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ORIGINAL ARTICLE

Neoadjuvant *in situ* gene-mediated cytotoxic immunotherapy improves postoperative outcomes in novel syngeneic esophageal carcinoma models

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Esophageal carcinoma is the most rapidly increasing tumor in the United States and has a dismal 15% 5-year survival. Immunotherapy has been proposed to improve patient outcomes; however, no immunocompetent esophageal carcinoma model exists to date to test this approach. We developed two mouse models of esophageal cancer by inoculating immunocompetent mice with syngeneic esophageal cell lines transformed by cyclin-D1 or mutant $HRAS^{G12V}$ and loss of p53. Similar to humans, surgery and adjuvant chemotherapy (cisplatin and 5-fluorouracil) demonstrated limited efficacy. Gene-mediated cytotoxic immunotherapy (adenoviral vector carrying the herpes simplex virus thymidine kinase gene in combination with the prodrug ganciclovir; AdV-tk/GCV) demonstrated high levels of *in vitro* transduction and efficacy. Using *in vivo* syngeneic esophageal carcinoma models, combining surgery, chemotherapy and AdV-tk/GCV improved survival ($P=0.007$) and decreased disease recurrence ($P<0.001$). Mechanistic studies suggested that AdV-tk/GCV mediated a direct cytotoxic effect and an increased intra-tumoral trafficking of CD8 T cells (8.15% vs 14.89%, $P=0.02$). These data provide the first preclinical evidence that augmenting standard of care with immunotherapy may improve outcomes in the management of esophageal carcinoma.
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Introduction

Esophageal cancer is the seventh most common cause of cancer death among men in the United States. At diagnosis, approximately 50% of patients have advanced, incurable disease, which is associated with a 5-year overall survival of 6%.[1] Surgery offers the best opportunity for long-term survival; however, only 25% of patients diagnosed qualify for surgical resection. Even with surgery, the 5-year survival rate of this cohort is only 32%.[1] The poor survival is due to the high postoperative recurrence rate ranging from 60 to 80%. To address the issue of recurrences, adjuvant chemotherapy and radiation have been utilized.[2–4] Standard chemotherapy regimen consists of concurrent cisplatin (cis) and 5-fluorouracil (5-FU). Clearly, there is a compelling need to improve the management of surgical candidates by incorporating new modalities into the treatment paradigm.

One alternative treatment approach that has been suggested is immunotherapy. Gene-mediated cytotoxic immunotherapy (GMCI) constitutes a promising type of antitumor immunotherapy, and is based on the introduction of a gene encoding an enzyme capable of transforming a non-toxic prodrug into a cellular toxin that kills dividing cells in an immunogenic manner. The most well-developed approach utilizes a replication-defective adenoviral vector encoding for the HSV*tk* protein (AdV-tk). The HSV*tk* gene monophosphorylates anti-herpetic prodrugs, such as ganciclovir (GCV), that are further phosphorylated by endogenous cellular kinases into active triphosphate nucleotide analogs. These are incorporated into cellular DNA and cause cell death.[5] In addition to its enzymatic effects, AdV-tk/GCV (adenoviral vector carrying the herpes simplex virus thymidine kinase gene in

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combination with the prodrug ganciclovir) treatment stimulates a strong local and systemic immune response characterized by a T-helper type 1 cytokine cascade that manifests with increased interleukin-2, tumor necrosis factor-α, interferon-γ and interleukin-12 production.[6–13] AdV-tk/GCV increases cytotoxic T-lymphocyte infiltration[5] and increased tumor immunogenicity.[14] It also stimulates the arrival of natural killer cells[5] and increases their activity level around neoplastic cells.[15] Overall the approach has demonstrated a good safety profile and efficacy in several tumor types, including mesothelioma, melanoma and others.[16–23] This treatment strategy has reached clinical trials for several other malignancies such as prostate cancer,[18,24] ovarian cancer[17] and gliomas.[19]

AdV-tk suicide gene therapy has several potential advantages for the treatment of esophageal cancer. First, tumors can be accessed directly by endoscopy in humans, thus allowing for intra-tumoral injection. Secondly, evidence suggests that this form of immunotherapy synergizes well with the chemotherapy and radiotherapy, which is typically employed in patients with esophageal carcinoma.[16,24–26] Finally, GMCI generates a systemic immune effect that has the potential to minimize distant disease that commonly occurs with esophageal malignancies.[16,26,27]

Unfortunately, effective preclinical evaluation of adjuvant immunotherapy strategies for esophageal carcinoma has been slow, primarily due to the lack of high-throughput animal models. Recent reports have described reliable transgenic murine systems, which develop orthotopic lesions, and mimic genetic alterations observed in human esophageal carcinogenesis. One such immunocompetent model is the ED-Ls-cyclin-D1;p53$^{-/-}$ model described by Opitz *et al*.[28] However, these models have many drawbacks, including (1) long periods for tumor development, (2) inability to monitor disease in real time, (3) expensive housing and breeding protocols and (4) lack of access for surgical excision. With these considerations in mind, syngeneic flank tumor models provide an optimal mechanism to study adjuvant immunotherapy strategies in hosts with intact immune systems.

Our aim in this study was to investigate the efficacy of GMCI in esophageal cancer. Because syngeneic models have not been reported for esophageal cancer, we first developed a readily transplantable syngeneic murine model derived from previously described transgenic mice. We then tested the clinical effect of AdV-tk in combination with surgery and standard of care chemotherapy (cis/5-FU) in our syngeneic murine models of esophageal cancers. We confirmed the efficacy of the standard approach using chemotherapy and surgery, but found the vast majority of animals recurred and ultimately died of disease, similar to clinical outcomes observed in humans. We therefore developed a multimodal approach combining immunotherapy with surgery and chemotherapy and showed that it was superior to surgery with adjuvant chemotherapy alone. GMCI further decreased recurrences and prolonged survival. We found that *preoperative* AdV-tk/GCV with chemotherapy and surgery generated a sustained local and systemic CD8 T-cell response *postoperatively*. These data suggest that immunotherapeutic approaches may be a reasonable modality for patients with resectable esophageal cancer.

Materials and methods

Animals

Female C57Bl/6 mice (B6, Thy1.2) were purchased from Charles River Laboratories. Female NOD/SCID/γ-chain knockout (NSG) mice that were bred at the Wistar Institute (Philadelphia, PA) were used. All mice were maintained in pathogen-free conditions and used for experiments at ages 8 week or older. Recognized principles of laboratory animal care (NIH Publication No. 85-23, revised 1985) were followed, and the Animal Use Committees of the Children's Hospital of Philadelphia, the Wistar Institute and the University of Pennsylvania approved all protocols.

Cell lines

AKR is an esophageal cancer cell line established from the ED-L2-cyclin-D1;p53$^{-/-}$ mouse as described previously.[28,29] We also used an independent p53-null murine esophageal cell line (HNM007) transformed by HRAS^{G12V} (ref. 30). In total, 0.5×10^6 of AKR or HNM007 cells were injected subcutaneously into the flanks of mice.

For *in vitro*, studies, we used several human esophageal cancer cell lines (TE-1 and SEG-1) established from human squamous cell carcinomas of the esophagus.[31,32] A549 is a human non-small-cell lung cancer (NSCLC) that we used as a control for *in vitro* assays.[13] All cell lines were maintained in D10 media (Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine and 1% penicillin and streptomycin (P/S)), and cultured at 37 °C in a humidified incubator containing 5% CO_2.

