

T-cell Engager-armed Oncolytic Vaccinia Virus Significantly Enhances Antitumor Therapy

Feng Yu¹, Xingbing Wang¹, Z Sheng Guo², David L Bartlett², Stephen M Gottschalk^{1,3,4,5} and Xiao-Tong Song^{1,3}

¹Center for Cell and Gene Therapy, Texas Children's Hospital, The Methodist Hospital, Baylor College of Medicine, Houston, Texas, USA; ²Department of Surgery, University of Pittsburgh Cancer Institute, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; ³Department of Pathology and Immunology, Baylor College of Medicine, Houston, Texas, USA; ⁴Department of Pediatrics, Baylor College of Medicine, Houston, Texas, USA; ⁵Texas Children's Cancer Center, Texas Children's Hospital, Baylor College of Medicine, Houston, Texas, USA

Oncolytic vaccinia virus (VV) therapy has shown promise in preclinical models and in clinical studies. However, complete responses have rarely been observed. This lack of efficacy is most likely due to suboptimal virus spread through the tumor resulting in limited tumor cell destruction. We reasoned that redirecting T cells to the tumor has the potential to improve the antitumor activity of oncolytic VVs. We, therefore, constructed a VV encoding a secretory bispecific T-cell engager consisting of two single-chain variable fragments specific for CD3 and the tumor cell surface antigen EphA2 (EphA2-T-cell engager-armed VV (EphA2-TEA-VV)). *In vitro*, EphA2-TEA-VV's ability to replicate and induce oncolysis was similar to that of unmodified virus. However, only tumor cells infected with EphA2-TEA-VV induced T-cell activation as judged by the secretion of interferon- γ and interleukin-2. In coculture assays, EphA2-TEA-VV not only killed infected tumor cells, but in the presence of T cells, it also induced bystander killing of noninfected tumor cells. *In vivo*, EphA2-TEA-VV plus T cells had potent antitumor activity in comparison with control VV plus T cells in a lung cancer xenograft model. Thus, arming oncolytic VVs with T-cell engagers may represent a promising approach to improve oncolytic virus therapy.

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INTRODUCTION

Oncolytic vaccinia viruses (VVs) are promising anticancer agents owing to their ability to infect, replicate in, and lyse tumor cells and spread to other tumor cells in successive rounds of replication.^{1,2} Oncolytic VVs' major mode of action is the destruction of tumor cells, which may then induce antigen-specific T-cell responses against the tumor thereby targeting metastatic disease even after local injection of virus. Oncolytic VV therapy has shown promise in preclinical models and in clinical studies. Complete clinical responses have, however, been rarely observed.³⁻¹⁰ This is most likely due to the limited virus spread through the tumor and limited activation of antitumor T-cell responses by oncolytic VVs.¹⁻¹⁰

T cells play a critical role in controlling tumor growth. There is increasing evidence that T cells are able to control tumor growth and survival in cancer patients, both in early and late stages of the disease. For example, adoptive transfer of T cells has been demonstrated to effectively treat disseminated tumors, including Hodgkin's lymphoma, nasopharyngeal carcinoma, neuroblastoma, and melanoma.¹¹⁻¹⁵ However, tumor-specific T-cell responses are difficult to induce and sustain in cancer patients and are limited by numerous immune escape mechanisms of tumor cells selected during immunoeediting.^{16,17} T-cell engager (CD3-scFv, single-chain variable fragment) provides an alternative approach to engage T cells for cancer therapy. CD3-scFv has been used in bispecific T-cell engagers (BiTEs) that consist of a CD3-scFv and a scFv specific for a tumor cell surface antigen.¹⁸⁻²⁶ Clinical studies have shown that infusion of CD19-specific BiTEs results in highly effective killing of tumor cells in patients with non-Hodgkin's lymphomas and B-cell precursor acute lymphoblastic leukemia.²⁴⁻²⁶

In this article, we report the development of a T-cell engager-armed oncolytic VV (TEA-VV) encoding secretory bispecific T-cell engagers (TEs) that bind both to human CD3 and a tumor cell surface antigen EphA2 (EphA2-TEA-VV). Upon infection of EphA2-positive tumor cells, EphA2-TEA-VVs activated T cells and induced T-cell killing of viral infected and noninfected tumor cells, indicative of "bystander killing". *In vivo*, EphA2-TEA-VV plus T cells had greater antitumor activity than that of unarmed VV plus T cells in a lung cancer xenograft model.

RESULTS

TEs induce killing of tumor cells

To investigate the ability of TEs to induce bystander killing of tumor cells, we first generated recombinant lentiviral vectors expressing a secretory bispecific EphA2-specific TEs (EphA2-TE; Lv-EphA2-TE), a membrane-bound CD3-specific TE (Lv-mTE), or GFP (Lv-GFP) (**Supplementary Figure S1A**). The EphA2-positive lung cancer cell line A549 was transduced with Lv-EphA2-TE, Lv-mTE, or Lv-GFP. Virus-infected A549 (GFP⁺) cells were mixed with noninfected A549 (GFP⁻) cells and cocultured with nonstimulated peripheral blood mononuclear cells (PBMCs). After 48 hours, the presence of tumor cells was determined using immunofluorescence and fluorescence-activated cell sorting (FACS) analysis

Correspondence: Xiao-Tong Song, Center for Cell and Gene Therapy, BCM505, Baylor College of Medicine, Houston, TX 77030-2316, USA. E-mail: xsong1@bcm.edu

(Supplementary Figure S1B–D). Flow analysis demonstrated that Lv-EphA2-TE induced killing of both virus-infected A549 (GFP⁺) and noninfected A549 (GFP⁻) cells, whereas Lv-mTE only induced killing of virus-infected A549 (GFP⁺) cells. These results indicate that the lentivirus encoding the secretable EphA2-TE induced bystander killing of noninfected tumor cells.

Generation of EphA2-TEA-VV and GFP-VV

We generated double-deleted VVs (vvDD, Western Reserve strain) expressing EphA2-scFv-CD3-scFv (EphA2-TEA-VV) or GFP (GFP-VV) (Figure 1a). To investigate whether EphA2-TEA-VV-infected tumor cells express secretory bispecific EphA2-TE, A549 cells were transduced with EphA2-TEA-VV or GFP-VV at a multiplicity of infection (MOI) of 5. After 24 hours, media were collected and mixed with unstimulated human PBMCs followed by FACS analysis using EphA2-FITC (EphA2 protein labeled with fluorescein isothiocyanate) as a probe to detect CD3-bound EphA2-TE. CD4⁻ and CD8⁺ T cells were positive for EphA2-FITC indicating that EphA2-TEs are secreted from infected A549 cells and are able to bind CD3 and EphA2 (Figure 1b).

