzyme-linked immunosorbent assay (ELISA) (**c**, **d**). The values are expressed as the mean + SEM. *Denotes for significant induction of IFNβ above the untreated control ($P < 0.05$), which was analyzed with one-way ANOVA.

immunocompetent mice-bearing TC-1 or LKRM2 tumors before and after vector administration. FACS analysis confirmed the successful depletion of CD8 T-cells in these mice prior to virus administration (**Supplementary Figure S6a**). Similar to the data with immunodeficient mice, depletion of CD8 had no effect on the overall VV-induced antitumor activity in TC-1 model; we saw 48% versus 50% decreases in tumor growth in undepleted and CD8-depleted mice, respectively (**Figure 4c,e**). VV-induced antitumor activity in LKRM2 was dampened, but still significant, when CD8 T-cells were depleted: we saw a 48% decrease in tumor size in intact animals, but only a 20% decrease in tumor size in the CD8-depleted mice (**Figure 4d,f**). However, VV.mIFNβ completely failed to inhibit lung metastases in CD8-depleted mice (**Supplementary Figure S5**).

To further explore the immunologic mechanisms in the LKRM2 model, we also depleted other immune cell types after administration of VV.mIFNβ and assessed the influence the antitumor response. Anti-CD4, anti-asialo-GM, and anti-Ly6G antibodies were given to mice to deplete CD4 cells, NK cells, and neutrophils, respectively. Successful depletion was demonstrated with FACS analyses (**Supplementary Figure S6**). However, no effect on therapeutic efficacy was seen in these immune subset-depleted mice (**Supplementary Figure S7**). These data thus indicate that induction of antitumor CD8 T-cells by VV.mIFNβ in the LKRM2 model, but not CD4 cells, NK cells, or neutrophils, play an important (but not exclusive role) in the antitumor activity.

The role of IFNβ transgene in the overall antitumor activity of VV

Given our previous data with Ad.mIFNβ showing that almost all of the antitumor activity was due to expression of the transgene,[3,4] we had originally assumed that VV.mIFNβ would show more therapeutic benefits than the control VV.Luc vector. To test this hypothesis, immunocompetent mice bearing established TC-1 or LKRM2 tumors were given either VV.Luc or VV.mIFNβ intravenously. In contrast to our experience with adenovirus, we saw the antitumor effects were quite similar between the VV.Luc "control" vector versus the VV.mIFNβ vector in both TC-1 and

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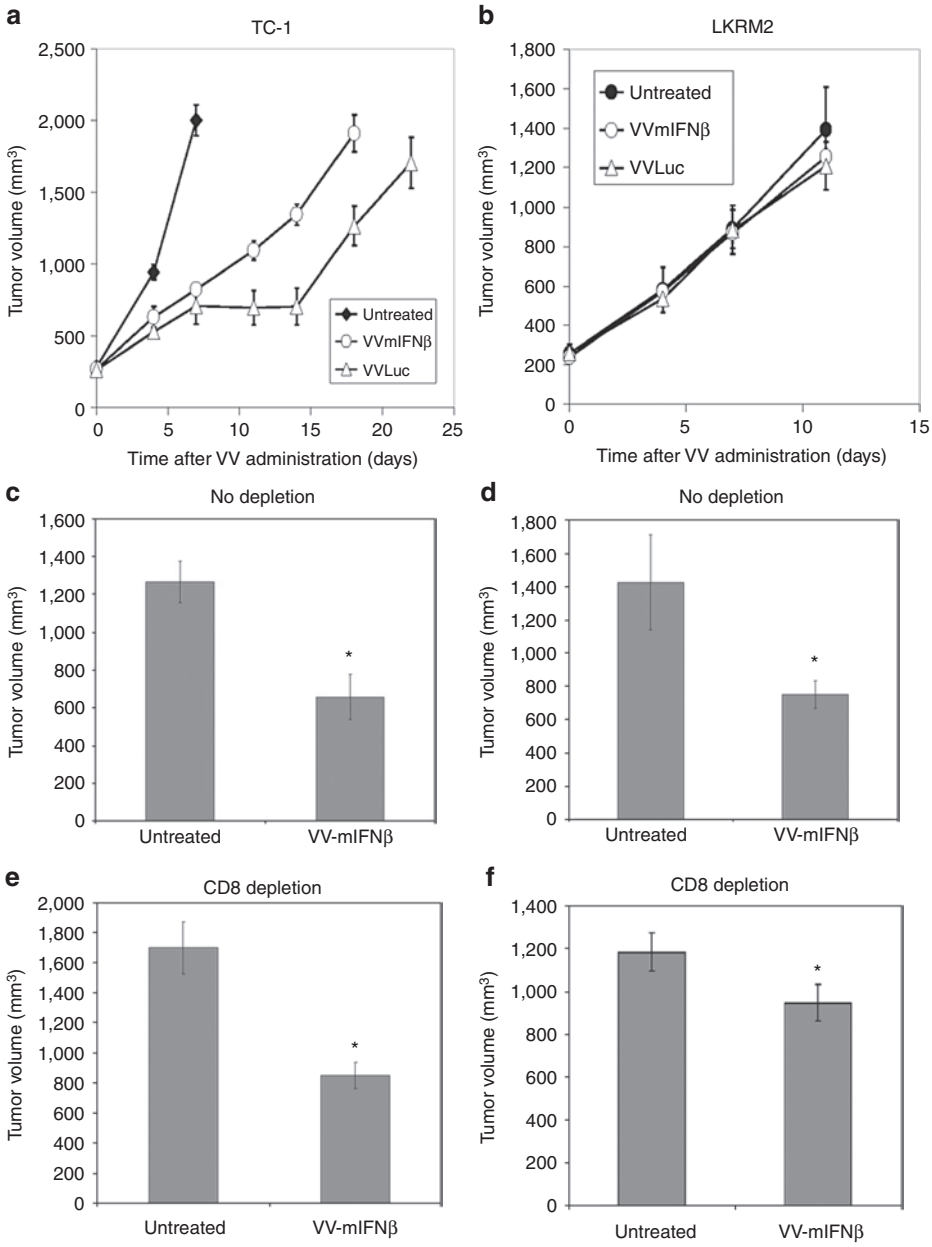

