Preclinical Evaluation of a Replication-Deficient Recombinant Adenovirus Serotype 5 Vaccine Expressing Guanylate Cyclase C and the PADRE T-helper Epitope

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There is an unmet need for improved therapeutics for colorectal cancer, the second leading cause of cancer mortality worldwide. Adjuvant chemotherapy only marginally improves survival in some patients and has no benefit in others, underscoring the clinical opportunity for novel immunotherapeutic approaches to improve survival in colorectal cancer. In that context, guanylate cyclase C (GUCY2C) is an established biomarker and therapeutic target for metastatic colorectal cancer with immunological characteristics that promote durable antitumor efficacy without autoimmunity. Preliminary studies established non-replicating human type 5 adenovirus (Ad5) expressing GUCY2C as safe and effective to induce GUCY2C-specific immune responses and antitumor immunity in mice. This study characterized the biodistribution, immunogenicity, and safety of a vector expressing GUCY2C fused with the human $CD4^+$ T helper cell epitope PADRE (Ad5-GUCY2C-PADRE) to advance this vaccine into clinical trials in colorectal cancer patients. Ad5-GUCY2C-PADRE levels were highest in the injection site and distributed *in vivo* primarily to draining lymph nodes, the liver, spleen and, unexpectedly, to the bone marrow. Immune responses following Ad5-GUCY2C-PADRE administration were characterized by PADRE-specific $CD4^+$ T-cell and GUCY2C-specific B-cell and $CD8^+$ T-cell responses, producing antitumor immunity targeting GUCY2C-expressing colorectal cancer metastases in the lungs, without acute or chronic autoimmune or other toxicities. Collectively, these data support Ad5-GUCY2C-PADRE as a safe and effective vaccination strategy in preclinical models and position Ad5-GUCY2C-PADRE for Phase I clinical testing in colorectal cancer patients.

Keywords: colorectal cancer, GUCY2C, vaccine

INTRODUCTION

Guanylate cyclase C (GUCY2C), one of a family of homologous proteins, is selectively expressed by intestinal epithelial cells,[1,2] where it catalyzes the production of cGMP following binding of its paracrine hormones guanylin and uroguanylin.[2] GUCY2C is normally expressed in intestinal epithelium and neurons of the hypothalamus[3] and substantia nigra,[4] but not by other normal tissues.[1,2,5,6] GUCY2C is also found in all colorectal cancers, and a subset of gastric, esophageal, and pancreatic cancers.[1,5–8] Limited expression in normal tissues and universal overexpression by primary and metastatic colorectal cancer (CRC) makes GUCY2C a useful biomarker for lymph node metastases in CRC patients[6,9] and potential chemotherapeutic[10] and immunotherapeutic[11–13] target in humans with GUCY2C-expressing cancers.

Development of a specific vaccine has leveraged the structurally distinct GUCY2C extracellular domain ($GUCY2C_{ECD}$), which is absent from other guanylate cyclase isoforms.[13] Indeed, while catalytic domains across family members share ~50% homology, the extracellular domain of GUCY2C exhibits <20% homology with other proteins and is antigenically unique.[2,13] In mouse models, replication deficient (E1 and E3 deleted) human serotype 5 adenovirus (Ad5) expressing the extracellular

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DOI: 10.1089/hgtb.2016.114

domain of GUCY2C produced GUCY2C-specific $CD8^+$ T-cell responses, but no $CD4^+$ T- or B -cell responses.[11,13] Because GUCY2C-deficient mice produced robust GUCY2C-specific $CD4^+$ T- and B -cell responses,[11,13] their absence in wild-type mice reflected self-tolerance, a complex program of immune cell selection mechanisms that eliminate or suppress self-reactive immunity to prevent autoimmunity.[14,15] Importantly, Ad5-GUCY2C vaccination producing $CD8^+$ T-cell responses protected mice with metastatic colorectal cancer in the lungs and liver, primary sites of colorectal cancer metastasis in humans, without autoimmune toxicity in GUCY2C-expressing tissues.[11,13]

Subsequent studies of GUCY2C tolerance uncovered a previously unknown mechanism of self-tolerance mediated by selective $CD4^+$ T-cell tolerance with preservation of self-antigen-specific $CD8^+$ T and B cells.[12] Indeed, selective $CD4^+$ T-cell tolerance was the primary mechanism limiting vaccine efficacy targeting GUCY2C, tyrosinase-related protein 2 (Trp2) and Her2 in colorectal, melanoma, and breast cancers, respectively.[12] Importantly, cancer vaccine efficacy could be restored by adding a foreign (non-self) $CD4^+$ T-cell epitope to these antigens, optimizing antitumor immunity.[12] Initially, a $CD4^+$ T-cell epitope restricted to mouse $H\text{-}2^b$ molecules was fused to GUCY2C to confirm proof-of-concept.[12] To translate those findings into a clinical vaccine approach, this study explored the biodistribution, immunogenicity, and safety of Ad5-GUCY2C-PADRE, containing the human $CD4^+$ T-cell epitope PADRE (PAn DR Epitope), which can be advanced into Phase I clinical trials in colorectal cancer patients.

