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Adenoviral Based Immunotherapy Provides Local Disease Control in an Orthotopic Murine Model of Esophageal Cancer

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Abstract

Despite recent advances in the development of novel therapies, esophageal carcinoma remains an aggressive cancer associated with a poor prognosis. The lack of a high throughput, reproducible syngeneic animal model that replicates human disease is partly responsible for the paucity of novel therapeutic approaches. In this report, we present the first successful syngeneic, orthotopic model for esophageal cancer. This model was used to test an established adenoviral-based tumor vaccine. We utilized a murine esophageal cancer cell line established from the EDL2-cyclin D1;p53^{-/-} mouse that was transduced to express a viral tumor antigen, the Human Papilloma Virus (HPV) E7 protein. The tumor was established in its natural microenvironment at the gastroesophageal (GE) junction. Tumor growth was consistent and reproducible. An adenoviral vaccine to E7 (Ad.E7) induced an E7-specific population of functionally active CD8⁺ T cells which trafficked into the tumors and retained cytotoxicity. Ad.E7 vaccination reduced local tumor growth and prolonged overall survival. These findings suggest that orthotopic tumor growth is a reasonable preclinical model to validate novel therapies.

Keywords

Surgery; Esophageal Carcinoma; Immunotherapy; Animal Models; Orthotopic Implantation; CD8⁺ T Lymphocytes

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Introduction

Esophageal cancer is one of the most commonly diagnosed cancers in the United States (1) and is associated with an increasing incidence worldwide (2). In 2008, 500,000 people were diagnosed with, and 400,000 people died of, esophageal cancer worldwide (2). In the United States alone, it ranks in the top ten for cancer-related mortality, mainly due to the high rate of locally advanced disease at presentation (3). As a result, this disease is associated with a dismal prognosis and estimated 5-year survival rates are less than 15% (3).

Currently the standard of care for patients includes a combination of radiation therapy, chemotherapy (typically a combination of platinum-based agents and 5-fluorouracil), and surgery (4-6). However, with the recognition of tumor cells' ability to actively resist and evade immune destruction, recent efforts have focused on developing immunotherapies to augment the endogenous immune system to combat this disease. Clinical evidence, although limited, supports the notion that esophageal cancer is immunogenic. Several early trials of targeted monoclonal antibodies and immuno-gene therapy have shown survival differences (7, 8). In addition, there have been limited case reports of immunotherapy for esophageal cancer using approaches such as lymphokine-activated killer cells (9, 10) and adoptive immunotherapy (11).

One of the major hurdles to developing novel therapies for esophageal cancer has been the lack of a high throughput, orthotopic, syngeneic model. Most experiments have been performed using human esophageal cancer cell lines in immunodeficient mice or have used tumor lines from alternative disease types and extrapolated the results to esophageal cancer (12-14). In response, our group was the first to develop a syngeneic murine model of esophageal cancer (15, 16), which we used to demonstrate the effectiveness of neoadjuvant *in situ* gene-mediated cytotoxic immunotherapy. However, this model was predicated on the direct injection of murine esophageal cancer cells into the flank location.

The shortcoming of this model, as with all non-orthotopic models, is the inability to replicate the environment of the primary tumor site and patterns of metastatic spread. Both of these differences may have an impact on the response to experimental therapies. For studies on early stage tumors prior to metastasis, re-creating the primary tumor site is critical. The microenvironments of tumors injected subcutaneously have been shown to be significantly different than the natural *in situ* microenvironments (17-19), which in turn affected the response to treatment (20). Replication of the patterns of metastatic spread is also important, especially for studies in advanced stage disease. Along these lines, the "soil and seed" hypothesis correctly highlights the idea of the necessary compatibility between specific tumor cells ('seed') and their metastatic niches ('soil') (21). This hypothesis, however, presupposes that tumor cells with metastatic potential were generated and that their patterns of dispersal throughout the body enable them to find their niches. Orthotopic models of disease best address these assumptions, allowing tumors to develop the full range of "seeds" found in the highly heterogeneous human cancers and to disperse these seeds along their native lymphovascular routes. Thus, we sought to develop an orthotopic animal model of esophageal cancer.

In this report, we present the first successful syngeneic, orthotopic model of esophageal cancer for the preclinical evaluation of a novel adenoviral-based immunotherapy. We first describe the development of the AKR-E7 cell line and characterize its growth in the orthotopic location; together, these results confirm that our model is easily reproducible, consistent, and inexpensive. Then, we demonstrate its utility in the evaluation of an active immunotherapeutical approach: cancer-antigen vaccination with an adenovirus expressing the protein E7.