Figure 4 The role of host immune system on the antitumor efficacy of vaccinia virus (VV) vectors. (**a**) TC-1 and (**b**) LKRM2 tumors were inoculated into NSG immunodeficient mice. When tumor reached ~200 mm^3, VV.Luc and vaccinia viral vector-expressing mIFNβ (VV.mIFNβ) were then injected into mice intratumorally and tumor size was monitored twice a week. The role of $CD8^+$ cells on VV-mediated antitumor effect was also investigated by inoculating (**c**, **e**) TC-1 and (**d**, **f**) LKRM2 cells into syngeneic immunocompetent mice. Intraperitoneal injection anti-CD8 antibody was used to deplete $CD8^+$ cells. The timepoint chosen for the comparison was 10 days post-VV administration. The values on the bar charts are expressed as the mean + SEM ($n = 6$/group). *Denotes for significant induction of interferon-β (IFNβ) above the untreated control ($P < 0.05$), which was analyzed with Student's *t*-test.

LKRM2 tumor models (**Figure 5**). In addition, no difference was observed in inhibition of LKRM2 spontaneous lung metastases (**Supplementary Figure S8**).

To determine whether this observation was also true in another lung cancer model, we examined the anticancer efficacy of intravenous VV.Luc and VV.mIFNβ in an orthotopic lung cancer model driven by the activation of a *k-ras* mutation.[21] When examined 70 days after administration, animals given either VV.Luc or VV.mIFNβ had 100% survival when compared to 70% survival in control mice (**Supplementary Figure S9**). Neither treatment was curative, however, as all of VV-treated mice died by 100 days after vector administration, compared with the untreated mice who died within 80 days (data not shown).

Given the observation that VV can be immunogenic through its ability to induce inflammatory responses via type I IFNs and other mediators,[12] we measured the levels of eight different cytokines/chemokines, including IFNβ, IP-10 (CXCL10), I-TAC (CXCL11), monocyte chemoattractant protein-1 (MCP-1) (CCL2), tumor necrosis factor-α, interleukin-6, macrophage inflammatory protein 2 (MIP-2) (CXCL2), and KC (CXCL1), in both cell culture and

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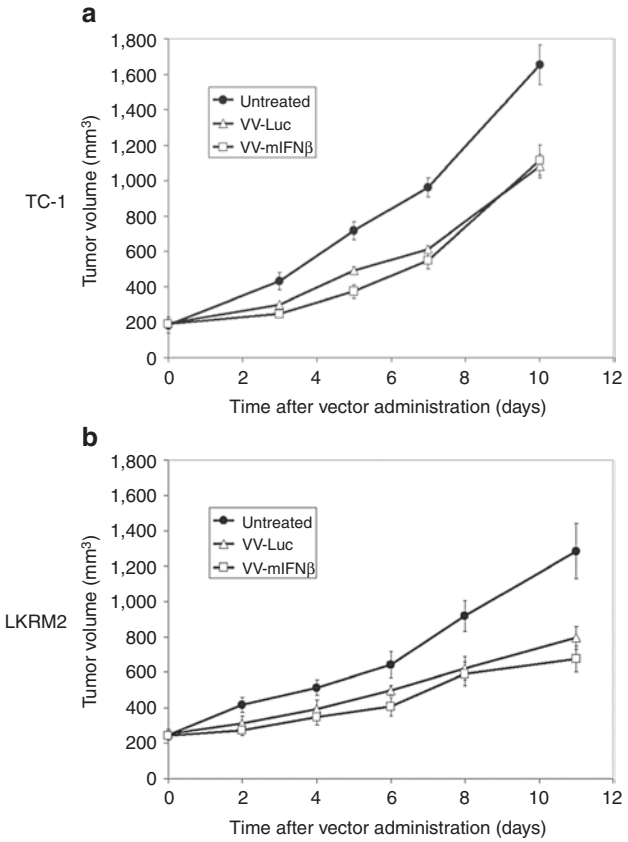

Figure 5 The role of interferon-β (IFNβ) transgene in the antitumor efficacy of our vaccinia virus (VV) vector. (**a**) TC-1 and (**b**) LKRM2 tumors were inoculated into the right flanks of syngeneic mice. When tumor reached ~200 mm^3, VV.Luc and VV.mIFNβ were then injected into mice intravenously and tumor size was monitored twice a week. The values are expressed as the mean + SEM (n = 6/group).

Table 1 Cytokine induction by VV.Luc or VV.mIFNβ in TC-1 cell culture

		6 Hours		48 Hours	
Cytokines	Untreated	Luciferase	IFNβ	Luciferase	IFNβ
IFNβ	45	410[a]	1,353[a]	334[a]	1,545[a]
IP-10	140	2,409[a]	4,379[a]	2,324[a]	4,309[a]
I-TAC	17	60	4,228[a]	87	33
MCP-1	184	766[a]	2,454[a]	2,632[a]	2,943[a]
TNFα	ND	ND	ND	ND	ND
IL-6	5	17	520[a]	142[a]	990[a]
MIP-2	119	155	163	129	138
KC	32	35	33	26	33

Abbreviations: IL-2, interleukin 2; IFNβ, interferon-β; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein 2; ND, not detectable; TNFα, tumor necrosis factor α; VV.Luc, VV-expressing luciferase; VV.mIFNβ, vaccinia viral vector-expressing mIFNβ.
The unit is expressed as pg/ml of cytokines per million cells. All the s.e. (not shown) are within 15% of the mean values.
[a]Denotes for statistically significant above the untreated control, based on one-way ANOVA analyses ($P < 0.05$).