MATERIALS AND METHODS

Vector design and manufacturing

Mouse (NP_001120790) and human (NP_004954) GUCY2C sequences were aligned (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/hgtb) using Geneious v9 (Biomatters Ltd.). Codon-optimized cDNA encoding mouse GUCY2C residues 1-429 with a C-terminal PADRE epitope (Fig. 1A) was cloned into the E1 region of pAd/CMV/V5 (Life Technologies) containing E1- and E3-deleted human serotype 5 adenovirus (Ad5; Fig. 1B). Ad5-GUCY2C-PADRE vector used for these studies was produced under Good Laboratory Practice (GLP) conditions in HEK293 cells and purified by CsCl ultracentrifugation at the Baylor College of Medicine in the Cell and Gene Therapy Vector Development Lab. Ad5-GUCY2C-PADRE vector was tested for replication-competent adenovirus (negative), sterility (negative), mycoplasma (negative), and host cell DNA contamination (negative), before employing it in preclinical studies.

In vitro GUCY2C-expression experiments (dose–response and time course) were carried out in 293A (Life Technologies) and A549 (ATCC) cells, respectively. Virus was added to the cultures at the indicated doses, and culture supernatants were collected at the indicated time points. Relative GUCY2C levels were quantified in supernatants by Western blot using MS20 mouse anti-GUCY2C monoclonal antibody[3,10,16] and HRP-conjugated goat anti-mouse secondary antibody (Jackson Immuno Research Laboratories).

Study design

The maximum tolerated dose (MTD) and 90-day biodistribution, toxicity, and immunogenicity studies (Figs. 2–5) were performed at WuXi AppTec and conducted in compliance with GLP for Non-Clinical Laboratory Studies. The *ex vivo* immunogenicity analysis component was conducted at Thomas Jefferson University and Cellular Technology Ltd. in compliance with the study plan and Standard Operating Procedures (non-GLP). All other studies were performed at Thomas Jefferson University.

It was anticipated that Ad5-GUCY2C-PADRE would be administered to patients at a dose of 10^{11} vp, reflecting the ability of this dose to overcome pre-existing Ad5-specific immunity in humans receiving Ad5-based HIV vaccines.[17] A dose of 10^{11} vp in an 80 kg human would be 1.25×10^9 vp/kg. Here, Ad5-GUCY2C-PADRE administrations employed doses of approximately 4×10^8 vp, 10^{10} vp, and 10^{11} vp (1.7×10^{10} vp/kg, 4.25×10^{11} vp/kg, and 4.25×10^{12} vp/kg in a 24 g mouse).

Mice and Ad5-GUCY2C-PADRE administrations

Except for CB6F1/J studies, all other studies employed 8- to 9-week-old C57BL/6 mice of both sexes (Harlan Sprague Dawley). Females were nulliparous and not pregnant. A sufficient number of replacement animals were also immunized to replace animals lost during the study, ensuring that five mice/group/sex were available for necropsy and blood and tissue harvest at all time points. Replacement animals not required during the in-life phase were euthanized without further evaluation. For each animal, vehicle control or Ad5-GUCY2C-PADRE was administered intramuscularly as two 50 μL injections, one in each of the two hind limbs using a Hamilton syringe. All doses were prepared by dilution in formulation buffer including the control group (0 vp), which received formulation buffer only (20 mM Tris-HCl, pH 8.0, 25 mM NaCl, 2.5% glycerol).