Materials and Methods

Animals

Female C57Bl/6 mice (B6, Thy1.2) were purchased from Charles River Laboratories. 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 (22, 23). To generate AKR-E7, AKR cells were infected with a retroviral vector coding E7 protein of HPV-16, pBABE-puro-HPV16-E7, which was a gift from Dr. Schlegel Richard (24). To generate AKR-E7-Luciferase (AKR-Luc), AKR-E7 cells were infected with a retroviral vector coding firefly luciferase, pFB-neo-Luc, was described previously (25). After gene-transduction with the retroviruses, drug selection was carried out for 7 days with puromycin (2 µg/ml) or G418 (300 µg/ml). The tumorigenic TC1 cell line was derived from mouse lung epithelial cells immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene (26) and provided by Dr. Steven Albelda (University of Pennsylvania, Philadelphia, PA). This line is highly tumorigenic in C57BL/6 mice and will expand in the murine flank.

Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum, 1% (vol/vol) L-glutamine, and 1% (vol/vol) penicillin/streptomycin, and cultured at 37°C in a humidified incubator containing 5% CO₂. Cells were tested regularly and maintained negative for *Mycoplasma* spp.

RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

Detection of mRNA transcripts was performed as previously described (27). Briefly, after culture in growth medium for 2 days, TC1, AKR and AKR-E7 cells were harvested and homogenized in TRIzol Reagent (Invitrogen, Carlsbad, CA). Three µg of RNA was reverse transcribed using 0.5 µg oligo (dT) (Promega, Madison, WI), 1 mmol/L deoxynucleotide triphosphates, 10 mmol/L dithiothreitol (Clontech), and 10 units SuperScript III reverse transcriptase in 1× First-Strand Buffer (Clontech, Palo Alto, CA) with 20 µl final volume for 60 minutes at 50°C, 15 minutes at 70°C and 5 minutes at 99°C. Primers were obtained from the literature or designed using standard protocols. Primer sequences can be obtained from the authors on request. cDNA concentrations from each pool were normalized using

glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control gene. PCR products were electrophoresed in a 1.5% agarose gel containing ethidium bromide.

Orthotopic Esophageal Cancer Animal Model

To establish orthotopic esophageal carcinomas at the gastroesophageal (GE) junction, C57BL/6 mice were first anesthetized using weight appropriate dosing of ketamine (100mg/kg) and xylazine (10mg/kg). Mice were then placed in the supine position, and the abdomen was shaved and cleaned using alcohol preps. The peritoneal space was entered via a small incision (0.5-1cm) just inferior to the left costal margin. Once in the peritoneal cavity, the body of the stomach was located. The stomach was gently pulled inferiorly and through the incision to reveal the GE junction. Once the junction was visualized, 0.5×10^6 AKR, AKR-E7, or AKR-E7-Luc cells (suspended in 50 μ L of solution: 25 μ L of X-Vivo™ 15 media [Lonza; Walkersville, MD] and 25 μ L Matrigel [BD Biosciences; Franklin Lakes, NJ]) were injected directly into the esophageal wall using a 27-gauge insulin syringe. Careful consideration was given to minimize injecting the cells directly into the esophageal lumen. Once the syringe was removed, a q-tip was applied to the injection site for 30 seconds to minimize spillage in to the cavity. The stomach was then repositioned into its native position in the peritoneal cavity. The peritoneum and abdominal muscles were closed together using a running 4-0 silk suture. The skin was closed after using discontinuous 4-0 silk suture. Weight appropriate buprenorphine (0.1 mg/kg) was given at the time of surgery and 6 hours later as postoperative analgesia.

Luciferase-based in vivo imaging

Mice bearing orthotopic AKR-luciferase tumors were imaged using *in vivo* IVIS bioluminescent imaging (Caliper Life Sciences; Hopkington, MA) at multiple time points as previously described by our group (28).

Immunotherapy and cancer vaccination

An adenoviral vector expressing the HPV-E7 protein under control of a cytomegalovirus promoter (Ad.E7) was provided by Hildegund Ertl (Wistar Institute, Philadelphia, PA) (29) and subsequently produced in the University of Pennsylvania Viral Core Facility. Non tumor-bearing animals were vaccinated subcutaneously (s.c.) in one flank with 1×10^9 plaque-forming units (pfu) of Ad.E7 vector. Seven days following the initial vaccination, mice received a booster vaccine of 1×10^9 pfu of Ad.E7. Tumor-bearing animals were vaccinated 5 days after tumor-injection s.c. in one flank with 1×10^9 pfu of Ad.E7 vector. Five days following the initial vaccination, mice received a booster vaccine of 1×10^9 pfu of Ad.E7. Control animals received the same doses of a control virus (Ad.LacZ) produced by Advantagene (Auburndale, MA). All vectors were suspended in 100 μ L of normal saline.