Table 2 Cytokine induction by VV.Luc or VV.mIFNβ in LKRM2 cell culture

		6 Hours		48 Hours	
Cytokines	Untreated	Luciferase	IFNβ	Luciferase	IFNβ
IFNβ	33	108[a]	735[a]	74	391[a]
IP-10	19	36	2,899[a]	72	351[a]
I-TAC	29	16	33	11	8
MCP-1	142	2,481[a]	1,766[a]	769[a]	660[a]
TNFα	ND	ND	ND	ND	ND
IL-6	ND	2	45	99	129
MIP-2	41	42	44	39	32
KC	528	536	512	510	414

Abbreviations: IL-2, interleukin 2; IFNβ, interferon-β; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein 2; ND, not detectable; TNFα, tumor necrosis factor α; VV.Luc, VV-expressing luciferase; VV.mIFNβ, vaccinia viral vector-expressing mIFNβ.
The unit is expressed as pg/ml of cytokines per million cells. All the s.e. (not shown) are within 15% of the mean values.
[a]Denotes for statistically significant above the untreated control, based on one-way ANOVA analyses ($P < 0.05$).

in mice after infecting with VV.Luc or VV.mIFNβ at an MOI of 1. We saw significant increases in levels of IFNβ, interferon gamma-inducible protein-10 (IP-10), and MCP-1 in both cell lines with both vectors (**Table 1** and **2**), although the levels of IFNβ, IP-10, and I-TAC were the highest in the supernatants harvested from TC-1 cells treated with VV.mIFNβ.

Interestingly, in the mouse tumors, VV.Luc and VV.mIFNβ induced similar amount of many cytokines, including I-TAC, MCP-1, interleukin-6, MIP-2, and KC in tumors derived from both cell lines (**Table 3** and **4**). The main differences were in the levels of IFNβ and IP-10, especially in the TC-1 tumors. These results indicate that the VV.*TK*$^-$*/B18R*$^-$*/Luc* vector itself, appears to induce a significant amount of inflammatory responses, including an IFN response, within our lung cancer models. This may help to explain the lack of difference in therapeutic responses seen between VV.Luc- and VV.mIFNβ-treated groups.

We have preliminary data suggesting that it is the lack of the *B18R* gene that may be important in inducing inflammation in the VV.luc vector. Although not definitive, since the vector backbones are slightly different, we did note that another luciferase producing VV vector that retained the *B18R* gene, but had deletions of the VV growth factor (*VGF)* and *TK* genes (VV.(*VGF*$^-$/*TK*$^-$)-Luc), completely lost its antitumor effect in LKRM2 tumor model, although some efficacy was still seen in TC-1 model (**Supplementary Figure S10**). While this vector was able to replicate in both TC-1 and LKRM2 cells both *in vitro* and *in vivo* (**Supplementary Figure S11**), it did not induce much chemokine response in the infected tumors (**Supplementary Table S1**); in fact, we actually observed a reduction in most of chemokines tested within the infected tumor tissues. We are currently conducting more careful comparisons of VV's with and without the *B18R* gene to study the importance of virus-induced inflammation as part of the overall antitumor efficacy mediated by VV.

VV.mIFNβ, but not VV.Luc, augments the efficacy of immunotherapy

Given the ability of both vectors to strongly induce intratumoral production of inflammatory cytokines (like IFNβ) and T-cell attracting chemokines (such as IP-10), we hypothesized that if antitumor

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Table 3 Cytokine induction by VV.Luc or VV.mIFNβ in TC-1 tumors

		6 Hours		48 Hours	
Cytokines	Untreated	Luciferase	IFNβ	Luciferase	IFNβ
IFNβ	112	456	13,423[a]	1,006	11,152[a]
IP-10	3,647	8,732	27,020[a]	6,531	8,960
I-TAC	1,054	1,989[a]	1,631	1,656	822
MCP-1	6,974	15,202[a]	27,012[a]	27,375[a]	29,611[a]
TNFα	12	12	43	53	74
IL-6	16,250	32,026[a]	40,948[a]	38,149[a]	36,322[a]
MIP-2	2,311	4,249	5,443	6,795	3,909
KC	1,893	2,246	3,722	2,756	3,689

Abbreviations: IL-2, interleukin 2; IFNβ, interferon-β; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein 2; ND, not detectable; TNFα, tumor necrosis factor α; VV.Luc, VV-expressing luciferase; VV.mIFNβ, vaccinia viral vector-expressing mIFNβ.
The unit is expressed as pg/ml of cytokines per million cells. All the s.e. (not shown) are within 15% of the mean values.
[a]Denotes for statistically significant above the untreated control, based on one-way ANOVA analyses ($P < 0.05$).

Table 4 Cytokine induction by VV.Luc or VV.mIFNβ in LKRM2 tumors

		6 Hours		48 Hours	
Cytokines	Untreated	Luciferase	IFNβ	Luciferase	IFNβ
IFNβ	32	971	10,735[a]	747	4,171[a]
IP-10	393	755	1,023[a]	923[a]	1,224[a]
I-TAC	1,154	3,697[a]	3,595[a]	2,828[a]	2,763[a]
MCP-1	11,414	18,668[a]	19,914[a]	18,968[a]	17,739[a]
TNFα	24	21	73	36	27
IL-6	5,072	38,028[a]	30,593[a]	10,093	8,932
MIP-2	4,086	3,277	2,875	4,539	3,411
KC	2,255	20,141[a]	14,932[a]	12,734[a]	12,265[a]

Abbreviations: IL-2, interleukin 2; IFNβ, interferon-β; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein 2; ND, not detectable; TNFα, tumor necrosis factor α; VV.Luc, VV-expressing luciferase; VV.mIFNβ, vaccinia viral vector-expressing mIFNβ.
The unit is expressed as pg/ml of cytokines per million cells. All the s.e. (not shown) are within 15% of the mean values.
[a]Denotes for statistically significant above the untreated control, based on one-way ANOVA analyses ($P < 0.05$).