Chemotherapy

Cis was purchased from APP Pharmaceuticals (Schaumburg, IL) and 5-FU was purchased from Sigma-Aldrich (St Louis, MO). Cis and 5-FU were administered intraperitoneally (i.p.) once a week to mice at 3 and 85 mg kg^{-1}, respectively. Doses were chosen based on human equivalent dosing. GCV was purchased from Roche Laboratories Inc. (Nutley, NJ) and was dosed at 50 mg kg^{-1}, once per day for 5 days, beginning at 2 days following intratumoral AdV-tk injection. GCV was suspended in 200 µl normal saline for i.p. injections.

Vectors

A non-replicating serotype 5 adenovirus containing the herpes simplex virus thymidine kinase gene driven by a Rous sarcoma virus long terminal repeat promoter in the region of the deleted E1 wild-type adenoviral genes (AdV-tk) was used. Control adenoviral vector contained the *LacZ* gene (Ad.LacZ) in the same position. Both were produced by Advantagene (Auburndale, MA). AdV-tk was produced in accordance with good manufacturing

practices (21 CFR210 and 211). The vector has been characterized for purity and potency and approved for clinical use. The viral particle (v.p.) per infectious unit ratio was 10:1. Animals bearing AKR tumors were treated with intratumoral injections of 1×10^{10} v.p. of AdV-tk or Ad.LacZ. After 48 h, GCV therapy was begun.

Determination of in vivo *'bystander effect'*

To study the *in vivo* bystander effect, NSGs were injected with AdV-tk-infected AKR cells mixed with varying numbers of uninfected cells to achieve ratios of infected: uninfected cells of 1:1, 1:5 and 1:10. In all circumstances, the number of AKR cells totaled 0.5×10^6. At 6 days after injection, when all tumors measured approximately 25 mm^3 in diameter, the animals were treated with i.p. GCV (50 $mg\,kg^{-1}$ per day) or saline daily for 7 days. Tumor growth was measured for the next 20 days.

Surgery

Surgery was performed on mice bearing flank tumors using an established partial resection model.[33] Briefly, mice bearing flank tumors were anesthetized using intramuscular ketamine (80 $mg\,kg^{-1}$) and xyalazine (10 $mg\,kg^{-1}$), and then shaved with hair clippers. A 1–2 cm incision was made immediately adjacent to the tumor. Resections were completed using standard blunt dissection. To mimic a positive margin, approximately 10% of the tumor burden was left at the tumor margin. Careful consideration was given to preserve blood supply to the remaining tumor volume. Occasionally, it was necessary to remove skin that was adherent to the tumor. Skin closure was performed using sterile silk 4-0 sutures. Buprenorphine (0.2 $mg\,kg^{-1}$) was administered at the time of surgery and 6 h after as postoperative analgesia. Preoperative treatment was unknown to the investigator performing surgery and making tumor measurements. The Animal Use Committee of the University of Pennsylvania and the Wistar Institute approved all protocols in compliance with the Guide for the Care and Use of Laboratory Animals.

Determination of adenoviral transduction efficiency

To demonstrate *in vitro* adenovirus transduction in esophageal cancer cell lines, AKR, HNM007, TE-1, SEG-1 and A549 (a human NSCLC with known transducibility)[13] were transduced with adenoviral vector carrying a luciferase reporter gene driven by a cytomegalovirus promoter (Ad.luciferase). Equal numbers of cells were transduced with varying concentrations of Ad.luciferase. Ad.luciferase was allowed to infect AKR cells in Dulbecco's modified Eagle's media supplemented with 5% fetal bovine serum, P/S and L-glutamine (D5) for 4 h at 37 °C. Cells were washed with phosphate-buffered saline, and then D5 media were added for a 20-h incubation period. At the end of incubation, cells were washed twice with phosphate-buffered saline, and then lysed with 100 µl per well of luciferase assay lysis buffer (Promega Corporation, Madison, WI) for 30 min at room temperature on a standard shaker. The lysed cells were transferred to a standard white-walled 96-well luminometer plate (80 µl per well). A measure of 100 µl of Luciferase Assay Reagent (Promega) was added to each well and immediately read in a standard luminometer at a wavelength of 490 nm. Results are expressed as luminescence (relative light units) vs vector concentration (v.p. per ml).

In vitro *cell viability assays*

To evaluate and compare the direct antiproliferative effect of AdV-tk/GCV on various murine cell lines, cells were transduced with AdV-tk or control adenovirus (Ad.LacZ) in T25 flasks at 1×10^6 v.p. per ml. Cultures were incubated for 24 h. After this incubation period, cells were seeded in quadruplet in 96-well plates (100 µl media per well) at a density of 2×10^3 cells per well and 20 µl of medium was added containing varying concentrations of GCV (0–2000 µM). Fresh medium containing prodrug was plated daily for up to day 5. Survival of infected cells compared with uninfected cells was measured when uninfected cells reached confluency (usually after 5–8 days after infection) using the MTT (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay, which is a colorimetric test for the quantitation of cell viability and proliferation (Cell Titer 96TM Aqueous Nonradioactive MTT Cell Proliferation Assay; Promega). The percentage of viable cells were determined using the following formula:

$$\text{Viable cells}\,(\%) = (A_{490}\,\text{of untreated} - A_{490}\,\text{of treated}) / (A_{490}\,\text{of untreated}) \times 100\%$$

In vivo *depletion of CD8 T cells*

To deplete CD8 T cells during treatment with AdV-tk in our AKR models, mice received i.p. injections of 200 µg of monoclonal antibodies purified from the anti-CD8 hybridoma 53-6.7 (obtained from the American Type Culture Collection, Manassas, VA). Injections were administered 1 day before beginning AdV-tk/GCV protocols. Thereafter, a maintenance dose of antibody was injected i.p. every 3 days throughout the entire experimental period to ensure depletion of CD8 T cells. Depletion was confirmed by flow cytometry of splenic suspension.

Immunohistochemical studies

Mice were euthanized at which time tumors were harvested and frozen in Tissue-Tek OCT compound (Sakura Finetek USA Inc., Torrance, CA) to be stored at 80 °C, and 5 µm sections were cut. Monoclonal antibodies against CD8 T cells (anti-CD8) were obtained from BD Biosciences and immunohistochemical staining was performed according to established protocols. Tumor cell infiltrate was quantified by counting the number of positively stained cells in four high-powered ($\times 400$) fields. Five slides for each specimen were analyzed.

Flow cytometric analysis of tumors

For flow cytometric analysis, tumors were removed from euthanized mice and minced into fine pieces in digestion

buffer containing 0.1 mg ml^{-1} DNase I and 2.0 mg ml^{-1} collagenase type IV (Sigma, St Louis, MO). Samples were incubated in digestion buffer at 37 °C for 30 min, filtered through a 70-μm filter and washed twice with R10. Fc receptors were blocked with anti-mouse CD16/CD32 antibodies (BD Biosciences Pharmingen, San Diego, CA). Following one wash with phosphate-buffered saline plus 2% fetal bovine serum (staining buffer), cells were incubated for 30 min at 4 °C with appropriate antibodies obtained from BD Biosciences PharMingen and used at the indicated dilutions for flow cytometry: CD45-PerCP (1:200) and CD8-APC (1:200). Samples were then washed and resuspended in staining buffer or fixed in 2% paraformaldehyde. Flow cytometry was completed using a Becton Dickinson FACS Calibur flow cytometer (San Jose, CA), and analyzed using the FlowJo software (Ashland, OR).