EphA2-TEs expression does not impair the ability of VV to replicate and induce tumor cell lysis

To demonstrate that EphA2-TEs do not impair the ability of EphA2-TEA-VV to replicate, CV-1 cells, A549 tumor cells, and normal human skin fibroblasts were infected with EphA2-TEA-VV, GFP-VV, or parental VV (VSC20). Infection of CV-1 (Figure 2a) and A549 (Figure 2b) with EphA2-TEA-VV, GFP-VV, or VSC20 yielded similar amounts of virus at various time points. By contrast, all three viruses replicated poorly in normal human skin fibroblasts (Figure 2c). Thus, EphA2-TEs do not interfere with VV replication. Next, we compared the ability of EphA2-TEA-VV or GFP-VV to induce tumor cell lysis in the absence of human T cells. A549 cells were transduced with EphA2-TEA-VV or GFP-VV at increasing MOIs (0, 0.01, 0.1, 1, or 5) and 48 hours post virus infection, A549 cell viability was determined using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. A549 cells were killed with increasing MOIs regardless of the VV used (Figure 3a). There was no difference between EphA2-TEA-VV and GFP-VV, indicating that the expression of

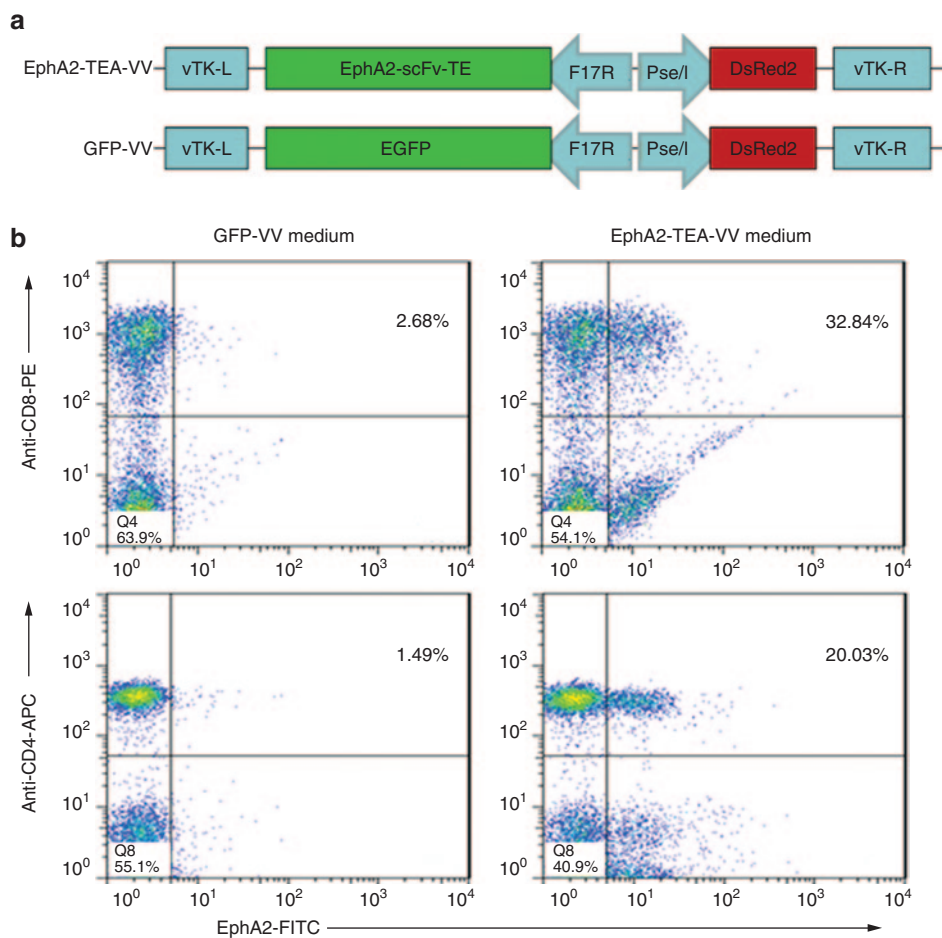


Figure 1 The EphA2-TEA-VV express functional EphA2-T-cell engagers. **(a)** Scheme of expression cassettes encoding EphA2-scFv-CD3-scFv (EphA2-TEA-VV) or GFP (GFP-VV). **(b)** EphA2-TEA-VV-infected cells secrete bispecific EphA2-scFv-CD3-scFv with the ability of binding to T cells and EphA2. A549 tumor cells ($1 \times 10^6/2$ ml/well in 6-well plate) were infected with EphA2-TEA-VV or GFP-VV at a multiplicity of infection of 5. The culture media was collected after 24 hours of incubation and added to 1×10^6 unstimulated peripheral blood mononuclear cells (PBMCs), followed by staining with EphA2-FITC, CD8-PE, and CD4-APC. EphA2-FITC, EphA2 protein labeled with fluorescein isothiocyanate; EphA2-TEA-VV, EphA2-T-cell engager-armed vaccinia virus; PE, phycoerythrin; scFv, single-chain variable fragment.

EphA2-TEs does not interfere with the ability of VV to induce tumor cell lysis.

EphA2-TEA-VVs redirect human T cells to EphA2-positive A549 cells

To determine whether EphA2-TEA-VVs redirect human T cells to A549 cells, cells were infected with EphA2-TEA-VV at increasing MOIs (MOI 0.001, 0.01, 0.1, or 1). Next, human unstimulated T cells isolated from PBMCs using CD4/CD8 microbeads were added to A549 cells at a T-cell to A549 ratio of 5:1. At 24, 48, 72, or 96 hours post virus infection, A549 viability was determined using MTS assay. A549 cells infected only with EphA2-TEA-VVs served as controls. EphA2-TEA-VV by itself induced cell killing in a dose-dependent manner. However, even at the highest MOI tested, 15% of tumor cells were still alive 96 hours postinfection. Adding human T cells to the culture significantly ($P < 0.05$) increased antitumor effects with all tumor cells being killed within 96 hours postinfection at MOIs of 0.1 and 1 (Figure 3b).

To confirm that the enhanced lytic activity of EphA2-TEA-VV depends on the secretion of EphA2-TEs, A549 cells were infected with EphA2-TEA-VV or GFP-VV at an MOI of 0.1. Human T cells were added as described above, and 24 or 48 hours post virus infection, A549 cell viability was determined using MTS assay. Only EphA2-TEA-VV displayed enhanced oncolytic activity in the presence of human T cells at 24 (EphA2-TEA-VV vs. GFP-VV, 75 vs. 100%) and 48 hours (EphA2-TEA-VV vs. GFP-VV, 35 vs. 81%) (Figure 3c). This finding was confirmed for a panel of EphA2-positive cancer cell lines (H1299, H1975, U373, and LM7) (Supplementary Figure S2A).²⁷

EphA2-TEA-VVs activate T cells

To determine whether EphA2-TEs secreted by EphA2-TEA-VV not only redirect T cells to tumor cells but also activate human T cells, A549 cells were infected with EphA2-TEA-VV or GFP-VV at an MOI of 1 or 0.1. Unstimulated human PBMCs were added as described above, and 24 or 48 hours post virus infection, cell culture media were collected to determine the presence of proinflammatory cytokines using enzyme-linked immunosorbent assay. Unstimulated human PBMCs were activated by EphA2-TEs as judged by the production of proinflammatory cytokines such as interferon- γ (IFN- γ) and interleukin-2 (IL-2) in the cell culture supernatant of EphA2-TEA-VV-infected A549 and T cells, compared with that of GFP-VV-infected A549 and T cells ($P < 0.05$). T cells produced little to no IFN- γ and IL-2 in response to GFP-VV-infected A549 cells (Figure 4). These results were confirmed for EphA2-positive cell lines H1299 and U373 (Supplementary Figure S2B) and indicate that T-cell activation depends on the expression of EphA2-TEs by tumor cells.