T-cells were first generated by a vaccine, subsequent administration of the VV vectors might serve to attract these cytotoxic T-cells to the tumors and enhance antitumor efficacy. To test this hypothesis, we combined a cancer vaccine for TC-1 tumors (which express HPV-E7 antigen), specifically an adenovirus-expressing HPV- E7 antigen (Ad.E7),[22] with our two VV vectors. A "prime" dose of Ad.E7 was injected to mice subcutaneously when tumor sizes were around 100–150 mm^3, and a "boost" dose was given a week later to generate activated T-cell response against TC-1 tumor antigen. A single intravenous injection of VV vector was administered 2 days after the second dose of Ad.E7, in an attempt to generate a chemokine gradient to attract the activated T-cells to the tumor site. Controls were also done with administration of just VV.luc or VV.IFNβ alone.

Two days later, we performed real-time PCR in a subgroup of tumors to look for CD8 mRNA message. We found that both VVs significantly increased the amount of CD8 mRNA, however, VV.IFNβ induced a much higher amount of CD8 mRNA in TC-1 tumors (22-fold compared to Ad.E7 tumors) than did the VV.Luc vector (a ninefold increase compared to Ad.E7 tumors (**Figure 6a**). The remainder of the tumors were harvested 2 weeks after VV administration (**Figure 6b**). Although all the treatments led to significantly smaller tumors than untreated controls, addition of VV.mIFNβ, but not VV.Luc, was able to significantly enhance the effect of this immunotherapy.

In summary, our data showed that the IFNβ transgene was associated with increased trafficking of CD8 T-cells into animals that had been vaccinated and was able to enhance the efficacy of this form of immunotherapy.

DISCUSSION

We report here that an attenuated VV-expressing IFNβ may be a promising therapeutic agent to treat lung cancer due to its ability to replicate and lyse tumors cells, to make high levels of IFNβ through either viral replication or induction of host immune responses, and its ability to induce other types of acute inflammatory responses within tumors. Moreover, this viral vector, unlike adenovirus, had efficacy after intravenous injection, suggesting that it can potentially be given systemically to target diffuse disease. However, this study also illustrates a number of interesting issues relating to the complexity of how these vectors exert their antitumor effects.

The first issue relates to the differential ability of VV to replicate within different tumor cell lines. The factors that determine whether an oncolytic vector will replicate well within tumors are still not known for certain. For some viruses, the presence or absence of an intact IFN-response pathway is highly predictive. Viral nucleic acids are often recognized by innate pattern recognition receptors such as Toll-like receptors, RIG-I-like receptors, double-stranded RNA-activated protein kinase, and melanoma differentiation-associated protein 5.[23] Binding of viral nucleic acids to these receptors result in type I IFN secretion with subsequent binding to the IFN receptor in an autocrine and paracrine fashion, that initiates the expression of a cohort of IFN-stimulated genes to inhibit virus replication through multiple mechanisms.[24] Numerous studies have demonstrated that many tumor cells have increased susceptibility to oncolytic viruses, including recombinant VV, if they have an impaired ability to respond to IFNs.[11,25,26] In our previous study with vesicular stomatitis virus (VSV), we were able to predict with a high degree of certainty whether the tumors would be susceptible to VSV-mediated oncolysis by the presence or absence of an intact IFN-response pathway. Although we did observe a small decrease in VV replication when our tumor cells were infected with VV.mIFNβ vector compared with the VV.Luc vector (**Figure 2a**), in contrast to our findings with VSV, IFN-responsiveness did not seem to be the major factor predicting whether or not the VV would replicate. This is consistent with considerable literature showing that VV is fairly resistant to inhibition by IFN, likely due to an extremely robust set of viral genes that inhibit all aspects of the IFN-response pathway (reviewed in ref. 27). Both TC-1 and LKRM2 are both somewhat IFN-responsive (with regard to supporting VSV growth), but were very different in their VV permissiveness. We also tested a mouse mesothelioma cell line, AB12, in which the IFN-response pathway was completely inactivated. To our surprise, the replication of VV in AB12 cells or tumors was limited, very similar to the pattern we saw in LKRM2 (data not shown).

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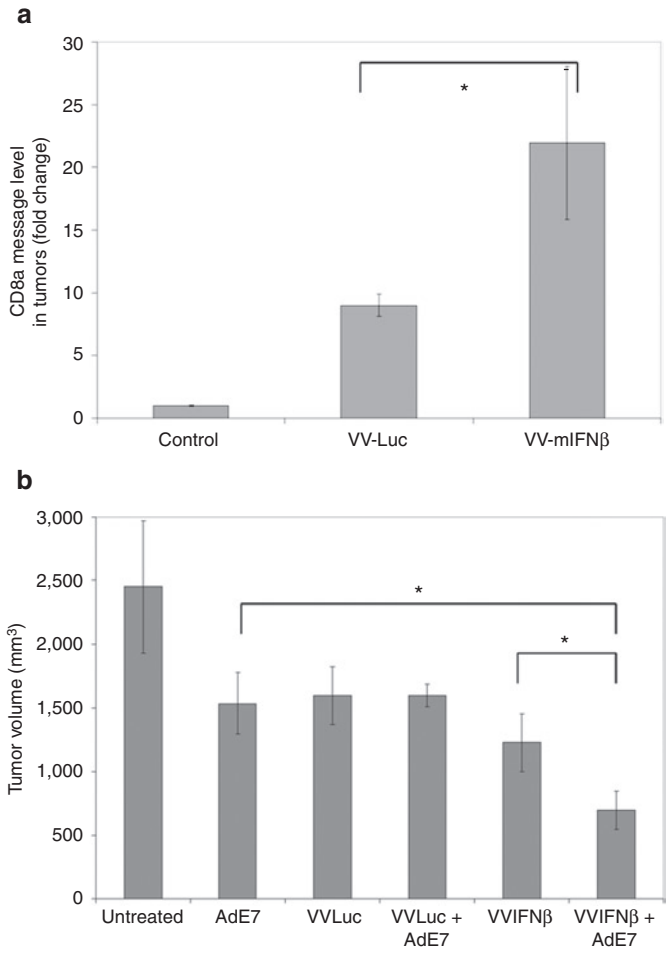