In vivo depletion of CD8 T cells

To deplete CD8⁺ T cells, mice were injected intraperitoneally (i.p.) with monoclonal antibodies (mAbs) purified from the anti-CD8⁺ hybridoma 53-6.7 (obtained from the American Type Culture Collection). Mice were given 200 μ g of monoclonal antibody i.p. suspended in 200 μ L PBS. Injections were administered 1 day after the second adenoviral

vaccination. 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 single cell suspensions of splenocytes (data not shown).

Flow cytometric analysis of tumors

For flow cytometric analysis, tumors were removed from euthanized mice and minced into 1-2 mm fine pieces in digestion buffer containing 0.1 mg/mL DNase I and 2.0 mg/mL collagenase type IV (Sigma; St. Louis, MO). Samples were incubated in digestion buffer at 37°C for 30 minutes, filtered through a 70 µm filter, and washed twice with DMEM supplemented with 10% FBS (vol/vol). Fc receptors were blocked with anti-mouse CD16/CD32 antibodies (BD Biosciences PharMingen). Following one wash with staining buffer (PBS supplemented with 2% FBS [vol/vol]), cells were incubated for 30 minutes at 4°C with appropriate antibodies obtained from BD Biosciences PharMingen (San Diego, CA) and used at the indicated dilutions for flow cytometry: CD45-PerCP (1:200), CD8-APC (1:200), or allophycocyanin-labeled H-2D^b tetramers (1:500 dilution) loaded with E7 peptide (RAHYNIVTF), that were obtained from the National Allergy and Infectious Diseases Tetramer Core Facility. Samples were then washed and resuspended in staining buffer for immediate flow cytometric analysis. Flow cytometry was completed using a Becton Dickinson FACS Calibur flow cytometer (BD Biosciences; Franklin Lakes, NJ), and analyzed using FlowJo software (Ashland, OR).

Measurement of Intracellular IFN γ Levels

For intracellular cytokine staining studies, cells were resuspended in medium before being plated in 24-well plates and incubated for 18 hours in 5% CO₂/humidified air at 37 °C. Ten µg of brefeldin A (Sigma Aldrich; St. Louis, MO) was then added to each well to immobilize the interleukins in the Golgi apparatus. After 18 hours of incubation, cells were pretreated with DMSO (Sigma Aldrich; St. Louis, MO) vehicle control for 2 hours. Fifty ng/mL of phorbol myristate acetate (PMA) and 0.5 µg/mL of ionomycin were then added for 4 hours. Cells were then fixed with 2% paraformaldehyde and incubated at 37 °C for 15 minutes in the dark. Cells were washed and permeabilized in 0.5% saponin for 30 minutes on ice in the dark. After permeabilization, cells were washed, resuspended in 0.5% saponin, and stained with IFN- γ (BD Biosciences; Franklin Lakes, NJ).

Statistical analyses

Unless otherwise noted, data comparing differences between two groups were assessed using unpaired Student's *t* test. Comparisons with more than two groups were conducted using ANOVA with appropriate *post hoc* testing. Survival data for treatment groups were compared using the log-rank statistic. Differences were considered significant when *p*<0.05. Data are presented as mean (standard error of the mean), unless otherwise noted.

Results

Generation of a syngeneic esophageal cancer cell line with genetic expression of the HPV E7 protein

Our group was the first to develop a syngeneic murine model of esophageal cancer, termed AKR (16). AKR was established from the ED-L2-cyclin D1;p53^{-/-} mouse as described previously (23). In order to test the hypothesis that an adenoviral-based antitumor vaccination strategy is effective in inhibiting orthotopic tumor growth, AKR cells were stably transduced with a retroviral vector encoding a known viral tumor antigen, the HPV E7 protein (30). As shown in Figure 1a, AKR-E7 cells express E7 transcripts at similar levels compared to the positive control, TC1, a known E7-expressing lung cancer cell line. Furthermore, C57BL/6 mice do not mount an immune response against AKR-E7, as it demonstrates consistent, reproducible growth curves in the flank (Figure 1b). Together, these results suggest that AKR-E7 represents a viable syngeneic esophageal cancer cell line whose expression of a known viral tumor antigen has no impact on tumor growth.