Winn assay for the detection of tumor-neutralizing cells

Splenocytes were isolated from spleens (2 mice per group) that were harvested 3 days after the first dose chemotherapy was administered to mice receiving AdV-tk/surgery/chemo and Ad.LacZ/surgery/chemo protocols. CD8 T cells were separated from the total splenocyte pool using MACS isolation system (CD8a [Ly-2] mouse MicroBeads; Miltenyi Biotec, Auburn, CA). The isolated cells consisted of greater than 90% CD8 T cells, as confirmed by fluorescence-activated cell sorter analysis. Fresh AKR cells were mixed with CD8 T cells (Winn assay) in a ratio of 3 CD8 T cells:1 AKR tumor cell. The resulting mixture (1.5×10^6 CD8 T cells:0.5×10^6 AKR tumor cells) was injected subcutaneously into the flanks of five naïve C57Bl/6 mice. A control group of five mice with AKR cells alone (0.5×10^6 cells) were injected in an analogous manner. Tumor size was assessed over the next 7 days.

Statistical analyses

For flow cytometry, immunohistochemistry and flank tumor volume studies comparing differences between two groups, we used unpaired Student's *t*-tests. For studies comparing more than two groups, analysis of variance with appropriate *post hoc* testing was implemented. Kaplan–Meier curves were utilized to determine postoperative median survival. Postoperative survivals (defined as the time from surgery to the time that flank tumor volume reached 1500 mm^3) for treatment groups were compared using the log-rank statistic. Differences were considered significant when $P<0.05$. Data are presented as mean (s.e.), unless otherwise noted.

Results

Treatment of esophageal cancer model with Cis/5-FU and surgery has limited efficacy

To examine the efficacy of the current standard of care chemotherapy regimen for esophageal cancer in our models, we evaluated human equivalent dosing of cis/5-FU in the murine esophageal carcinoma model, AKR. Moderate-sized flank tumors (250 mm^3) were treated with human equivalent dosing of Cis (3 mg kg^{-1}) and 5-FU (85 mg kg^{-1}). Animals were given two doses of chemotherapy, 1 week apart, i.p. We observed a reduction in the tumor volume or delay of tumor growth from chemotherapy administration (Figure 1a). By day 19, the mean tumor volume was 775 mm^3 in mice randomized to control treatment vs 398 mm^3 in mice randomized to cis/5-FU ($P=0.03$) (Figure 1b). All mice ultimately required being killed owing to excessive tumor burden. These results were confirmed with repeat experiments. Increasing concentrations of cis/5-FU were toxic to animals, and did not significantly alter tumor growth kinetics (data not shown). Results in our AKR model were similar to those observed in humans, confirming that the model accurately reflects the limited efficacy of cis/5-FU in esophageal cancer.

Next, the efficacy of cis/5-FU in decreasing postoperative local recurrences in the AKR model was evaluated. Again, C57Bl/6 mice were injected with AKR cells in the subcutaneous flank. Large flank tumors ($\sim$500 mm^3) were incompletely resected to mirror the scenario of a positive margin that may occur following surgery. On postoperative day 3, animals were randomized to treatment with cis/5-FU or normal saline injections. Animals were given two doses of chemotherapy or saline, 1 week apart. In both groups, all mice undergoing surgery were observed to have locally recurrent disease, although we observed a temporary reduction in tumor volume after chemotherapy administration (Figure 1c). At postoperative day 10, the mean tumor volume of the control mice was 835 vs 402 mm^3 in mice randomized to surgery vs chemotherapy following surgery, respectively ($P=0.03$) (Figure 1d). Similar to non-surgical models, tumors promptly returned to normal growth and ultimately overcame all mice following chemotherapy cessation. These experiments demonstrated that chemotherapy has marginal benefits in preventing disease progression in a positive margin setting, and that the model reflects human clinical experiences.

Taken together, these experiments demonstrate that the AKR esophageal cancer model resembles human esophageal malignancies in terms of its modest susceptibility to cis/5-FU chemotherapy regimen. In addition, these results suggest that the AKR model may be a candidate esophageal carcinoma model to test preclinical models involving standard chemotherapy treatment and surgical intervention.

Human and murine esophageal carcinoma cells are sensitive to AdV-tk/GCV

There have been conflicting reports on the efficiency of serotype 5 adenovirus-transducing esophageal carcinomas.[34,35] Thus, we sought to test serotype 5 adenovirus transduction in murine and human esophageal cancer cell lines in an *in vitro* setting. Using Ad.luciferase, a serotype 5 adenovirus similar to AdV-tk, we transduced two mouse cell lines (AKR and HNM007) and two human esophageal cancer lines (SEG-1 and TE-1) at vector concentrations ranging from 0 to 2.5×10^9 v.p. per ml. A known susceptible human NSCLC (A549)[13] was used as a