Figure 6 Vaccinia viral vector-expressing mIFNβ (VV.mIFNβ) enhances the efficacy of immunotherapy by recruiting more CD8 T-cells into tumors than VV.Luc. TC-1 tumor cells were inoculated into the right flanks of C57Bl6 mice. When tumors reached 150 mm^3, the first dose of Ad.E7 [10^9 plaque-forming units (pfu)] was given to the mice contralaterally to their flank tumors. A second boost dose of Ad.E7 vaccine was given 5 days later to induce antitumor immune response, and a single intravenous injection of VV.mIFNβ or VV.Luc was given to mice. Two days after **vaccinia virus (VV)** injection, some tumors were harvested to check for CD8 expression by (**a**) real-time PCR. The remainder of the tumors were measured over time, and the time point chosen was 2 weeks after VV administration for comparison (**b**). The values are expressed as the mean + SEM (n = 6/group). *Denotes significant difference between two groups, analyzed with Student's t-test ($P < 0.05$).

Ras activation has been demonstrated by many groups to enhance viral infection and replication of many viruses, including VV, VSV, HIV, and reovirus.[28–30] However, both cell lines used in this study have a mutated *ras* gene.[21,31] We are thus still unclear why we see the differential ability of VV to replication in our tumor cell lines. A better understanding of this question may help us to predict which patients might have the best antitumor efficacy with this virus.

An issue of even more interest relates to the observation that even though the VV.mIFNβ vector showed equivalent antitumor efficacy in two different lung cancer animal models (**Figure 1**), the mechanisms responsible for the effects seemed to be completely different. In the TC-1 model, VV.mIFNβ was able to efficiently replicate and produce high and prolonged levels of IFNβ (**Figures 2b–d** and **3a,c**). However, the antitumor efficacy seemed to be almost entirely due to the oncolytic effects, as there was no loss of efficacy in profoundly immunodeficient mice (**Figure 4a**). In contrast, in the LKRM2 model, VV was not able to replicate well, but produced moderately high levels of IFNβ in tumors (**Figures 2b–d** and **3b,d**). In this model, the antitumor effects seen appeared to be primarily CD8 T-cell-mediated, because all the efficacy was lost in immunodeficient mice and was significantly dampened in CD8-depleted mice (**Figure 4b,e,f**; **Supplementary Figure S8**), while other immune cells, such as CD4 T-cells, NK cells, and neutrophils, seemed to play little role in the VV-mediated antitumor activity.

A previous study using the same vector showed that the addition of IFNβ to the B18R-deleted virus increased efficacy to a small extent, but had the greatest effect on the clearance of the virus from normal tissues, rather than on increased antitumor effects.[11] In our study, it was of interest that we saw virtually identical antitumor effect when comparing the VV.mIFNβ vector with the control VV.Luc vector lacking the *B18R* gene (**Figure 5**). An explanation for this might be expected in the TC-1 model which was primarily, but not solely, dependent on viral replication. In this case, it might even be expected that the IFNβ produced by the VV.mIFNβ would actually decrease replication (and thus efficacy). As discussed above, however,

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the presence of IFNβ seemed to have only minimal antiviral effects. Less expected was the observation that the VV.Luc vector seemed just as efficacious as the VV.mIFNβ vector in the LKRM2 model (**Figure 5a**), where antitumor immune responses were responsible for efficacy. This observation is similar to that recently reported in a study of VSV encoding CD40L[32] where no difference was observed between VSV.GFP and VSV.CD40L. We believe the data in **Tables 1–4** provide a possible explanation for this finding of equivalent efficacy. In the LKRM2 model, both vectors induced initial innate immune responses marked by upregulation of number of proinflammatory cytokines and chemokines. Although the VV.mIFNβ vector induces more IFNβ and IP-10 at an early time point, a threefold increase in IFNβ was stimulated by the VV.Luc vector at 6 hours. Similar levels of strong induction of MCP-1, KC, and interleukin-6 were seen in both vectors at early and late time points. In contrast, when we studied a different VV control vector with a different backbone, deleted in the *VGF* and *TK* genes, we saw relatively little or no antitumor effect (**Figure 6b**) and no induction of proinflammatory cytokines (**Supplementary Table S1**).

These data suggest that this ability to induce intratumoral inflammation with subsequent generation of a CD8 T-cell-mediated antitumor response may be dependent on the viral backbone. In this study, we speculate that the lack of the *B18R* gene may have played just as important a role as the expression of the *IFNβ* transgene. Like all other viruses, VV encodes proteins that block the innate immune response to viral infection.[27] B18R, a soluble type I IFN-binding protein, can sequester the type I IFN produced in response to viral infection.[33,34] Our vector was purposely engineered to delete this key protein to prevent inactivation of the *IFNβ* transgene.[11] However, it appears that just the loss of this protein in a VV not carrying other transgenes enhances antitumor immune responses. Although there are very few studies using the B18R-deleted VV variant, a recent paper by Waibler and the colleagues[35] showed that the wild-type VV could inhibit systemic type I IFN responses, whereas the B18R-deleted variant actually induced IFN. We show here, for the first time, that this B18R-deleted VV not only induced IFNβ, but also stimulated production of IFN-inducible chemokines (*i.e.*, IP-10 and I-TAC) and other chemokines and cytokines that might aid in its antitumor efficacy. This is consistent with predictions that "more new functions will be found for already known genes" involved in blocking the IFN pathway.[27] Future investigations to examine the role of B18R gene expression or to regulate the VV-induced IFN response will be helpful for vector development, since our observations are limited by the fact that we did not directly compare the VV.(*B18R+/TK⁻*) and VV.(*B18R⁻/TK⁻*) vectors.