Establishment of AKR-E7 tumor cells at the GE junction

Given the inherent advantages of orthotopic models in replicating the primary tumor site and patterns of metastatic spread, we sought to develop the first such model by testing the ability of AKR-E7 cells to grow in an orthotopic location at the GE junction (Figure 2a). After injection into the esophageal wall, tumors were allowed to grow for 24 days. Mice were sacrificed at various time intervals to analyze tumor growth.

After the technique was established in our lab, we injected 60 mice, with 82% demonstrating tumors at times of necropsy. We appreciated rapid, but consistent, growth of these tumors after injection, with sizes of approximately 20mg at Day 4 and 360mg at Day 14 (Figure 2b, left panel). Lesions were confined to the point of tumor-injection (Figure 2b, right panel). Microscopically, these tumors invaded the lumen of the esophagus and eventually obliterated the muscular layers (Figure 2c). We did not, however, observe direct invasion of the esophageal mucosa. This most likely reflects the route of injection, in which we deliberately avoided tumor cell delivery into the lumen (or near the lumen in an attempt to avoid esophageal perforation). No animals had appreciable evidence of dysphagia or significant weight loss (data not shown).

Although reliable, monitoring lesions required periodic animal sacrifice. Such an approach is time-consuming and expensive, especially when evaluating anti-neoplastic therapies in the pre-clinical setting. To address this concern, AKR-E7 tumor cells were stably transduced to express firefly luciferase (AKR-E7-Luc) to allow for non-invasive, *in vivo* monitoring of disease. As in our previous model, AKR-E7-Luc tumor cells were implanted in the GE junction of wild-type mice. Growth was monitored using *in vivo* IVIS bioluminescent imaging. We appreciated steady growth of these lesions for the first 7-10 days (Figure 2d), however, the fluorescence ultimately regressed presumably due to immunogenic properties of the luciferase gene product in the C57BL/6 mouse. When the animals were euthanized, the tumors were viable at the GE junction but were clearly not bioluminescent.

Together, these results demonstrate that the AKR-E7 esophageal cell line grows in a reproducible and predictable manner in the orthotopic location.

Vaccination of naïve non tumor-bearing animals with tumor-associated antigen prevents tumor establishment

Our group has shown that vaccination with Ad.E7 prevents the growth of another E7-expressing lung adenocarcinoma cell line, TC1. We showed that this effect was the direct result of the production of E7-specific CD8⁺ T cells with cytotoxic function (31). Thus, we sought to replicate these findings in order to validate our model.

First, we confirmed that Ad.E7 vaccination could generate E7-specific CD8⁺ T cells. Briefly, we vaccinated naïve non tumor-bearing mice with Ad.E7 (n= 8) or Ad.LacZ (n=9) both 10 and 3 days prior to injecting AKR-E7 cells into the GE junction. Of note, at the time of tumor cell implantation, a mouse from each group was euthanized to confirm the presence of E7 epitope-specific CD8⁺ T cells using tetramer staining techniques. As shown in Figure 3a, the Ad.E7-vaccinated animal generated more E7-specific CD8⁺ T cells than the Ad.LacZ control animal.

We subsequently measured tumor burden in these animals to determine if vaccination prevented the growth of AKR-E7 tumors. At Day 14 post tumor cell-injection, mice were euthanized and tumor presence evaluated. We appreciated growth in 14% (1/7) of tumors in the mice randomized to Ad.E7 vaccination compared to 75% (6/8) in mice randomized to control vaccination protocols ($p < 0.05$). Representative bloc specimens are provided in Figure 3b.

To confirm the role of vaccine-induced CD8⁺ T cells, we repeated the previous experiment in CD8⁺ T cell-depleted wild-type animals. Briefly, mice were vaccinated both 10 and 3 days prior to tumor challenge with Ad.E7 (n=15). These animals were then randomized to receive either anti-CD8 antibodies (n=8) or isotype control antibodies (n=7) 1 day after the second Ad.E7 dose was administered. AKR-E7 tumors were then implanted at the GE junction 2 days following anti-CD8 antibody administration (3 days following the second dose of Ad.E7). As a positive control, AKR-E7 cells were injected into the GE junction of mice receiving neither vaccination nor CD8-depleting antibodies (n=5). We also included a group of animals whose CD8⁺ T cells were depleted in the absence of vaccination (n=7). As before, Ad.E7 vaccination without CD8⁺ T cell-depletion prevented the growth of AKR-E7 tumors, as all 5 animals demonstrated an absence of tumor growth (0%). As predicted, the previously observed prevention of tumor growth associated with Ad.E7 vaccination was negated in the presence of CD8⁺ T cell-depletion; 88% (7/8) of animals who were neither vaccinated nor administered CD8-depleting antibodies and 100% (7/7) of animals whose CD8⁺ T cells were depleted in the absence of Ad.E7 vaccination developed tumors, as compared to 85% (6/7) of mice vaccinated with Ad.E7 alongside CD8⁺ T cell-depletion ($p = 0.89$). Furthermore, tumors were similar in weight among these three groups: 324 ± 64 mg, 350 ± 78 mg, and 345 ± 52 mg, respectively (Figure 3c).