To confirm that T-cell activation depends on the presence of EphA2 on the cells surface of tumor cells, we took advantage of K562, which are EphA2 negative, and K562-EphA2, which were genetically modified to express EphA2. A549 tumor cells were infected with EphA2-TEA-VV or GFP-VV at an MOI of 5. After 24 hours, supernatants were collected and added to a coculture of human PBMCs with K562-EphA2 or K562 (effector cell: tumor cell = 5:1). Only the combination of K562-EphA2 and supernatants of EphA2-TEA-VV-infected A549 cells induced T-cell

activation as judged by IFN- γ and IL-2 secretion, demonstrating that T-cell activation and tumor cell recognition not only depends on the presence of EphA2-TEs but also on the expression of EphA2 on tumor cells (Supplementary Figure S3A). This was confirmed in a cytotoxicity assay (Supplementary Figure S3B).

EphA2-TEA-VVs induce bystander killing of noninfected tumor cells

We next investigated the ability of EphA2-TEA-VVs to induce bystander killing of tumor cells. First, A549 cells were transduced with EphA2-TEA-VVs or GFP-VVs at various MOIs. The cell culture medium was collected and filtered with a 0.22 μm filter after 48 hours of culture. Then, noninfected A549 cells were cocultured with unstimulated PBMCs in the presence of the cell culture medium collected from the culture of VV-infected A549 cells. Forty-eight hours later, tumor killing was measured using MTS assay. A549 cells were only killed in the presence of cell culture medium of EphA2-TEA-VV-infected A549 cells, indicating bystander killing of tumor cells (Figure 5).

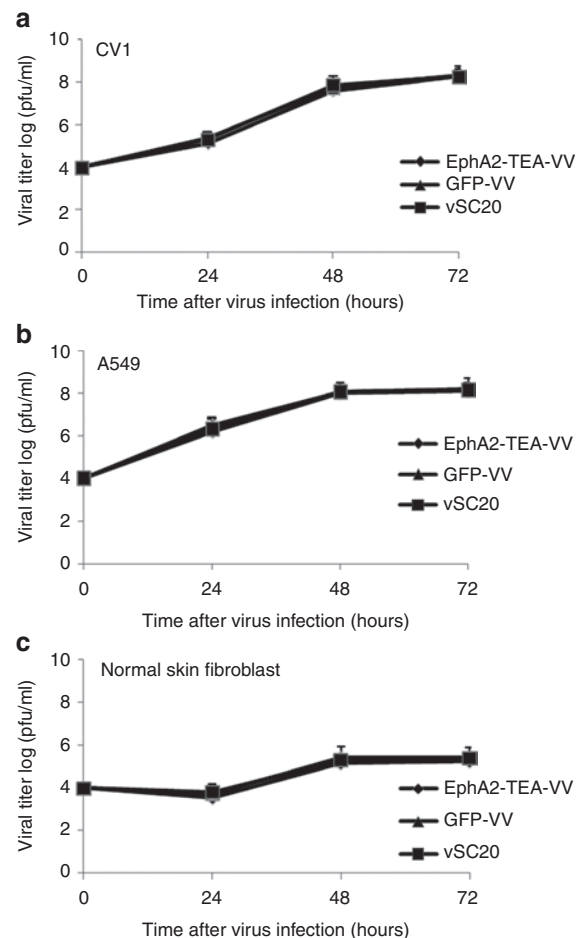


Figure 2 *In vitro* replication of EphA2-TEA-VV in CV-1 cells and normal human cells. The replication of EphA2-TEA-VV, GFP-VV, and vSC20 in (a) CV-1, (b) A549 tumor cells, and (c) normal human fibroblast are shown. Cells were infected at a multiplicity of infection of 0.1, and after 1, 2, or 3 days, viral titers were determined using plaque assays in CV-1 cells. EphA2-TEA-VV, EphA2-T-cell engager-armed vaccinia virus; pfu, plaque-forming units.