Although we did not see major differences in antitumor efficacy between the VV.Luc and the VV.mIFNβ vectors when given by themselves, we did note that the VV.mIFNβ vector was superior in attracting CD8 T-cells and augmenting antitumor efficacy when combined with a tumor vaccine in the TC-1 model (**Figure 6**). Although, the reason for this is not known for certain, the vectors did differ in the degree of induction of intratumoral IFNβ and in the IFN-dependent chemokine, IP-10, in the TC-1 model. Given the importance of type 1 IFNs and IP-10 in activating and attracting CD8 T-cells,[1,36,37] we speculate that these activities are responsible for the observed augmentation of efficacy of immunotherapy.

Vaccinia is know to be highly immunogenic, as seen during its use in the smallpox eradication program, and with its subsequent use in antigen-expressing vaccines for the treatment or prevention of a variety of diseases. However, it is also known to express multiple immune modulating virulence genes, many of which act as secreted decoy receptors for binding of Th1-associated cytokines.[27,35,38,39] It is therefore possible that the expression of these genes (including B18R) may limit the CTL response raised by the virus, and so could at least partially explain the lack of clinical success with vaccinia-based therapeutic cancer vaccines to date.[40] In order to enhance virally generated antitumor immune responses, there are a number of approaches being explored. One approach is to delete viral genes that encode for specific proteins to suppress host immunity.[33–35,39] The B18R gene, as discussed above, appears to be a good example.[33,34] A second approach involves addition of transgenes that can enhance the host immunity;[13,14,41] for example, the current promising VV vector-expressing GM-CSF in the phase II trials. Another strategy involves engineering tumor-associated antigens into the viral vector.[42] A final approach is illustrated by our study (**Figure 6**), where antitumor CD8 T-cells were generated with active immunotherapy (*i.e.*, a vaccine) and then the viral vector was used to induce cytokine or chemokines that would produce local inflammation (such as IFNβ and IP-10) leading to chemoattraction and potential activation or expansion of these vaccine-generated antitumor T-cells.

Our findings support the growing recognition of the complex interactions between oncolytic vectors and the immune system.[43] It is becoming increasingly clear that none of the available oncolytic viruses can effectively destroy large, established tumors through viral replication alone, but also need an accompanying antitumor immune response. If the immune reaction induced by the virus is too strong, replication can be totally inhibited and the immune response induced will be dominated by antiviral rather than antitumor responses. If the immune response to the virus is too weak, viral replication may be enhanced, but an effective antitumor immune responses may not be produced. As Prestwich and colleagues[44] have recently summarized, "the mechanisms of activity of oncolytic viruses involve direct oncolysis, vascular effects, and innate adaptive immunity. The extent of viral replication does not necessarily correlate with therapeutic efficacy. Immune interactions may be either beneficial or detrimental." Our data point out that differences in mechanisms can exist with the same virus, depending on the tumor cell line treated. This observation is also supported by a recent study showing that the same oncolytic VV can destroy the same tumor by different mechanisms depending on whether the mouse has been preimmunized or not.[16]

It should be noted that human cancer cells are, in general, more sensitive to direct oncolytic effect of VV than mouse cells in culture (data not shown). Whether or not VV-mediated antitumor activity in patients will be primarily due to oncolytic activity or immune effect still remains to be elucidated.

In conclusion, VV.mIFNβ was demonstrated here to be a promising therapeutic agent in two different lung cancer models when delivered intratumorally or intravenously. In one model, it

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has better effects than an adenovirus-expressing IFNβ. However, the mechanisms of antitumor effect were different. In one model, tumor killing was mediated by viral replication and oncolysis, whereas in the other model, efficacy was dependent on acquired antitumor immune response. We also found that a VV vector lacking the *B18R* gene was effective in inducing local tumor inflammatory response. The factors that enable VV to replicate in different lung cancer models is still not clear, but it was found not to be limited by the IFN-response status or *ras* mutation of the infected cells. An understanding of the complexity of virus-tumor-immune system interactions will be important to define as these vectors are moved into clinical trials in human cancer patients.

MATERIALS AND METHODS

Viruses. The VV.(*B18R*$^-$/*TK*$^-$) vectors encoding firefly *luciferase* (VV.Luc) or the *firefly luciferase* plus murine *IFNβ* (VV.mIFNβ) genes were generated as previously described.[11] In brief, the cloning plasmid pSC65 was modified so that the firefly *luciferase* gene was expressed from the strong synthetic early/late promoter (pSE/L) and the murine IFNβ (*IFNβ*) gene was expressed from the weak p7.5 early/late promoter. The plasmid was then used to insert the cDNA into the vaccinia *TK* gene of *B18R*-deleted strain of Western Reserve VV by homologous recombination. Successful recombination events were selected for by luciferase expression, and correct insertion of plaque-purified clones was verified by PCR. Viral stocks were then amplified in human HeLa cervical cancer cells, purified with 0.05 µm MicroKros filter (Cat no. X10S-100-04N; Spectrum Laboratories, Rancho Dominguez, CA) in addition to traditional sucrose cushion, and were titered using standard plaque assay in green monkey BSC-1 kidney cells. The VV (*VGF*$^-$/*TK*$^-$) vector encoding firefly luciferase was also generated in similar manner as previously described.[45] The E1/E3-deleted type 5 adenoviral vector-encoding murine IFNβ (Ad.mIFNβ was previously generated by the Vector Core of the Institute for Human Gene Therapy (University of Pennsylvania Medical Center).[46]

Cell lines. Mouse TC-1 lung cancer cells were derived from mouse lung epithelial cells immortalized with human papillomavirus-16 E6 and E7 and transformed with the c-Ha-ras oncogene,[31] and maintained in RPMI1640 medium (Gibco; Invitrogen, Carlsbad, CA) supplemented with 10 % fetal bovine serum (Georgia Biotechnology, Atlanta, GA), 2 mmol/l L-glutamine and 1% penicillin/streptomycin. The original mouse "LKR" cell line was obtained as a gift from Dr Friedberg (University of Pennsylvania School of Medicine). These cells were derived from an explant of a pulmonary tumor from an activated *Kras*G12D mutant mouse grown in Dr Tyler Jacks' lab at M.I.T. (Boston, MA).[21] The LKRM2 cells were derived in our lab from spontaneous lung metastases in a mouse bearing subcutaneous LKR tumor. LKRM2 cells are more aggressive than its parental LKR cells in terms of forming lung metastases in mice, and they were maintained in high glucose DMEM (Gibco; Invitrogen) supplemented with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin. Green monkey kidney BSC-1 epithelial cells and human HeLa cervical carcinoma cells were purchased from ATCC (Manassas, VA), and they were also maintained in DMEM with 10% fetal bovine serum, L-glutamine, and penicillin/streptomycin.