Thus, these results demonstrate that Ad.E7 vaccination generates tumor antigen-specific (E7-specific) CD8⁺ T cells with significant *in vivo* anti-tumor cytotoxicity.

Ad.E7 vaccination significantly inhibits the growth of established orthotopic esophageal tumors and prolongs survival

Similar to human esophageal carcinomas, we have previously shown that the AKR cells line is only moderately responsive to standard-of-care chemotherapy (cisplatin/gemcitabine) at equivalent human dosing (16). Thus, we hypothesized that active immunotherapy with a cancer vaccine might represent a superior therapeutic strategy.

We injected 0.5×10^6 AKR-E7 cells into the GE junction of C57Bl/6 mice (n=28). Five days after tumor-injection, animals were randomized to receive administration of 1×10^{10} viral particles of either Ad.E7 (n=14) or Ad.LacZ (n=14) in the flank. This initial administration was followed up with a booster vaccine 5 days later (i.e., Day 10 post tumor-injection). At Day 14, 7 animals from each group were sacrificed and their tumors isolated for analysis. By Day 14, we appreciated significant reduction in tumor growth in mice randomized to the Ad.E7 immunotherapy protocol compared to Ad.LacZ (control) (164 mg vs. 324 mg, respectively; $p < 0.05$) (Figure 4a).

Furthermore, we found that Ad.E7 immunotherapy conferred a survival advantage over Ad.LacZ (control) therapy. As shown in the Kaplan Meier survival analysis in Figure 4b, animals who received Ad.E7 immunotherapy demonstrated significantly prolonged survival compared to Ad.LacZ-vaccinated animals. While the first portion of control animals passed away at Day 21 with the remaining animals following shortly thereafter, the first portion of Ad.E7-treated animals did not pass away until a week later, at Day 28, with some animals surviving up to Day 42. Of note, we did not appreciate any cures of mice receiving Ad.E7, suggesting that this approach is only partially effective.

Thus, these results demonstrate that Ad.E7 vaccination, when employed as an immunotherapy in the treatment of esophageal cancer, has the potential to significantly reduce the rate of tumor growth and improve survival. In confirming our previous findings for small TC1 flank tumors (31), these results validate our AKR-E7 orthotopic model as a potential model for the evaluation of preclinical tumor vaccination therapies.

Ad.E7 vaccination augments antigen-specific tumor-infiltrating E7-specific CD8⁺ T cells

Our group has shown that the reduction in TC1 flank tumor growth associated with Ad.E7 vaccination is the direct result of the induction of cytotoxic CD8⁺ T cells that secrete IFN γ (31). To investigate if a similar mechanism was responsible for the reduction in tumor growth associated with our orthotopic esophageal cancer model, we analyzed tumors isolated from Ad.E7 and Ad.LacZ-treated tumor-bearing animals. Our main objective was to determine the total number of CD8⁺ T cells and E7-specific CD8⁺ T cells infiltrating the tumors. Tumor-bearing animals were vaccinated with Ad.E7 (n=7) or Ad.LacZ (n=7) on Day 5 post tumor-injection and received a booster on Day 10. Tumors were then harvested on Day 14 and enzymatically digested to generate a single-cell suspension for flow cytometry.

As shown in Figure 5, vaccination of AKR-E7 tumor-bearing animals with Ad.E7 resulted in a significant increase in tumor-associated CD8⁺ T cells compared to Ad.LacZ (control) (19% vs. 5.1%, respectively; $p < 0.05$) (top panels of Figure 5a and 5b). In addition to the

increased numbers of intratumoral CD8⁺ T cells found in Ad.E7-vaccinated animals, further analysis of these CD8⁺ T cells revealed marked differences in their antigen specificity and ability to produce IFN γ . E7-tetramer analysis revealed that treatment with Ad.E7 vaccination significantly increased the number of E7-specific CD8⁺ T cells compared to Ad.LacZ (2.8% vs. 0.35%, respectively; $p < 0.05$) (Bottom panels of Figure 5a and 5b). Furthermore, when primed with PMA and ionomycin, a higher percentage of the intratumoral CD8⁺ T cells from the Ad.E7-vaccinated animals secreted IFN γ (23.6% vs. 16.3%, respectively; $p < 0.05$). Thus, these results suggest that the mechanism by which Ad.E7 vaccination limits the rate of orthotopic AKR-E7 tumor growth is dependent on the induction of antigen-specific CD8⁺ T cells which demonstrate increased tumor infiltration and IFN γ production.