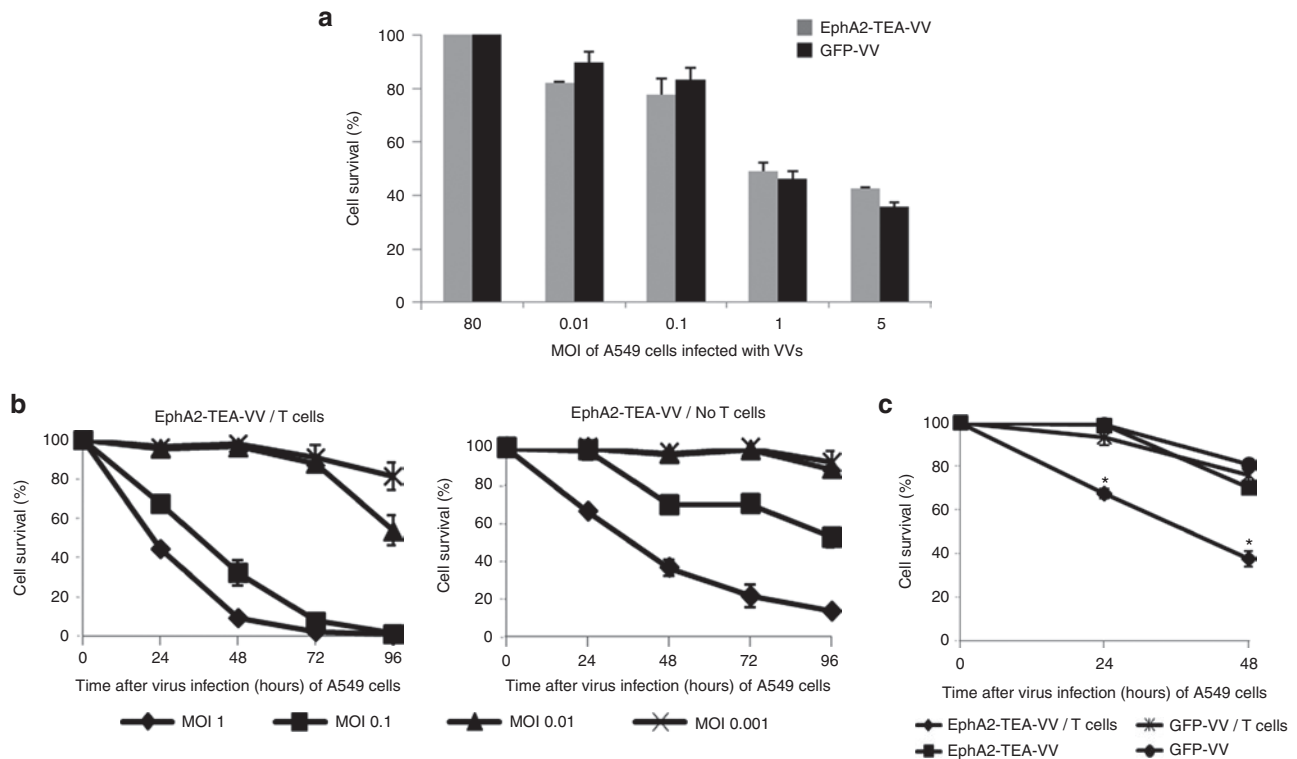


Figure 3 Lytic activity of EphA2-TEA-VV or GFP-VV against EphA2-positive A549 tumor cells. **(a)** A549 tumor cells were infected with increasing doses (multiplicity of infection (MOI) of 0.01, 0.1, 1, or 5) of EphA2-TEA-VV or GFP-VV. Cell viability at 48 hours postinfection was determined using MTS assays. EphA2-TEA-VV or GFP-VV exhibited comparable tumor lytic activity against A549 cells in the absence of human T cells. **(b)** Human T cells enhanced the oncolytic activity of EphA2-TEA-VV against EphA2-positive A549 cells. A549 tumor cells were infected with increasing MOIs (0.001, 0.01, 0.1, or 1) of EphA2-TEA-VV. Infected A549 cells were either cultured alone or in the presence of CD4/CD8 bead-isolated human T cells (T cells: A549 tumor cells = 5:1). Cell viability was determined using MTS assays at 24, 48, 72, and 96 hours postinfection. **(c)** A549 tumor cells were infected with EphA2-TEA-VV or GFP-VV at an MOI of 0.1. Infected A549 cells were either cultured alone or in the presence of CD4/CD8 bead-isolated human T cells (T cells: A549 cells = 5:1). Cell viability at 24 or 48 hours postinfection was determined using MTS assays (EphA2-TEA-VV vs. GFP-VV, $*P < 0.05$). EphA2-TEA-VV, EphA2-T-cell engager armed vaccinia virus.

EphA2-TEA-VVs have enhanced antitumor activity in A549 xenograft tumor model

To investigate the antitumor effects of EphA2-TEA-VV *in vivo*, we initially used a subcutaneous A549 tumor model. To establish A549 tumors, 2×10^6 A549 cells were mixed with unstimulated PBMCs from healthy donor and inoculated subcutaneously (s.c.) into the right flank of SCID-Bg mice, followed by an intraperitoneal (i.p.) injection of 1×10^8 plaque-forming units (pfu) of EphA2-TEA-VV or GFP-VV on day 0. A549/unstimulated PBMC-injected mice receiving phosphate-buffered saline (PBS) and mice that were only injected s.c. with A549 served as control. GFP-VVs moderately inhibited tumor growth compared with that in control mice. By contrast, mice that received EphA2-TEA-VVs showed a significant decrease in tumor growth, compared with mice that received GFP-VV or PBS (Figure 6a, EphA2-TEA-VV vs. GFP-VV $P < 0.0001$). This resulted in a significant increase in survival of mice that received both EphA2-TEA-VV and human PBMCs (Figure 6b, EphA2-TEA-VV vs. GFP-VV $P < 0.001$).

To determine whether EphA2-TEA-VV activated T cells within tumor, mice were injected with an admixture of A549 cells and carboxyfluorescein succinimidyl ester-labeled PBMCs followed by an i.p. injection of 1×10^8 pfu of EphA2-TEA-VV or GFP-VV on day 0. Tumor infiltrating cells were isolated on day 5 and subjected to

FACS analysis. Only EphA2-TEA-VV induced PBMC activation as judged by intracellular staining for IFN- γ ($n = 7$, EphA2-TEA-VV vs. GFP-VV, 5.19 ± 0.94 vs. $2.43 \pm 1.19\%$, $P < 0.01$) and IL-2 ($n = 7$, EphA2-TEA-VV vs. GFP-VV, 3.70 ± 0.88 vs. $1.60 \pm 0.86\%$, $P < 0.01$) (Figure 6c). However, there was no difference in the mean fluorescence intensity of carboxyfluorescein succinimidyl ester between EphA2-TEA-VV- and GFP-VV-injected animals, indicating that the observed cytokine production was not sufficient to induce T-cell proliferation.

To further investigate the antitumor effects of EphA2-TEA-VV in a systemic model, luciferase-expressing A549 cells (A549.eGFP.fLuc) were injected intravenously (i.v.) into SCID-Bg mice on day 0. On day 7, an admixture of 10×10^6 untreated PBMCs and/or 1×10^8 pfu of EphA2-TEA-VVs or GFP-VVs were administered i.v. The mice that received only PBMCs served as control. Quantification of bioluminescent imaging showed that VVs or GFP-VVs plus PBMCs only moderately inhibited tumor growth compared with controls. By contrast, mice that received EphA2-TEA-VVs plus PBMCs showed a significant decrease in tumor growth, compared with mice that received VVs or PBMCs, or GFP-VVs plus PBMCs (Figure 7, EphA2-TEA-VV plus PBMCs vs. GFP-VV, EphA2-TEA-VV, and GFP-VV plus PBMCs, $P < 0.05$). Thus, in both animal models, EphA2-TEA-VV plus PBMCs resulted in enhanced antitumor activity in comparison with GFP-VV plus PBMCs.