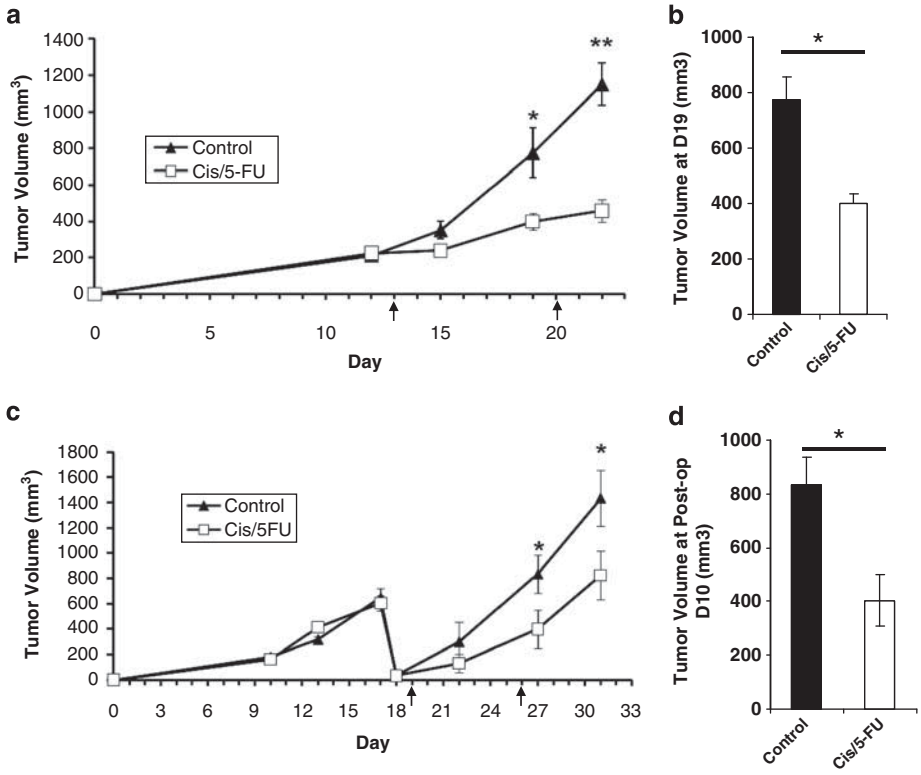


Figure 1 Cisplatin (Cis) and 5-fluorouracil (5-FU) in the murine esophageal carcinoma model, AKR. (**a**) Cis and 5-FU in AKR (no surgery). Cis/5FU was administered at days 13 and 19 in mice ($n=9$) bearing AKR flank tumors. Control mice ($n=8$) were administered normal saline at days 13 and 19 as a control. After chemotherapy protocol completion, tumors resumed normal growth rates and mice required being killed owing to tumor burden. (**b**) Bar graph demonstrating tumor volumes at day 19. (**c**) Postoperative Cis and 5-FU in AKR. Mice were administered cis/5-FU following surgery ($n=10$) on postoperative days 3 and 10. Control mice ($n=10$) received normal saline injections. As observed in mice not undergoing surgery, chemotherapy administration decreased tumor volume, but did not result in complete tumor eradication. All mice had recurrences, and ultimately were killed owing to excessive tumor burden. (**d**) Demonstration of flank tumor volumes at postoperative day 10. $^{*}P<0.05$; $^{**}P<0.01$; arrowheads signify time of chemotherapy administration.

positive control. Although AdV-tk/GCV therapy does not require 100% transduction efficiency to stimulate an immune response, some transduction is required to release tumor antigens and initiate an immunological response.

Both human lines (TE-1, SEG-1) and one mouse line (HNM007) showed transduction efficiencies comparable to A549, whereas the AKR cells were up to 100-fold less susceptible at each vector concentration (Figure 2a). These results showed that serotype 5 adenovirus constructs, including AdV-tk, have the potential to transduce adequately both human and murine esophageal carcinoma cells.

To further evaluate the efficacy of AdV-tk/GCV therapy *in vitro*, we first tested the most stringent model—the murine esophageal cancer model, AKR. AKR cells were transduced with AdV-tk or Ad.LacZ (control) at 1×10^{6} v.p. per ml and the cells were exposed to varying doses of GCV (range 0–2000 μM; note that human peak GCV serum concentrations are between 20 and 200 μM). The direct cytotoxic effect was evaluated by MTT assay (Figure 2b). GCV exerted cytotoxic effects on AdV-tk-transduced AKR cells in a dose-related manner. We observed that the half-maximal inhibitory concentration in this MTT assay was found to be at a GCV concentration of 200 μM, which is consistent with a low efficiency. These doses are similar to concentrations, we have used in NSCLC cell lines (unpublished data). We next carried out a similar assay for the HNM007 cell line (Figure 2c). We again observed a dose-dependent response; however, the half-maximal concentration was found to be at a GCV concentration of 20 μM, which was consistent with a slightly higher level of efficiency when compared to AKR.

These data provide evidence that AdV-tk/GCV is directly cytotoxic and potentially useful in the treatment of human esophageal carcinomas. In addition, the AKR model appears to be less susceptible to GCV and AdV-tk transduction; thus, it will likely provide a more stringent *in vivo* model.

AdV-tk/GCV is effective in in vivo *syngeneic murine models of esophageal cancer*

We next examined the efficacy of AdV-tk/GCV therapy in mice bearing flank AKR tumors. AKR tumor cells were injected subcutaneously into C57Bl/6 mice. When the established flank nodules reached a size of 250 mm^3, an intratumoral injection of 1×10^{10} v.p. of AdV-tk or Ad.LacZ (control) was administered. After 2 days, we

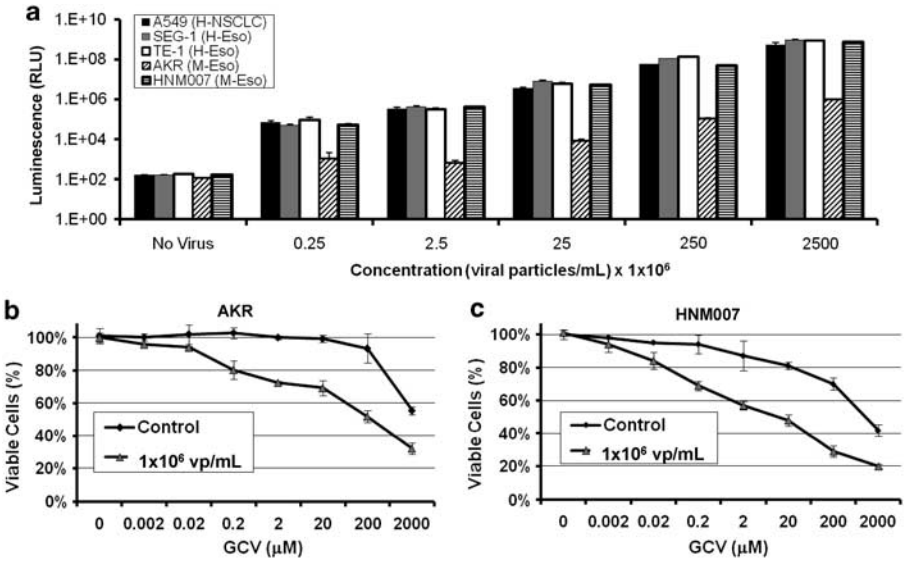


Figure 2 *In vitro* transduction and efficacy of type 5 adenovirus (Ad.luciferase) and AdV-tk/GCV (adenoviral vector carrying the herpes simplex virus thymidine kinase gene in combination with the prodrug ganciclovir) therapy for esophageal cancer models. (**a**) *In vitro* transduction of Ad.luciferase for esophageal cancer models. To determine *in vitro* transduction efficiency of human esophageal carcinomas (H-Eso) SEG-1, TE-1; murine esophageal carcinomas (M-Eso) AKR, HNM007; and A549 (human NSCLC line used as positive control) were transduced with Ad.luciferase at varying concentrations. Transduction efficiency was proportional to relative light units. SEG-1, TE-1 and HNM007 demonstrated high levels of transduction consistent with A549, whereas AKR was 10- to 100-fold more resistant to transduction. Next, *in vitro* efficacy of AdV-tk/GCV was investigated in the murine models of esophageal carcinoma (**b**) AKR and (**c**) HNM007. Cells were transduced at 1×10^6 viral particles per ml with AdV-tk or Ad.LacZ (control), and then treated with varying concentrations of the prodrug, GCV. The half-maximal inhibitory concentration for AKR cells was 200 μM and for HNM007 cells was 20 μM.

administered GCV at a dose of 50 μg i.p. daily, for 5 days. AdV-tk/GCV significantly inhibited tumor growth in this model; at day 26, the mean tumor volume of control mice was 1016 vs 338 mm^3 in mice receiving AdV-tk/GCV; $P = 0.004$ (Figures 3a and b). All mice randomized to AdV-tk/GCV appeared healthy, suffered no weight loss and tolerated the treatment well. However, no mice were cured, and tumors ultimately grew and animals required being killed owing to tumor burden.