Discussion

The incidence of esophageal cancer in the United States has increased more than any other cancer in the 21st century (2). Globally, it is increasingly recognized as a significant health problem, with over a half-million people dying from this cancer world-wide (32). With the current therapies available, the 5-year survival rate (for all stages combined) is dismal at 19% (2). The current standard of care for local disease includes combinations of radiation therapy, chemotherapy (typically a combination of platinum-based agents and 5-fluorouracil), and surgery (4-6). Despite recent advances in adjuvant therapies and surgical technique, less than 50% of those undergoing surgery will remain alive at 5 years (33). Based on these modest results, it is evident that the current models for both developing and testing therapeutic interventions have failed and innovative approaches that address these deficiencies are urgently needed.

Alternative approaches to adjuvant therapy exist and have been used in the treatment of other solid tumors. These include cancer vaccination therapy (31), photodynamic therapy (34), cryotherapy (35, 36), chemo-embolization (37), and immunotherapy (38). Despite the abundance of these alternative treatment modalities, their application to esophageal cancer has been slow. A significant reason for this delay is the lack of models allowing for novel therapeutic evaluation. Current animal models of esophageal cancer are expensive, time-intensive, and difficult to reproduce. For these reasons, no adequate animals for therapeutic evaluation currently exist. One such example has been the rat reflux model, created by performing a jejuno-esophagostomy with gastric preservation on Sprague Dawley rats (39). Unfortunately, the procedure is time-intensive, technically challenging, and poorly reproducible. An alternative model has utilized genetically engineered mice with p53 mutations (40). Nevertheless, tumors in this model have been slow to develop and appear with limited consistency (in less than 25% of mice). A third model involves explantation of esophageal cancer xenografts into immunodeficient mice (13, 14, 41, 42). Although this model produces consistent results, conclusions drawn from experimentation in immunodeficient hosts are not generalizable to the human population.

In order to study novel treatments for esophageal cancer, it is essential to have well-characterized models of esophageal cancer in immunocompetent animals. The ideal animal model should be cost-effective, technically feasible, consistent, and time-efficient. The

mouse provides an ideal animal to develop such a model. In addition to being relatively inexpensive and easily obtainable, the murine genome is quite similar to that of humans, suggesting that immunologic parallels are present (41). Furthermore, the mucosa of the murine esophagus is similar to that in humans, as they both consist of squamous cell epithelium. Murine models of other cancers, including mesothelioma, thymoma, and non-small cell lung cancers have been developed to grow in syngeneic, immunocompetent mice and can be directly injected into orthotopic and subcutaneous locations (31, 43, 44). These models have proved to be time efficient and have allowed for initial preclinical therapy evaluation. It is likely that syngeneic murine models would similarly be useful for drug discovery aimed at esophageal carcinoma.

Our group was the first to develop a syngeneic murine model of esophageal cancer (16). However, this model was predicated on the direct injection of murine esophageal cancer cells into the flank location. The major drawback of this model, and all subcutaneous models, is the inability to replicate the primary tumor site and patterns of metastatic spread (45). Indeed, Fidler and colleagues have demonstrated that the microenvironments of tumors injected subcutaneously are significantly different from the natural *in situ* microenvironments (17-19). These inherent differences may impact the response to novel therapeutic interventions. For example, Kuo et. al. have shown that orthotopic small-cell lung cancer (SCLC) tumors more accurately reflect the clinical effects of chemotherapy on human SCLC than those grown s.c., as cisplatin had significant effects on orthotopic tumors but not the same tumors grown s.c. (20). Thus, it is becoming increasingly clear that the use of models that more closely reproduce the tumor microenvironment will better predict clinical activity of new therapeutic agents (45).

Given the advantages of orthotopic models in replicating human disease, we sought to develop a novel orthotopic model of esophageal carcinoma using the AKR cell line. Similar to human esophageal carcinomas, we have demonstrated that the AKR cell line is only moderately responsive to standard of care chemotherapy (cisplatin/gemcitabine) at equivalent human dosing (16). In addition, we also observed that these models were similarly susceptible to *in vitro* adenoviral infection, opening the door as a potential model for preclinical evaluation of adenoviral driven immunotherapies. Thus, given these attributes, the AKR cell line represents the ideal selection for an orthotopic model of esophageal cancer.