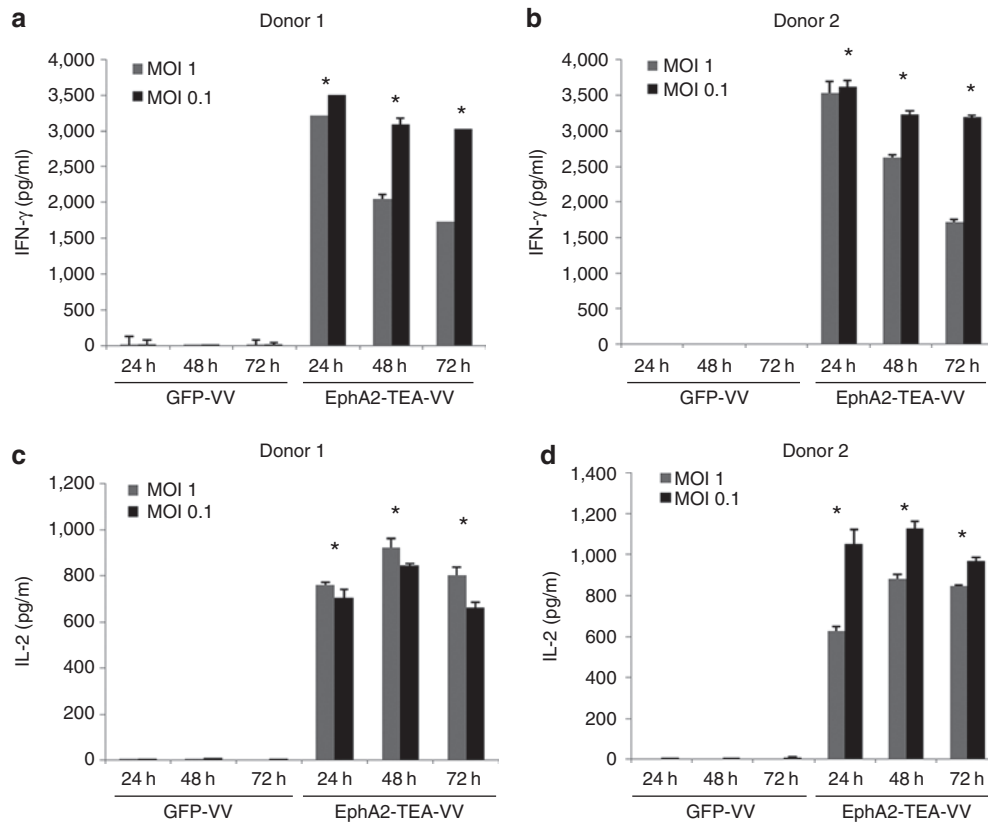


Figure 4 EphA2-TEA-VV activates human T cells. A549 cells were infected with EphA2-TEA-VV or GFP-VV at a multiplicity of infection (MOI) of 0.1 or 1. Infected A549 cells were cultured in the presence of human PBMCs (PBMCs: A549 cell ratio = 5:1). After 24, 48, or 72 hours, supernatants were collected, and (a,b) interferon- γ (IFN- γ) and (c,d) interleukin-2 (IL-2) production was determined using enzyme-linked immunosorbent assay (EphA2-TEA-VV vs. GFP-VV, * $P < 0.05$). EphA2-TEA-VV, EphA2-T-cell engager armed vaccinia virus; PBMCs, peripheral blood mononuclear cells.

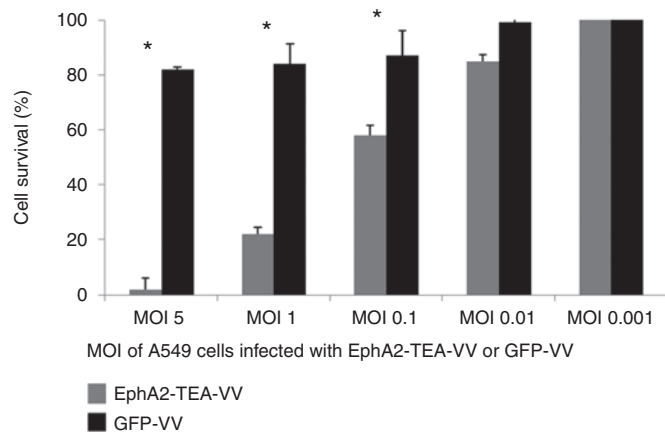


Figure 5 EphA2-TEs induce bystander killing of tumor cells. A549 cells were infected with EphA2-TEA-VV or GFP-VV at various multiplicity of infections (MOIs), and after 24 hours, supernatants were collected and used in a coculture assay of unstimulated peripheral blood mononuclear cells (PBMCs) and A549 cells (PBMCs: A549 cells ratio = 5:1). After 48 hours, tumor cell killing was measured by MTS assay (EphA2-TEA-VV vs. GFP-VV, * $P < 0.05$). EphA2-TEs, EphA2-T-cell engagers; EphA2-TEA-VV, EphA2-T-cell engager-armed vaccinia virus.

DISCUSSION

Oncolytic VVs have demonstrated promising results in the treatment of cancer in preclinical models and early clinical trials.¹⁻¹⁰

However, complete clinical responses were rarely observed, highlighting the need for further improvement of oncolytic VV therapy. In this study, we generated a novel T-cell engager-armed oncolytic VV (TEA-VV) designed to engage T cells for cancer therapy. To our knowledge, this is the first example to evaluate a TEA oncolytic VV in preclinical cancer models.

To enhance the antitumor efficacy of VVs, oncolytic VVs have been engineered to express a variety of transgenes encoding cytokines, chemokines, prodrug-converting enzymes, and antiangiogenic agents.^{3-10,28-33} One major aim of these modifications was to induce bystander killing of tumor cells that are not infected and/or killed by oncolytic VVs. For example, expression of a prodrug-converting enzyme by VVs is capable of converting a systemically administrated nontoxic prodrug to an active pharmacological agent within the tumor. Such an oncolytic VV (vvDD-CDSR) is currently under investigation in an early phase clinical trial. The most successful transgene so far has been the cytokine granulocyte macrophage colony-stimulating factor (GM-CSF).³⁻⁹ The GM-CSF-expressing VV JX-594 has shown exciting results in phase I/IIa clinical trials targeting hepatocellular cancers as local therapy and a variety of solid tumors as systemic therapy. Based on these results, JX-594 is currently being evaluated in global phase III clinical trials. However, the exact mechanism of how GM-CSF enhances the antitumor activity of VVs remains elusive. For example, whereas some studies have