To determine if a bystander effect occurred in AKR tumors growing *in vivo*, cells were infected with AdV-tk *in vitro* and mixed with various concentrations of non-transduced AKR cells 24 h after infection (under these conditions, no contamination of cells via residual adenovirus could occur). The cell mixtures were injected into the flanks of NSG mice (lack B cells, T cells, macrophages and NK cells) and allowed to grow to measurable size (day 6), at which time animals were then treated with i.p. GCV for 7 days. As shown in Figure 3c, all tumors containing AdV-tk-transduced cells demonstrated strong regression as compared to tumors consisting of uninfected AKR cells. The strongest effects were observed in tumors grown from 100% infected AKR cells; however, even those derived from mixtures of infected: uninfected cells at ratios 1:1, 1:5 and 1:10 displayed strong regression ($P < 0.01$) (Figure 3d). These experiments show that a strong cytotoxic bystander effect is operative in adenovirus-transduced AKR tumors *in vivo*.

Previous reports have described that acquired immune responses are a primary mechanism of the effects of GMCI systems.[12–14] To more precisely define this role in our AKR model and AdV-tk system, mice bearing established AKR flank tumors were randomized to four groups: (1) Ad.LacZ/GCV treatment (control); (2) Ad.LacZ/GCV with CD8 T-cell depletion; (3) AdV-tk/GCV; or (4) AdV-tk/GCV with CD8 T-cell depletion. Depletion of the T-cell population was confirmed weekly by analysis of spleen cells by flow cytometry (data not shown). Although mice receiving AdV-tk/GCV had smaller nodules after 26 days of tumor growth, effects were negated in the presence of CD8 T-cell depletion (Figures 3e and f). In fact, mice randomized to CD8 T-cell depletion protocols were observed to have more rapid growth, regardless of administration of AdV-tk/GCV and experienced more rapid growth (Figure 3e). These results confirm the role of CD8 T cells as an essential element in antitumor effects of AdV-tk/GCV protocols in our esophageal carcinoma models.

These experiments demonstrate *in vivo* evidence that AdV-tk/GCV therapy provides benefit with regard to tumor reduction in murine models of esophageal carcinoma. Our data in the NSG mice, as well as previous data using GMCI approaches, demonstrates that a bystander effect is observed when a sufficient number of tumor cells is infected. Given the rapid growth of the AKR line and the intratumoral delivery method, it is most likely that only a small percentage of tumor cells were infected; thus, only a small bystander effect was in wild-type mouse experiments. Although small, it appears that this mechanism of death generates a strong immunological response

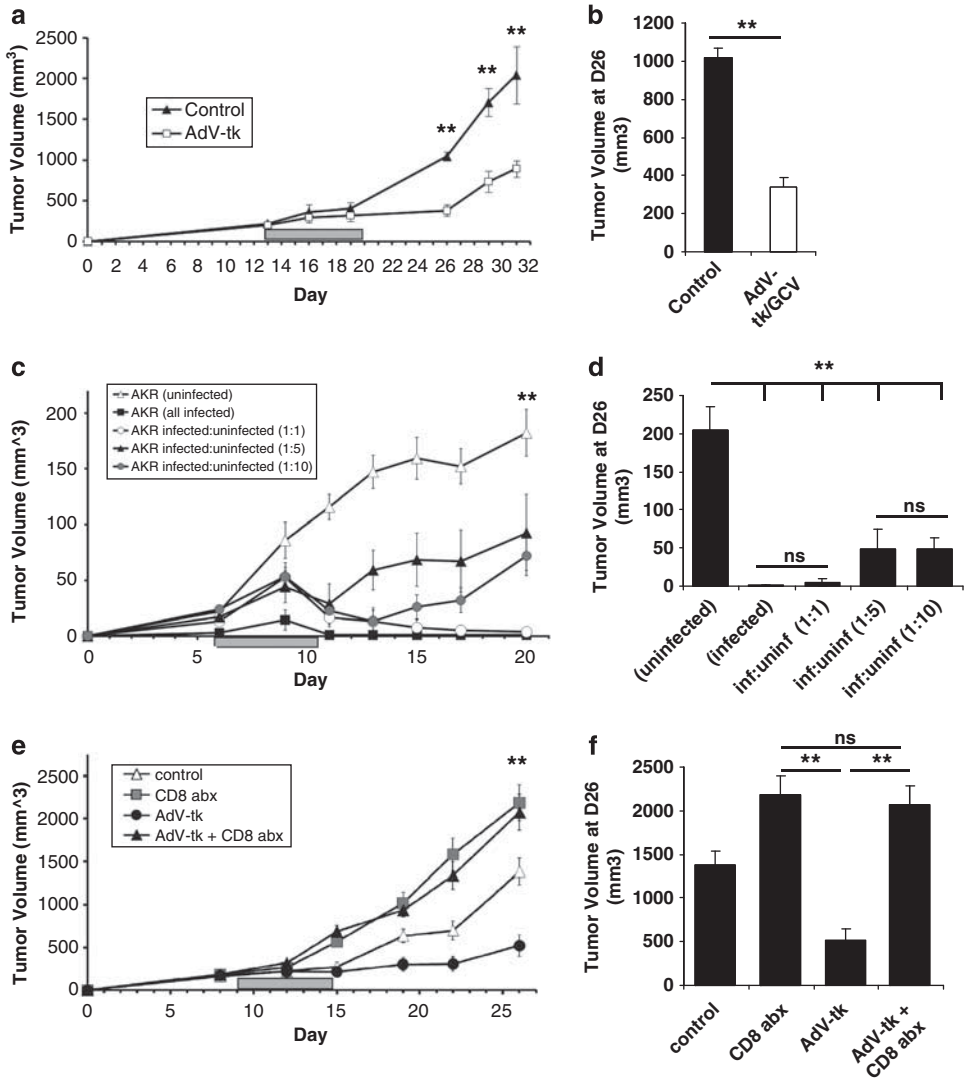


Figure 3 AdV-tk/GCV (adenoviral vector carrying the herpes simplex virus thymidine kinase gene in combination with the prodrug ganciclovir) treatment in murine model of esophageal cancer, AKR. (**a**) AdV-tk/GCV in AKR (no surgery). AdV-tk/GCV ($n=8$) or Ad.LacZ/GCV (control) ($n=8$) was administered intratumorally at day 13, and volume was recorded. AdV-tk/GCV provided a temporary, but significant reduction in tumor volume. (**b**) At day 31 after tumor cell inoculation, tumors treated with AdV-tk/GCV were less than 50% of the size of controls. The bystander effect. (**c**) AKR cells were infected with AdV-tk *in vitro* and mixed with uninfected AKR cells in varying ratios, and then injected into the flanks of NSG mice ($n=6$ for each group). At day 6 following inoculation, GCV was administered for 7 days. Mice bearing tumors originating from AdV-tk-infected cells grew slower in the presence of GCV. (**d**) Tumor volumes demonstrating a strong bystander effect at day 20. AdV-tk works in a CD8 T-cell-dependent manner. (**e**) C57Bl/6 mice bearing flank tumors were randomized into four groups: (1) Ad.LacZ/GCV treatment (control), (2) Ad.LacZ/GCV with CD8 T-cell depletion, (3) AdV-tk/GCV, or (4) AdV-tk/GCV with CD8 T-cell depletion. Effects of AdV-tk were eliminated in the absence of CD8 T cells. (**f**) Chart displaying the tumor volumes at day 26 following treatments evaluating AdV-tk/GCV dependence on CD8 T cells. *$P<0.05$; **$P<0.01$; shaded bar designates time over which AdV-tk/GCV therapy took place.

dependent on CD8+ T-cell trafficking. Taken together, these findings suggest an intricate interplay between cytotoxic effects (bystander effects) and a robust anti-tumor response driven by CD8 T cells.