In this report, we presented and validated the first successful syngeneic, orthotopic model of esophageal cancer in immunocompetent mice for the preclinical evaluation of novel immunotherapeutic approaches. We first described the generation of the AKR-E7 murine esophageal cancer cell line and characterized its growth in the orthotopic location (Figures 1-2). We provided evidence that this modified cell line yields tumors whose natural history is consistent and reproducible. Then, we demonstrated its utility in the evaluation of an active immunotherapeutic approach: cancer-antigen vaccination with an adenovirus expressing the protein E7. This adenoviral-based immunotherapy has been used by our lab in the past for the treatment of subcutaneous TC1 (a murine NSCLC cell line) flank tumors (31). Within this NSCLC model, our group was able to show that Ad.E7 vaccination significantly inhibited the growth of small tumors by inducing an antigen-specific,

functionally active CD8⁺ T cell population. Given this promising result, we implemented the Ad.E7-based immunotherapy in our novel orthotopic esophageal cancer model with the hopes of both generating evidence supporting immunotherapy in the treatment of esophageal cancer, while at the same time validating our model for the evaluation of novel preclinical therapies.

Our results indicate that Ad.E7 vaccination induced an E7-specific population of functionally active CD8⁺ T cells (Figure 3). These anti-tumor effector cells demonstrate an increased ability to infiltrate tumors and produce IFN γ upon stimulation in culture. More importantly, Ad.E7 vaccination significantly reduced the rate of established tumor growth and prolonged overall survival (Figures 4-5). These results favor further research into adenoviral-based immunotherapy for the treatment of esophageal cancer in combination with other agents or surgical resection (as a neoadjuvant or adjuvant therapy). Experiments are currently underway to determine if augmentation with chemotherapy or COX-2 inhibitors may enhance the anti-tumor response and further limit tumor growth and prolong survival.

Our study is not without limitations. The major limitation of our orthotopic model (and most orthotopic models of internal organ systems for that matter), is the inability to measure tumor response using traditional methods (diameter, volume, etc.) without animal sacrifice. We sought to address this limitation with two approaches. The first attempted to monitor tumor growth using the *in vivo* IVIS bioluminescent imaging. While we were able to appreciate steady growth of these lesions for the first 7-10 days (Figure 2d), these lesions ultimately regressed without any further intervention. Our hypothesis is that these lesions regressed due to immunogenic properties of the luciferase gene product, causing it to be rejected by the host's immune system. Our second attempt at non-invasive monitoring was predicated on using changes in total animal body weight as a proxy for tumor burden (data not shown). While a difference in weight between the Ad.E7-vaccinated group and control group on Day 15 was suggested by the data, the method has multiple problems. First, it is not likely to detect subtle tumor volume changes. Second, many other factors contribute to the overall weight of the animals, such as tumor associated anorexia (most likely responsible for the initial decrease in weight in both groups after tumor-injection), tumor-associated psychological changes, and underlying genetic predispositions. Thus, while promising, further investigation into noninvasive monitoring is warranted.

In conclusion, we have presented the first successful syngeneic, orthotopic model of esophageal cancer in immunocompetent mice for the preclinical evaluation of novel immunotherapeutical approaches. We have demonstrated its utility in the evaluation of an active immunotherapeutical approach: cancer-antigen vaccination with an adenovirus expressing the protein E7. These results validate our model as a reasonable model for testing novel preclinical therapies for esophageal cancer.

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Abbreviations

HPV	Human Papilloma Virus
Ad.E7	adenoviral vaccine to E7
AKR-Luc	AKR-E7-Luciferase
DMEM	Dulbecco's modified Eagle's medium
RT-PCR	reverse transcriptase-polymerase chain reaction
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GE	gastroesophageal
s.c	subcutaneously
pfu	plaque-forming units
Ad.LacZ	adenoviral vaccine to LacZ
mAbs	monoclonal antibodies
i.p.	intraperitoneally
PMA	phorbol myristate acetate
SCLC	small-cell lung cancer

Figure 1. Generation of AKR-E7, a syngeneic murine esophageal cancer line that expresses the known viral tumor antigen E7 and grows well *in vivo*

A. After stable transduction with a retroviral vector encoding the E7 gene, AKR-E7 cells express significant levels of E7 mRNA. TC-1 represents the positive control given its derivation from mouse lung epithelial cells immortalized with HPV-16 E7, while AKR serves as the negative control. **B.** When injected into the flanks of C57BL/6 mice, this model demonstrates consistent and predictable growth curves identical to the cell line of origin, AKR.