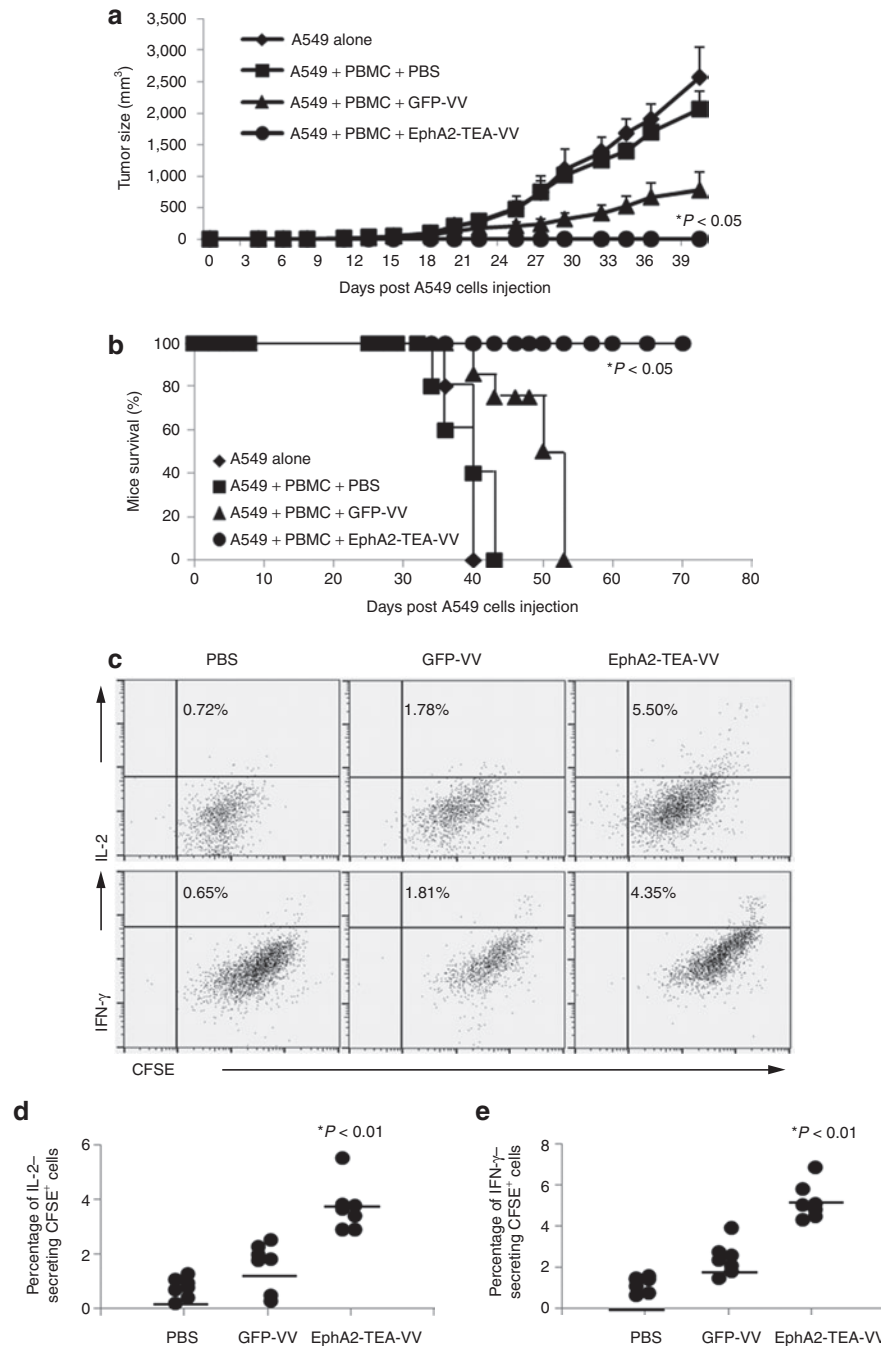


Figure 6 Administration of EphA2-TEA-VV in combination with peripheral blood mononuclear cells (PBMCs) results in an enhanced antitumor response and improved survival in A549 subcutaneous (s.c.) tumor model. 2×10^6 A549 cells were mixed with 1×10^7 unstimulated PBMCs and inoculated s.c. into the right flank of SCID mice on day 0, immediately followed by intraperitoneal (i.p.) injection of 1×10^8 plaque-forming units (pfu) EphA2-TEA-VV, GFP-VV, or phosphate-buffered saline (PBS). **(a)** Tumor size was measured using a caliper (EphA2-TEA-VV $n = 13$ vs. GFP-VV $n = 12$, **P* < 0.001). **(b)** Kaplan–Meier survival showed a significant survival advantage of mice treated with EphA2-TEA-VVs plus PBMCs (EphA2-TEA-VV $n = 13$ vs. GFP-VV $n = 12$, **P* < 0.0001). **(c–e)** 2×10^6 A549 cells were mixed with 1×10^7 CFSE-labeled unstimulated PBMCs and inoculated s.c. into the right flank of SCID mice on day 0, immediately followed by i.p. injection of 1×10^8 pfu EphA2-TEA-VV, GFP-VV, or PBS. Five days later, tumor tissues were dissected, and single cells suspensions were prepared for intracellular staining of interferon- γ (IFN- γ) and interleukin-2 (IL-2). **(c)** Representative fluorescence-activated cell sorting analysis of CFSE⁺ cells. **(d,e)** Summary data of all mice showing a significant increase of IFN- γ ($n = 7$, EphA2-TEA-VV vs. GFP-VV, 5.19 ± 0.94 vs. $2.43 \pm 1.19\%$, *P* < 0.01) or IL-2 ($n = 7$, EphA2-TEA-VV vs. GFP-VV, 3.70 ± 0.88 vs. $1.60 \pm 0.86\%$, *P* < 0.01) secreting PBMCs in EphA2-TEA-VV treated mice. EphA2-TEA-VV, EphA2-T-cell engager armed vaccinia virus.

shown that GM-CSF activates innate immunity and induces the generation of an adaptive antitumor response, other studies have highlighted the role of GM-CSF in inducing proliferation

of monocyte-derived suppressor cells, which suppress antitumor immune responses.³⁴ Compared with these approaches, our TEA-VV platform provides a straightforward mechanism