Neoadjuvant AdV-tk/GCV decreases postoperative cancer recurrences

We next evaluated the efficacy of the AdV-tk/GCV regimen in limiting postoperative local recurrences using the positive margin model. C57Bl/6 mice were again injected with AKR cells into the subcutaneous flank. At day 9 following tumor cell inoculation, when the tumors reached a volume of $\sim 250\,mm^3$, mice were administered intratumoral AdV-tk or Ad.LacZ at a concentration of 1×10^{10} v.p. After 2 days, GCV was administered for 5 days, followed by 'positive margin' surgery on day 17. All mice undergoing surgery were observed to have a local recurrence; however, recurrent tumor volumes in the group receiving AdV-tk/GCV were significantly reduced postoperatively (Figures 4a and b). By postoperative day 14, the mean recurrent tumor volume in control mice was 1434 vs $735\,mm^3$ in mice receiving neoadjuvant

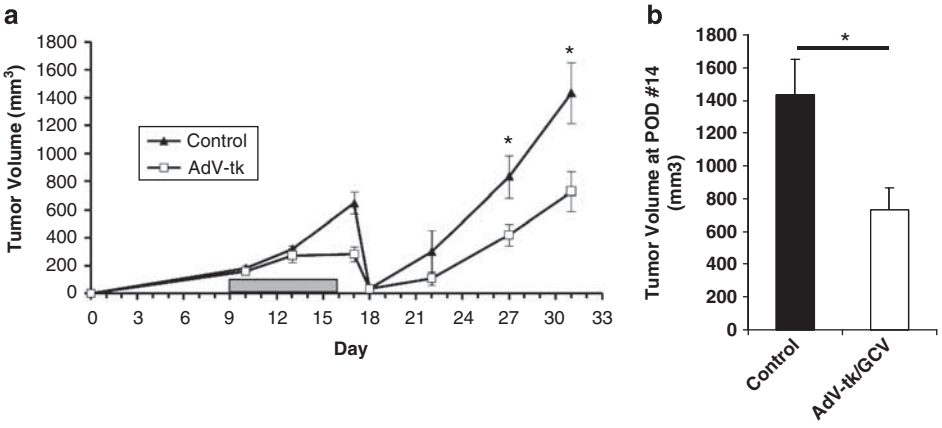


Figure 4 Neoadjuvant AdV-tk/GCV (adenoviral vector carrying the herpes simplex virus thymidine kinase gene in combination with the prodrug ganciclovir) for AKR. (**a**) AdV-tk/GCV ($n=8$) or Ad.LacZ/GCV (control) ($n=7$) was started on at day 10. Suboptimal surgery was performed on day 17, and postoperative local recurrent volumes were monitored. As in non-surgical models, administration of AdV-tk/GCV resulted in decreased tumor volumes at postoperative periods; however, all tumors recurred and ultimately grew to a point requiring animals to be killed. (**b**) Bar graph demonstrating tumor volumes at postoperative day 14. *$P<0.05$; **$P<0.01$; shaded bar designates time over which AdV-tk/GCV therapy took place.

AdV-tk/GCV therapy; $P=0.03$. Again, all mice randomized to AdV-tk/GCV appeared healthy. No mice were cured from this treatment, and all ultimately succumbed to disease burden. Although not curative as a monotherapy, these results suggest a potential adjuvant role for GMCI in limiting recurrent tumor growth in models of esophageal carcinoma.

Neoadjuvant AdV-tk/GCV followed by surgery and chemotherapy significantly decreases recurrent disease progression

Evidence in other tumor models indicates a synergy between AdV-tk/GCV and chemotherapy.[36–38] We hypothesized that AdV-tk/GCV followed by surgery and cis/5-FU would similarly provide superior effects in limiting postoperative recurrences with minimal additional toxicity. To test this, AKR cells were injected into the flanks of C57Bl/6 mice. Once flank tumors reached a volume of 250 mm^3, 1×10^{10} v.p. of AdV-tk or Ad.LacZ was given intratumorally. After 2 days, we administered i.p. GCV therapy daily for 7 days. Following completion of AdV-tk/GCV or Ad.LacZ/GCV therapy, surgery was performed, leaving approximately 10% of tumor volume. At postoperative days 3 and 10, cis/5-FU was administered in an i.p. manner as described previously.

As in the single-agent studies described above, we observed a modest reduction in tumor progression in animals receiving AdV-tk/GCV or chemotherapy as monotherapy. However, in mice randomized to combination therapy, we observed substantial additional reduction in tumor growth when compared to control mice and mice receiving monotherapies (Figure 5a). At postoperative day 14, recurrent tumor volumes of control mice, mice receiving AdV-tk/GCV, cis/5-FU or combination were 1434, 886, 744 and 345 mm^3, respectively ($P=0.007$) (Figure 5b). Although no mice were cured in any treatment groups, we observed almost a twofold increase in postoperative median survival in mice receiving combination therapy vs those receiving monotherapy (Figure 5c). By Kaplan–Meier analysis with log-rank testing, median postoperative survival for control, AdV-tk/GCV, cis/5-FU and combination groups was 18, 21.5, 24 and 34 days, respectively ($P<0.001$). Of note, all mice receiving combination therapy appeared healthy without signs of systemic toxicity throughout the duration of the experiment.

These results suggest that addition of AdV-tk/GCV with standard chemotherapy protocols and surgery result in superior outcomes following surgery. This regimen appears to be well tolerated and highly effective in postoperative survival, although no mice were cured. Even though no cures were observed, the significant increases in postoperative survival are encouraging and offer a potentially life-prolonging intervention for patients diagnosed with operable esophageal carcinoma.

Preoperative in situ *GMCI increases intratumoral trafficking of CD8 T cells postoperatively*

To further assess the mechanistic role of AdV-tk/GCV treatment, we performed immunohistochemical staining on selected tumor sections. Mice bearing flank AKR tumor were again treated with Ad.LacZ or AdV-tk/GCV, followed by surgery and postoperative cis/5-FU. Tumors were harvested at 3–5 days after the first dose of postoperative chemotherapy. In both groups, staining with hematoxylin and eosin revealed the presence of a large area of central necrosis with a diffuse infiltration of white cells into the tumor, whereas a peripheral rim of intact tumor tissue was noted (data not shown). Immunohistochemical CD8 staining demonstrated an increased intratumoral CD8 T-cell trafficking in mice receiving AdV-tk/GCV therapy vs those receiving Ad.LacZ/GCV; 13.6 vs 4.2 cells per high power field ($P=0.009$) (Figure 6a).