Figure 2. Development of a novel, immunocompetent, orthotopic esophageal cancer model employing the syngeneic AKR-E7 cell line

A. Relative anatomy of the esophagus and adjacent tissue landmarks. Arrows highlight the diaphragm (muscle separating the thoracic cavity from the abdominal cavity), the esophagus, and the gastroesophageal (GE) junction (where the stomach connects with the esophagus). Once the GE junction is mobilized through a 1cm left subcostal incision, 0.5×10^6 tumor cells are injected into the tissue wall. **B.** Orthotopic AKR-E7 growth curve and images of *in vivo* tumors (highlighted by the black arrows) prior to harvest at Day 4 and 14 post tumor-injection. Tumors demonstrated rapid growth and remained confined to the point of tumor injection. **C.** Representative H&E histological sections showing neoplastic tissue architecture and integration within the native esophageal wall (T = tumor, L= lumen, M = muscularis externa, St = Stomach). Tumors demonstrated invasive growth within the esophageal wall and eventual obliteration of muscular layers. **D.** Non-invasive *in vivo* IVIS bioluminescent imaging of luciferase-transduced AKR cells at the GE junction 9 days after injection.

Figure 3. Ad.E7 vaccination prevents the growth of AKR-E7 cells via the generation of cytotoxic E7-specific CD8⁺ T cells

A. Flow cytometric analysis to confirm the generation of E7-specific CD8⁺ T cells. Spleens were isolated from non tumor-bearing animals 3 days following the second vaccination with control (Ad.LacZ) or Ad.E7 virus and stained for the presence of E7-specific CD8⁺ T cells. Ad.E7 vaccination generated E7-specific CD8⁺ T cells while Ad.LacZ-vaccinated animals showed only scant background staining. **B.** To confirm the ability of Ad.E7 vaccination to prevent the growth of AKR-E7 cells, animals were vaccinated with Ad.E7 virus 10 and 3 days prior to tumor cell injection. Ad.E7 vaccination prior to tumor challenge significantly prevented the development of tumors compared to Ad.LacZ vaccination (* $p < 0.05$). **C.** To determine if CD8⁺ cytotoxic T cells were responsible for the prevention of tumor growth, we repeated the prior experiment in CD8⁺ T cell-depleted animals. CD8⁺ T cell-depletion completely negated the effect of vaccination, as there was no significant difference between those that were vaccinated with Ad.E7 but depleted of CD8⁺ T cells, those that were depleted of CD8⁺ T cells in the absence of vaccination, or those that were neither vaccinated nor depleted of CD8⁺ T cells (NS, $p > 0.05$).

Figure 4. Ad.E7 vaccination inhibits the growth of orthotopic AKR-E7 tumors and improves overall survival

A. Animals were vaccinated with either Ad.LacZ or Ad.E7 on Day 5 post tumor-injection and boosted on Day 10, as described in the Materials and Methods. Ad.E7 vaccination significantly slowed tumor growth compared to Ad.LacZ (control) vaccination ($*p<0.05$) on Day 14 post tumor-injection. Representative images of tumors from both Ad.E7-vaccinated and Ad.LacZ-vaccinated (control) animals at Day 14 are shown. **B.** Kaplan-Meier analysis with log-rank comparison showing that Ad.E7 vaccination resulted in significantly prolonged survival compared to Ad.LacZ vaccination (control) ($*p<0.05$).

Figure 5. Ad.E7 vaccination increases tumor-infiltrating E7-specific CD8⁺ T cells which secrete IFN γ

Tumor-bearing animals were vaccinated with Ad.E7 or Ad.LacZ on Day 5 post tumor-injection and received a booster on Day 10. Tumors were then harvested on Day 14 and enzymatically digested to generate a single-cell suspension for flow cytometry. **A.** Representative flow cytometry plots demonstrating gating techniques on single-cell suspensions derived from tumor tissue. Top panel shows percentages of CD8⁺ T cells from control or Ad.E7 tumors. Bottom panel shows percentage of total CD8⁺ T cells that were positive for E7. **B.** Bar graph representations of the percentages of CD8⁺ T cells among all live cells in tumors (top, * $p < 0.05$) and E7-specific CD8⁺ T cells among all tumor-infiltrating CD8⁺ T cells (bottom, * $p < 0.05$). **C.** Ad.E7 vaccination increased the percentage of CD8⁺ T cells secreting IFN γ after 6 hours of *in vitro* stimulation with PMA/ionomycin (* $p < 0.05$).