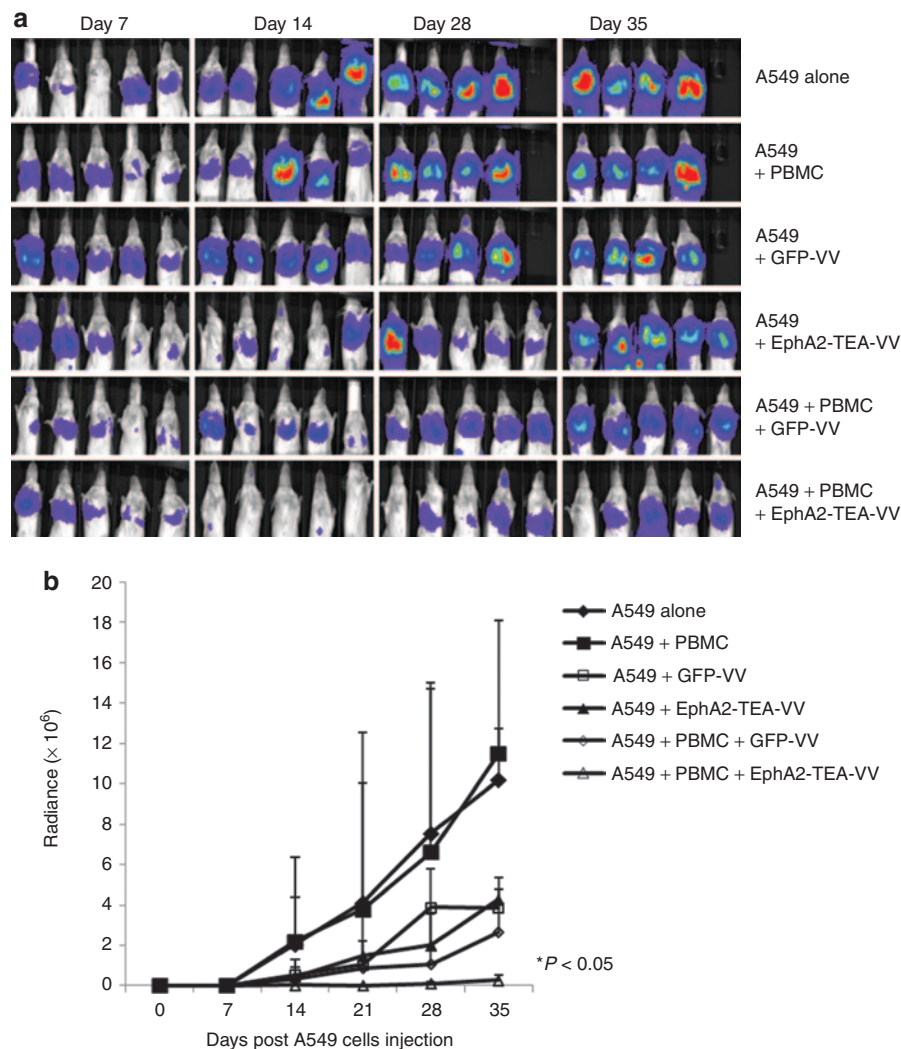


Figure 7 Administration of EphA2-TEA-VV in combination with peripheral blood mononuclear cells (PBMCs) results in an enhanced antitumor response in A549 intravenous (i.v.) lung cancer model. **(a)** 2×10^6 A549.eGFP.FLuc were injected i.v. into SCID mice on day 0 and treated with a single injection of mixed 1×10^7 unstimulated PBMCs and 1×10^8 plaque-forming units EphA2-TEA-VV or GFP-VVs i.v. on day 7. Tumor progression was followed by *in vivo* bioluminescence imaging. Images of representative animals are shown. **(b)** Solid lines represent each group of mice (EphA2-TEA-VV $n = 5$ vs. GFP-VV $n = 5$, * $P < 0.05$). EphA2-TEA-VV, EphA2-T-cell engager armed vaccinia virus.

by which TEA-VV exert its potent effects by utilizing adaptive immunity in a tumor antigen-specific manner. TEs expressed by TEA-VV are capable of engaging endogenous T cells and tumor cells, resulting in antigen-specific tumor lysis. In addition, we show that TEs induce the secretion of proinflammatory cytokines *in vitro* and *in vivo* that should reverse the immune inhibitory milieu, which exist in the majority of solid tumors.^{16,17}

Although EphA2-TEA-VV induced the production of IFN- γ and IL-2, we did not observe significant T-cell proliferation *in vitro* and *in vivo*. However, EphA2-TEA-VVs induced significant T-cell proliferation if the cell culture medium was supplemented with 100 U/ml human IL-2 (**Supplementary Figure S4**). Thus, the amount of IL-2 produced by T cells after EphA2-TEA-VV-activation (1 ng/ml of IL-2, equivalent to ~ 1 U/ml, **Figure 4c,d**) is not sufficient to induce T-cell proliferation. Currently, TEA-VVs only provide T-cell activation by binding to CD3. Additional genetic modification of TEA-VVs with transgenes that encode costimulatory molecules or cytokines³⁵⁻⁴¹ should result in

TEA-VVs that not only activate T cells but also induce robust T-cell proliferation.

Limitations of systemic cancer treatments are their adverse effects, pharmacological half-life, and/or limited tissue distribution. For example, although BiTEs have been shown to be effective for the treatment of CD19-positive malignancies, BiTEs have a very short half-life, necessitating continuous infusion.^{18-26,42} In addition, BiTEs do not actively accumulate at tumor sites, and systemic adverse events have been reported including within the central nervous system. These limitations could be potentially overcome by the use of oncolytic virus as a delivery system, because transgene expression from the VVs will occur mainly within the tumor.³⁴ Following virus infection of a cell, a cascade of virus protein, called early or late protein, is produced under the transcriptional control of early or late promoter individually. Generally, late promoters are activated after DNA replication of the virus in the cell, therefore allowing transgene expression selectively within the tumors. In this study, we choose the late vaccinia

viral promoter F17R, because studies have demonstrated that the F17R promoter is only activated after VV infection in tumor cells.⁴³ Thus tumor selective expression of transgene from VV will be further enhanced by the use of F17R promoter. The late expression of the T-cell engager will also allow for sufficient viral replication before T-cell activation and mediated tumor lysis.

We evaluated the therapeutic effects of EphA2-TEA-VVs in two murine xenograft models. EphA2-TEA-VVs plus human PBMCs induced complete remissions in all treated animals in the s.c. A549 model. Because the ability of human PBMCs to migrate to s.c. tumor sites in mice is limited, we injected s.c. an admixture of tumor cells and PBMCs as routinely done for the preclinical evaluation of BiTEs.^{18–21} To evaluate the antitumor activity of EphA2-TEA-VVs after i.v. administration of PBMCs, we used the systemic A549 lung cancer model. While in the systemic model, EphA2-TEA-VVs plus human PBMCs had potent antitumor response, all tumors progressed. This difference in efficacy in the two models is most likely due to several reasons. First, the systemic A549 model is a very aggressive tumor model, and we treated mice on day 7 after tumor cell injection. Second, TEA-VV exerts its antitumor effects in part by activating human T cells, which in the used xenograft model (SCID-Beige mice) persist only for 7–10 days postinjection.⁴⁴ As discussed above, introduction of additional transgenes into TEA-VVs to provide costimulatory signals and/or cytokines are potential strategies to improve T-cell activation and persistence *in vivo*.^{35–41} While we choose a xenograft model to demonstrate the benefit of arming VVs with TEs, we are currently developing an immune competent animal model to perform detailed mechanistic studies. Insight from this model will most likely enable to further improve the antitumor activity of TEA-VVs.

In conclusion, our findings provide preclinical evidence for the therapeutic potential of TEA-VVs. In this study, our EphA2-TEA-VVs activated human PBMCs and enhanced antitumor activity in preclinical models. Our T-cell engager arming strategy may be applicable to a broad range of oncolytic viruses currently under investigation, including oncolytic adenoviral vector, herpes simplex virus, reovirus, myxoma virus, poliovirus, vesicular stomatitis virus, measles virus, and Newcastle disease virus.

MATERIALS AND METHODS

Tumor cell lines. The non-small-cell lung cancer cell line A549 was purchased from the American Type Culture Collection (Manassas, VA). A549 cell line expressing the eGFP-Firefly luciferase (A549.eGFP.fluc) fusion gene was generated as previously described with a retrovirus vector encoding the eGFP.fluc.⁴⁴ Cell lines were grown in Roswell Park Memorial Institute (Thermo Scientific HyClone, Waltham, MA; Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (FCS) HyClone, Logan, UT) and 2 mmol/l GlutaMAX-I (Invitrogen, Carlsbad, CA). Cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C. The “Characterized Cell Line Core Facility” at MD Anderson Cancer Center, Houston, TX, performed cell line validation.