Animals. Pathogen-free C57BL/6 mice, hybrid (C57BL/6J female × 129P3/J male F1) mice, and NOD/SCID/IL2 receptor γ2 chain knockout (NSG) mice were purchased from Charles River Laboratories (Wilmington, MA), Jackson Labs (Bar Harbor, ME) and Wistar Institute (Philadelphia, PA) respectively. Animals used for all experiments were female mice between 6 and 10 weeks old, and were housed in a pathogen-free animal facility at Wistar Institute. All protocols were approved by the Animal Use Committees of the Wistar Institute and University of Pennsylvania, and were in compliance with the guide and care and use of animals.

Animal studies. To create flank tumors, mice were injected with 1.2×10^6 of TC-1 or 2×10^6 of LKRM2 cells subcutaneously on the right hind flank. Unless otherwise stated, a single intratumoral dose (10^8 pfu) in 100 µl of saline) of viral vectors (Ad.mIFNβ, VV.Luc, or VV.mIFNβ) was administered to mice when tumors reached ~200 mm^3 in size. For some experiments, VV vectors were injected through tail vein. Tumors were measured two to three times per week with calipers, and mice were monitored for toxicity. Mice were euthanized if toxicity was evident or tumor burden exceeded 2,500 mm^3. All experiments had at least five mice per group and were repeated at least two times.

The LKRM2 cell line was also used to study the effect of VV vectors on distant diseases, which has been previously described.[20] This cell line was produced from a spontaneous lung metastasis formed in a LKR tumor-bearing mouse, and after two *in vivo* passage cycles, a stable line was established with a reproducible pattern of spontaneous lung metastatic induction (>40% of untreated mice in 5 weeks). Hybrid (B6X129/J1) mice were injected on the right flank with 2×10^6 LKR-M cells. After flank tumors reached an average size of 200 mm^3, mice were randomized to the untreated group or VV-treated group. Forty days after tumor inoculation, the mice were killed and their lungs were excised, separated into discrete lobes, and weighed. Sections were cut from each of the five lobes and stained with hematoxylin and eosin. In each lung, the total number of nodules was counted, and the total tumor area measured and divided by the total area of the lung section, using Image J (National Institutes of Health, Bethesda, MD) software. These evaluations were performed by a technician blinded to the group origin of the lung.

To deplete CD8$^+$ T-cells systemically prior to administration of VV vectors, mice received intraperitoneal injections of 200 µg of purified monoclonal antibodies from the anti-CD8 hybridoma 53-6.72 (Harlan Bioproducts, Indianapolis, IN). Injections were administered 2 days before virus injection. Thereafter, a maintenance dose of antibody was injected intraperitoneally twice a week throughout the entire experimental period to ensure depletion. Cell depletion was confirmed by flow cytometry of splenic suspensions at different time points. We used the similar approach to deplete other immune subsets, including CD4, NK, and neutrophils. The anti-CD4 antibody was purified from anti-CD4 hybridoma GK1.5 (ATCC), and anti-asialo (GM1; NK) and anti-Ly6G (1A8; neutrophils) antibodies were purchased from Wako Pure Chemical Industries (Osaka, Japan) and BioXcell (West Lebanon, NH), respectively. Successful depletion was confirmed with flow cytometry from splenic population.

The orthotopic lung cancer model using intranasal Ad.Cre administration in transgenic K-ras mice has been previously described in detail.[4] Briefly, to activate the conditional oncogene and induce tumors, 100 µl of saline with 3×10^{10} particles of adenovirus containing Cre recombinase (Ad.Cre) were administered to LSL *KrasG12D* mice intranasally. Four weeks after instillation of Ad.Cre, mice were treated with 10^8 pfu of VV.Luc or VV.mIFNβ intravenously. Mice were checked daily to monitor their health status. Animals were sacrificed when they exhibited any sort of distress or shortness of breath.

For the combination studies with the Ad.E7 cancer vaccine, mice-bearing TC-1 tumors (~200 mm^3 in size) were vaccinated subcutaneously in the left flank (contralateral to the tumor) with 1×10^9 pfu of Ad.E7 vector.[22] Five days following the initial vaccination, mice received a booster vaccine of 1×10^9 pfu of Ad.E7 in the left flank. Two days later, VV-Luc and VV-mIFNβ were injected into corresponding mice intravenously via tail vein. Tumors were monitored three times weekly until they reached large size.

MTT assays. To perform MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assays, TC-1 and LKRM2 cells were plated in quadruplicate on 96-well plates (5,000 cells/well in RPMI or DMEM medium with 10% fetal bovine serum) and were infected with MOIs of 1 of the VV-Luc and VV-mIFNβ viruses. Viability was assessed 72 hours postinfection by

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developing the reaction assay per manufacturer's instruction (Promega, Madison, WI). Optical density was read at 570 nm and corrected using a background control value.