To further assess the intratumoral CD8 T-cell trafficking associated with AdV-tk/GCV therapy, we digested the

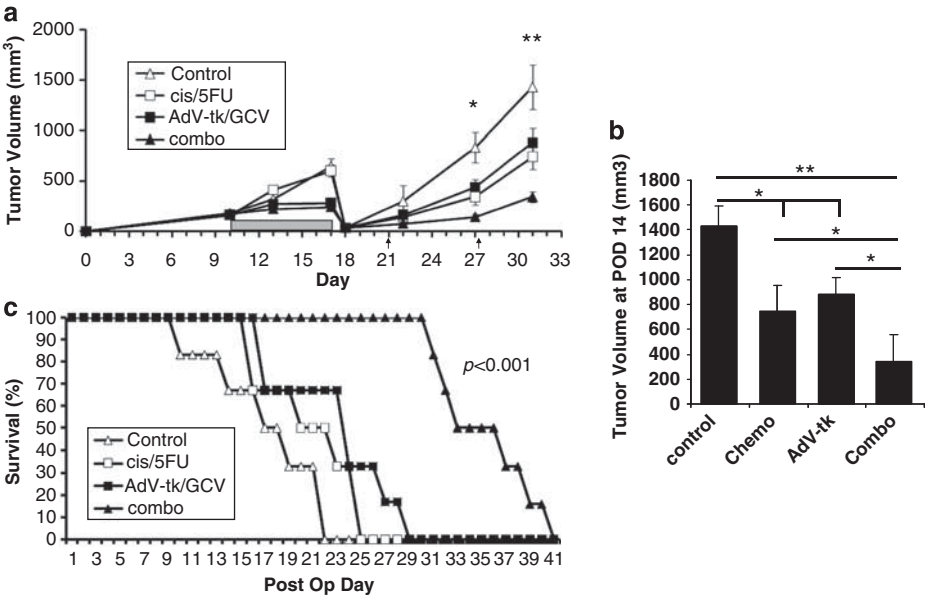


Figure 5 Combination of neoadjuvant AdV-tk/GCV (adenoviral vector carrying the herpes simplex virus thymidine kinase gene in combination with the prodrug ganciclovir) with postoperative cisplatin (cis)/5-fluorouracil (5-FU) is effective in AKR. (**a**) Flank tumor growth with combination adjuvant therapy. AdV-tk/GCV or Ad.LacZ/GCV (control) was begun at day 10. Surgery was completed on day 17, followed by cis/5-FU administration (days 20 and 27). Seven mice were randomized to each of the four treatment protocols. Postoperative volumes were recorded, and significant decreases were observed in mice randomized to treatment arms, with combination therapy providing the most significant reduction at day 31 (postoperative day 14); $P=0.007$. (**b**) Bar graph further describing dramatic reduction in recurrent tumor burden following protocols incorporating AdV-tk/GCV with postoperative cis/5-FU. (**c**) Kaplan–Meier analysis with log-rank comparison demonstrating postoperative survival benefits associated with adjuvant combination therapy administration. *$P<0.03$; **$P<0.01$; shaded bar designates time over which AdV-tk/GCV therapy took place; arrowheads designate timing of cis/5-FU.

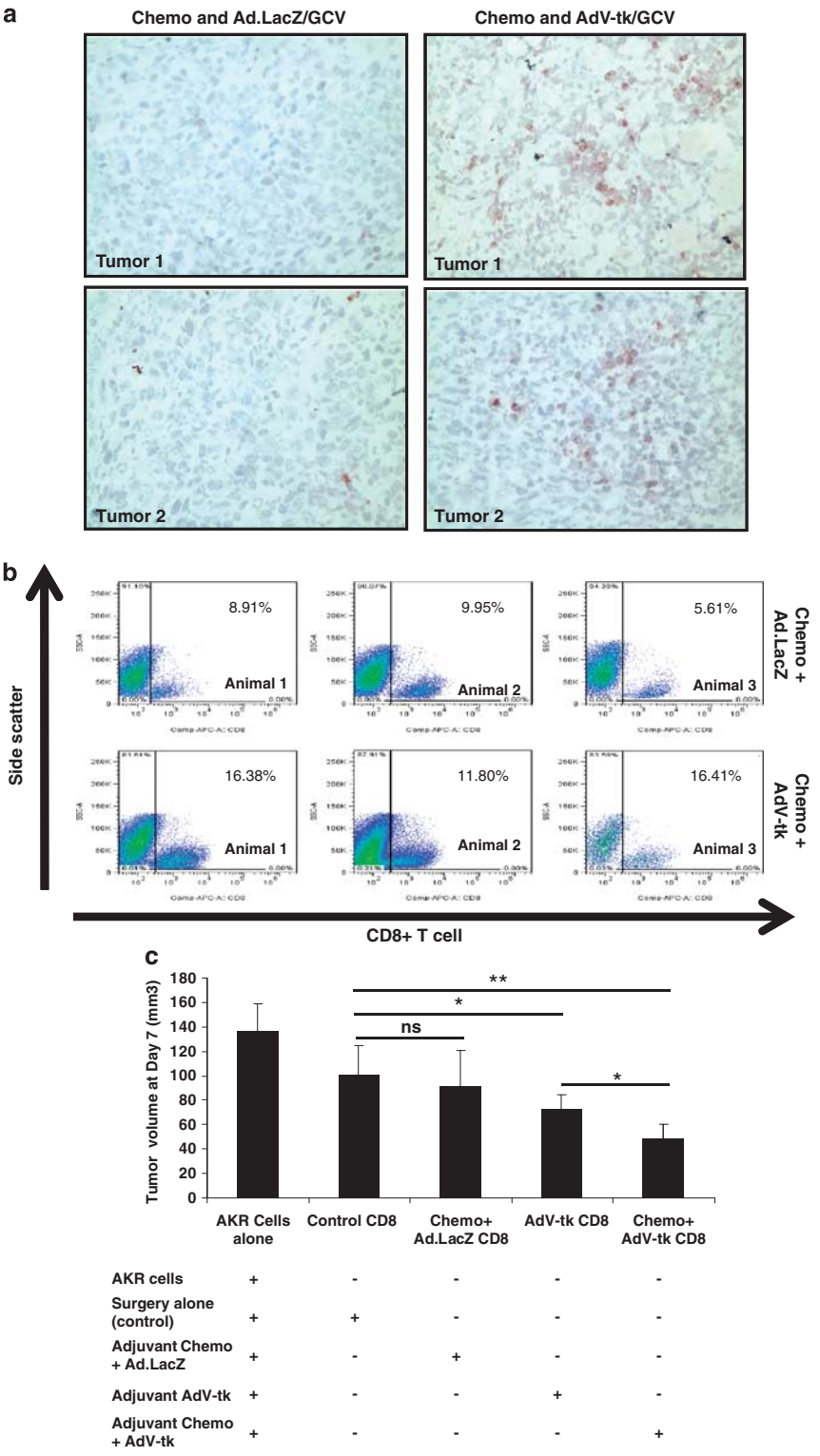
residual tumors and performed fluorescence-activated cell sorter analysis. At the same time, tumors were harvested for staining, and were also processed for flow cytometric analysis. We noted that mice receiving AdV-tk/GCV had a significant increase in the percentage of CD8 immunocytes (8.15% vs 14.89%; $P=0.02$) of CD8 cells (Figure 6b). To confirm that CD8 T cells were responsible for this antitumor effect, we isolated CD8 T cells from mice 3 to 5 days following the first dose of postoperative chemotherapy. These CD8 T cells were injected along with fresh AKR tumor cells (in a ratio of 3 CD8 T cells:1 AKR cell) into the flank of tumor-naïve C57bl/6 mice. AKR cells were injected alone in five mice to tumor cell viability. The growth of these nodules was then observed for 7 days. We found that CD8 T cells obtained from mice receiving both neoadjuvant AdV-tk/GCV and postoperative chemotherapy were better able to neutralize AKR cells as compared to mice receiving either monotherapy alone ($P=0.02$) (Figure 6c). These results suggest that neoadjuvant *in situ* GMCI decreases tumor burden primarily by augmentation of antitumor CD8 T-cell function.