Mononuclear cells. Blood samples from healthy donors were obtained in accordance to protocols approved by the Institutional Review Board of Baylor College of Medicine. Peripheral blood was processed over Ficoll gradients, and the resulting PBMCs were cultured in Roswell Park Memorial Institute 1640 (Thermo Scientific HyClone, Waltham, MA; Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated FCS and 2 mmol/l GLUTAMAX.

Production of VV. We generated double-deleted VVs (vvDD, Western Reserve strain) expressing EphA2-scFv-CD3-scFv (EphA2-TEA-VV) or GFP (GFP-VV) by recombination with a pSEL shuttle plasmid encoding CD3 scFv^{18–26} or EphA2-scFv²⁷ or GFP into the TK gene of the VSC20 strain of WR VV (Figure 1).^{45–50} The inserted TEs were expressed under the transcriptional control of the F17R late promoter to allow for sufficient viral replication before T-cell activation.³⁴ All VVs used in this study were derived from the WR strain. Two low pathogenic mutants containing deletions in the TK and VGF genes (vvdd) have been previously described. Viral stocks were prepared as previously reported. Briefly, dividing CV-1 cells were infected with 200 pfu (MOI: ~0.0005) of VSC20, GFP-VV, or TEA-VV in 1 ml of minimum essential medium-2.5% FCS for 2 hours at 37°C. Dulbecco's modified Eagle's medium-10% FCS was added, and cells were incubated until harvesting at 24, 48, and 72 hours after infection. After a single freeze-thaw cycle, virus was quantified by plaque titering on CV-1 cells as described previously.⁵⁰

Flow cytometry. For immunophenotyping, cells were stained with fluorescein-conjugated monoclonal antibodies (Becton Dickinson, San Jose, CA) directed against CD3-, CD4-, CD8-, or FITC-labeled EphA2 protein. Isotype controls were immunoglobulin G1-fluorescein isothiocyanate (IgG1-FITC; BD) and IgG1-phycoerythrin (IgG1-PE; BD). Cells were collected and washed once with PBS containing 1% FBS (Sigma, St. Louis, MO; FACS buffer) before the addition of antibodies. Cells were then incubated for 30 minutes on ice in the dark, washed once, and fixed in 0.5% paraformaldehyde/FACS buffer before analysis. For each sample, 10,000 cells were analyzed using FACSCalibur instrument (BD, Becton Dickinson, Mountain View, CA) with the Cell Quest Software (Becton Dickinson) or with FCS Express software (De Novo Software, Los Angeles, CA).

Enzyme-linked immunosorbent assay. Tumor cells were infected with either EphA2-TEA-VV or GFP-VV at different MOIs followed by coculturing with unstimulated PBMCs. At different time points, supernatants were collected and run on enzyme-linked immunosorbent assay with DuoSet enzyme-linked immunosorbent assay development kit (R&D systems, Minneapolis, MN) according to the manufacturer's guidelines.

MTS assay. Tumor cells were plated in a 96-well tissue culture plate at 1×10^4 cells per well (100 μ l) and incubated overnight at 37°C. The tumor cells were infected with VVs at indicated MOIs in 2.5% FBS medium for 2 hours followed by culturing in complete medium. All samples were measured in triplicate. Unstimulated human PBMCs were incubated at 37°C for 2 hours to remove adherent cells. In some experiments, unstimulated nonadherent human PBMCs or CD4/8 microbead-selected T cells were added to the culture at effector: target ratio of 5:1. Plates were incubated at 37°C for 48 or 72 hours. The nonadherent human PBMCs or CD4/8 microbead-selected T cells were removed, and the wells were washed gently twice with PBS. The viability of tumor cells was then determined using a MTS formazan viability assay (Promega, Madison, WI). Optical density of the wells was read at 490 nm on a plate reader (Molecular Devices VERSAmax, Sunnyvale, CA (Molecular Devices)). The mean viability of the virus infected tumor cells for each viral dilution was calculated as a percentage relative to the control wells treated with media alone (100% survival) \pm SEM.

A549 tumor models. All animal experiments followed a protocol approved by the Baylor College of Medicine Institutional Animal Care and Use Committee. Nine- to 12-week-old SCID Beige mice were purchased from Jackson, Bar Harbor, ME.

A549 i.v. lung model. 2×10^6 A549.eGFP.fluc cells in PBS were injected i.v. on day 0. To determine the antitumor efficacy of EphA2-TEA-VV, mice were treated with an i.v. administration of either 1×10^8 pfu GFP-VV or 1×10^8 pfu EphA2-TEA-VV mixed with 1×10^7 human unstimulated PBMCs on day 7 posttumor challenge. Animals receiving unstimulated PBMCs alone or PBS served as controls. Isoflurane anesthetized animals were imaged. A constant region-of-interest was drawn over the

chest of the animal, and the intensity of the signal was measured as total photons/s/cm²/steradian. Mice were euthanized when they met euthanasia criteria (weight loss, signs of distress) in accordance with the Center for Comparative Medicine at Baylor College of Medicine.

A549 s.c. tumor model. 2×10^6 A549 cells alone or mixed with unstimulated PBMCs from healthy donors in 100 μ l of PBS were injected s.c. on day 0, immediately followed by i.p. administration of either 1×10^8 pfu GFP-VV or 1×10^8 pfu EphA2-TEA-VV. Animals receiving PBS served as controls. Tumor size was measured using a caliper.

To monitor T cells within tumor tissue, 2×10^6 A549 cells were mixed with 1×10^7 carboxyfluorescein succinimidyl ester-labeled unstimulated PBMCs and inoculated s.c. into the right flank of SCID mice on day 0, immediately followed by i.p. injection of 1×10^8 pfu EphA2-TEA-VV, GFP-VV, or PBS. Five days later, tumor tissues were dissected, and single cells suspensions were prepared for intracellular staining of IFN- γ and IL-2.

Statistical analysis. All *in vitro* experiments were performed in triplicate. Measurement data were presented as mean \pm SD. The differences between means were tested by nonparametric Mann-Whitney *U* test or Bonferroni's multiple comparison *t*-test. The significance level used was *P* < 0.05. For the mouse experiments, tumor radiance data were log-transformed and summarized using mean \pm SD at baseline and multiple subsequent time points for each group of mice. Changes in tumor radiance from baseline at each time point were calculated and compared between groups using *t*-test. Survival determined from the time of tumor cell injection was analyzed by the Kaplan-Meier method and by the log-rank test.

SUPPLEMENTARY MATERIAL

Figure S1. EphA2-specific T-cell engagers induce tumor cells killing.

Figure S2. T cells recognize EphA2-TEA-VV-infected EphA2-positive H1299, H1975, U373, or LM7 tumor cells.

Figure S3. T cells recognize EphA2-TEA-VV-infected EphA2 expressing K562-EphA2 cells, but not parental, EphA2-negative K562 cells.

Figure S4. EphA2-TEA-VVs induced significant T-cell proliferation in the presence of 100U/ml human IL2.

Supplementary Materials and Methods.

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