Plaque assays. To evaluate the ability of VV-mIFNβ to replicate in lung cancer cells, TC-1 and LKRM2 cells were infected at an MOI of 1, and after a 2-hour absorption period, the inoculum was removed, and the monolayer washed with phosphate-buffered saline (PBS). The cell lysates and supernatants were collected at various time points (6, 24, 48 and 72 hours; unless stated otherwise), and titered with standard plaque protocol on BSC-1 cells and visualized with 0.05% crystal violet (wt/vol). For tumor samples from mice, TC-1 and LKRM2 tumors were harvested at various timepoints following intratumoral injection of VV-mIFNβ. Tumors were then homogenized in Tris–HCl buffer (pH 9) with tissue grinder (Tissuemiser; Fisher Scientific, Waltham, MA) till all material was in suspension. Tumor lysates were subjected to three freeze-thaw cycles and centrifuged to pellet debris. The resulting supernatants were then tittered on BSC-1 cells.

RNA extraction and real time-PCR. To collect total RNA for reverse transcription-PCR after VV.mIFNβ infection, TC-1, and LKRM2 cells (10^6 cells/3 ml/well) were seeded in 6-well plate, incubated overnight, and then infected with VV.mIFNβ at an MOI of 1. Two hour postinfection, the viral inoculum was removed, cells were washed twice with PBS, and 2.5 ml of supplemented RPMI medium was applied in each of the flasks. At various timepoints the culture medium was removed, cells were washed twice with PBS, and one ml of TRIzol Reagent (Invitrogen) was added to each well to lyse cells. Total RNA were isolated using the protocol provided by the manufacturer, followed by removal of contaminating genomic DNA by DNAse I treatment (Roche Molecular Biochemicals, Indianapolis, IN). High-quality RNA was confirmed by running an aliquot of each sample on a denaturing formaldehyde/agarose/EtBr gel.

Quantitative analysis of mRNA expression was performed using real-time reverse transcriptase-PCR. Three micrograms of total RNA were reverse transcribed to cDNA, using Oligo$(dT)_{15}$ primer (Promega) and SuperScript III reverse transcriptase (Invitrogen), following the protocol provided by the manufacturer. Synthesized cDNA was normalized to β-actin or GAPDH mRNA levels. Relative VV *K1L* gene expression (fold-change in VV.mIFNβ-treated samples versus control) was determined, which gives a 131-base pair PCR amplified product. The sequence for forward primer of *K1L* gene is: GGACACGTGGATATGATGATTCTC, and the one for the reverse primer is: TGAACAGAGCCTGTAACATCTCAAT. The annealing temperature for this primer pairs is 60 °C. Each sample was run in quadruplicate and the experiment was repeated at least once using the using Smart Cycler System (Cepheid, Sunnyvale, CA).

Cytokine production measured with enzyme-linked immunosorbent assays. For culture supernatants, TC-1 and LKRM2 cells (10^6) were plated in 6-well plates in triplicate and were incubated in 3 ml of supplemented RPMI or DMEM medium overnight. On the next day, the cells were infected with different VV vectors at MOI 1 in a total volume of 1 ml. After 2 hours, the inoculum was aspirated, cells were washed with PBS twice and 3 ml of culture medium were applied. The culture supernatants were collected, spun for 10 minutes at 13,000 r.p.m. to remove detached cells and kept at −80 °C till cytokine analyses. For tumor samples from mice, TC-1 and LKRM2 tumors were harvested at various timepoints. Tumors were weighed, and lysis buffer (PBS with 1% Triton-X (FisherBiotech, Fair Lawn, NJ), 1 mmol/l phenylmethanesulfonyl fluoride (cat no. P7626-5G; Sigma-Aldrich, St Louis, MO) plus other protease inhibitors (Complete Mini Protease inhibitors; cat no: 13999600; Roche Diagnostic, Mannheim, Germany) were added according to the tumor weight (100 mg/ml of lysis buffer). All the enzyme-linked immunosorbent assay protocols provided by the manufacturers were followed: including IFNβ (cat no: 42400-1; PBL Interferonsource, Piscataway, NJ), IP-10 (cat no: Duoset DY466; R&D Systems; Minneapolis, MN), CXCL11 (cat no: Duoset DY572; R&D Systems), MCP-1 (cat no: 555260; BD Biosciences, Franklin Lakes, NJ), KC (cat no: Duoset DY453; R&D Systems), tumor necrosis factor-α (cat no: 555268; BD Biosciences), interleukin-6 (cat no: 555240; BD Biosciences) and MIP-2 (Duoset DY452; R&D Systems).

Statistical analyses. For the reverse transcriptase-PCR and flank tumor studies comparing differences between two groups, we used unpaired Student *t*-tests. For flank tumor studies comparing more than two groups, we used one-way ANOVA with appropriate *post hoc* testing. Kaplan–Meier data were analyzed using Epi Info software developed by Centers for Disease Control and Prevention (Atlanta, GA). Differences were considered significant when $P < 0.05$. Data are presented as mean ± SEM.

SUPPLEMENTARY MATERIAL

Figure S1. Spontaneous lung metastases formed in LKRM2 flank tumor model.

Figure S2. Effect of Ad.mIFNβ and VV.mIFNβ on spontaneous lung metastases in LKRM2 model.

Figure S3. Effect of different routes of VV.mIFNβ administration on spontaneous lung metastases in LKRM2 model.

Figure S4. IFNβ production by Ad.mIFNβ and VV.mIFNβ in AB12 mouse mesothelioma cells.

Figure S5. Effect of CD8 depletion on VV.mIFNβ-induced lung metastases growth inhibition.

Figure S6. Flow cytometry analyses of splenic population upon depletion of various immune subsets.

Figure S7. Effect of CD4, NK, and neutrophil depletion on VV.mIFNβ-mediated antitumor response.

Figure S8. Comparison of VV.Luc- and VV.mIFNb-induced growth inhibition on spontaneous lung metastases.

Figure S9. VV.Luc and VV.mIFNβ induced moderate antitumor response in a mutated *k-ras* orthotopic model.

Figure S10. Loss of antitumor efficacy of VV vector with *VGF/TK* deleted backbone.

Figure S11. Replication of VV.(*VGF*$^-$/*TK*$^-$).Luc *in vitro* and *in vivo*.

Table S1. Chemokine induction by VV.(VGF$^-$/TK$^-$).Luc in TC-1 and LKRM2 tumors.

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