Discussion

To our knowledge, this report marks the first study utilizing immunotherapy in a syngeneic murine model of esophageal cancer. In this report, we demonstrate that neoadjuvant GMCI in conjunction with surgery and cis/5-FU has a strong effect in impeding recurrent tumor growth and improving postoperative survival in our novel murine models of esophageal cancer. The mechanism underlying these benefits arises from (1) direct cytotoxic effects and (2) augmentation of adaptive host immune system locally at the site of surgical resection. Similar studies examining the role of adjuvant immunotherapies for esophageal cancer have been lacking owing to the paucity of high-throughput esophageal cancer models, which can be readily utilized for preclinical evaluation.

Immunotherapy approaches augment the host's adaptive immune system to eliminate residual cancer cells, which may persist after debulking with standard therapies. This approach is attractive as a surgical adjuvant for a number of reasons. First, immunotherapy is most successful for small tumor burdens. Therefore, esophageal resection and chemoradiation eliminate large tumor burden, whereas immunotherapy offers the potential to eliminate residual disease.[39,40] Second, immunotherapy has minimal toxicity, lack of dependence on rapidly dividing cells, and the ability to generate long-standing antitumor memory. Third, immunotherapy has recently been demonstrated to synergize with chemotherapy and radiotherapy to augment the antitumor effect of each individual modality.[24,38]

The development of immunocompetent syngeneic murine models is critical for the study of adjuvant



immunotherapy, as there is an array of evidence suggesting that a complex set of immune responses that occur during the perioperative period: (i) the generation of an endogenous antitumor immune response (concomitant immunity);[41] (ii) the well-known phenomenon of perioperative immunosuppression;[42] and (iii) the immunological consequences associated with wounds and wound healing.[43] Our recent development of two syngeneic esophageal carcinoma lines, AKR and HNM007, has allowed us to examine immunotherapies, and ultimately

Figure 6 Neoadjuvant AdV-tk/GCV (adenoviral vector carrying the herpes simplex virus thymidine kinase gene in combination with the prodrug ganciclovir) with postoperative cis/5-FU increases CD8 T-cell lymphocyte infiltration and systemic populations more effectively than Ad.LacZ/GCV with cis/5-FU. (**a**) Representative immunohistochemistry staining for CD8 T-cell trafficking (tissue was obtained from tumors of two different mice per group). Flank tumors were harvested, sectioned and stained for CD8 T cells following the first dose of chemotherapy. Tumor immunohistochemical staining revealed increased CD8 cells in mice receiving combination AdV-tk/GCV with cis/5-FU vs Ad.LacZ/GCV with cis/5-FU (13.6 vs 4.2 cells per high power field (HPF); $P=0.009$). (**b**) Representative fluorescence-activated cell sorter (FACS) tracings for intratumoral CD8 T cells (three representative samples per group). Tumors infiltrates were analyzed by flow cytometry following the administration of the first dose of chemotherapy. Flow cytometry tracing revealed an increased percentage of intratumoral leukocytes composed of CD8 T cells in those mice receiving AdV-tk/GCV with cis/5-FU vs Ad.LacZ/GCV with cis/5-FU ($P=0.02$). (**c**) Winn assay results reveal that combination of AdV-tk/GCV with chemotherapy functions in a CD8 T-cell-dependent manner. CD8 T cells from mice receiving combination treatments were superior in neutralizing fresh tumor cells *in vivo* when compared to those CD8 T cells harvested from mice receiving postoperative chemotherapy and surgery alone, and tumor cells alone ($n=5$ per group). $^{*}P<0.05$; $^{**}P<0.01$; shaded bar designates time over which AdV-tk/GCV therapy took place.

determine their effects with the presence of these surgical variables. Our AKR model incorporates both genetic (cyclin-D1 overexpression and p53 deletion) and physiological (resistance to standard platinum-based chemotherapy regimen) similarities to human esophageal carcinomas.

Until recently, the combination of immunotherapy with chemotherapy has not been investigated because of theoretical fears that the antiproliferative effects of chemotherapy (seen clinically as post-chemotherapy leukopenia) would eliminate antitumor directed lymphocytes. An increasing number of preclinical and clinical studies, including work by our own group,[38] have recently shown that immunotherapy is not only compatible, but may act synergistically with certain chemotherapies. Our observations suggest that such a cooperation exists between AdV-tk/GCV and standard cis/5-FU used in esophageal cancer management. With consideration of our findings, it is conceivable that other immunotherapy approaches may also offer alternative approaches to manage patients with esophageal cancer, without adding unnecessary toxicities.

The mechanisms responsible for this unique combination between immunotherapy and subsequent chemotherapy have not been fully explored. Our group has provided evidence suggesting that this markedly augmented effect could partially be explained by a 'prime and boost' phenomenon.[38] To further describe, viral immunogene therapy initially induces effector antitumor T cells (the prime). After the initial elimination of tumor cells, chemotherapy agents serve to augment (the boost) the immune response by providing additional antigen and tumor danger signals to antigen-presenting cells that re-activate memory antitumor T cells, and altering the tumor microenvironment.[38] We provide additional evidence that immunotherapy (GMCI) synergizes with chemotherapy (cis/5-FU) and surgery and reduces recurrent tumor growth and enhances survival in our murine models of esophageal carcinoma. In addition, following treatment of esophageal flank tumors with preoperative AdV-tk/GCV, we noted increased trafficking of CD8 T cells into recurrent tumors by immunohistochemistry. These data support the notion that AdV-tk/GCV functions by an immune pathway. In addition to local effects, previous studies suggest that systemic CD8 T cells also increase, which reflects the ability of preoperative therapy to generate prolonged antitumor responses.

In summary, this report describes the first successful study demonstrating cooperative effects of immunotherapy with standard chemotherapy and surgery for esophageal carcinoma using our newly developed syngeneic murine models. We have found that this treatment protocol provides both significant decreases in disease progression and postoperative survival, with minimal morbidity and mortality. The primary effects of this regimen are elicited via increases in CD8 T-cell trafficking within the tumor. We also present initial *in vitro* evidence supporting the notion that a human response will mirror (and potentially supersede) our murine preclinical findings, as the human esophageal cancer cells were more susceptible to AdV-tk-mediated killing than murine tumor cells. Although additional studies are likely required to most efficiently translate these results to the clinical arena, the results nonetheless demonstrate that protocols incorporating inmmunotherapies have the potential to dramatically improve outcomes for those diagnosed with esophageal cancer.

Conflict of interest

Estudardo Aguilar-Cordova is the CEO of Advantagene and Laura Aguilar the CFO of Advantagene.

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