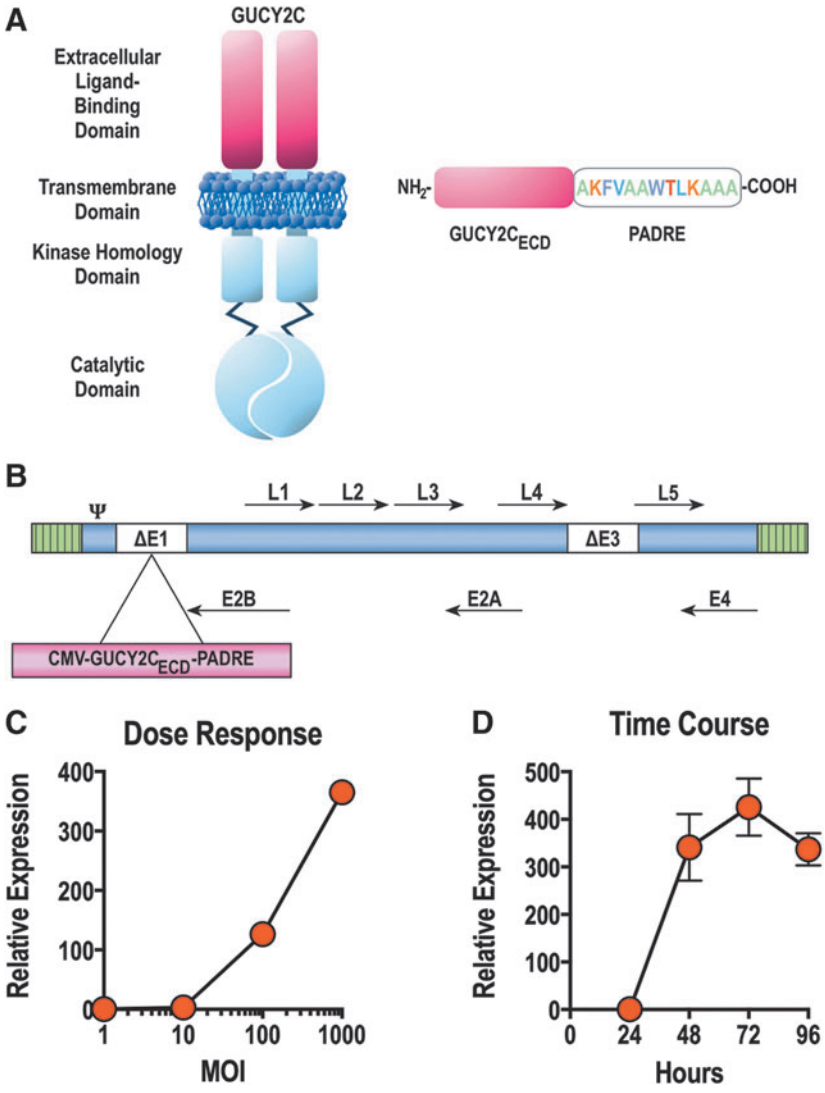

Figure 1. Ad5-GUCY2C-PADRE design and antigen expression. (**A**) GUCY2C is a membrane-spanning enzyme possessing an extracellular ligand-binding domain, intracellular cGMP-producing catalytic domain, and intervening regulatory domains. The extracellular domain (ECD) of GUCY2C was employed in the vaccine design and included the PADRE epitope on its COOH-terminus. (**B**) $GUCY2C_{ECD}$-PADRE was inserted into the E1 region of E1/E3-deleted Ad5. (**C**) HEK293 cells were transduced with Ad5-GUCY2C-PADRE at a multiplicity of infection (MOI) of 1–1,000 for 48 h. Supernatants were analyzed for GUCY2C-PADRE expression by Western blot. Densitometry analysis was employed to quantify relative expression to dose 0 vp. (**D**) A549 cells were transduced with Ad5-GUCY2C-PADRE at a MOI of 10,000 for 24–96 h, and supernatants were analyzed for GUCY2C-PADRE expression by Western blot. Densitometry was employed to quantify expression relative to time 0 h.

Biodistribution

Blood was collected by cardiac puncture into K_2EDTA tubes and frozen. Tissues were collected at necropsy and flash frozen. DNA was isolated using a DNeasy Blood and Tissue Kit (Qiagen). TaqMan quantitative PCR (qPCR) was performed in triplicate on 1 μg of DNA from each tissue (maximum of 10 μL of DNA sample per reaction) using a custom Ad5-GUCY2C-PADRE primer-probe set. Due to tissue-specific qPCR interference observed during assay validation, a 1:5 dilution was applied to blood, the bone marrow, draining lymph nodes, injection site, the stomach, and ovaries prior to analysis. Linearized Ad5-GUCY2C-PADRE plasmid was employed as positive control standard for copy number quantification and reagent and process controls were employed as negative controls. The Limit of Quantification (LOQ) was defined as the lowest DNA standard, which could be reliably detected above the reagent control in at least two out of the three replicates. Data and statistical analyses for the biodistribution study are described below in Statistical Methods.

Toxicity

Mortality and moribundity checks were performed daily. Clinical observations (1 and 3 h post dosing, then daily), body weight measurements (weekly),

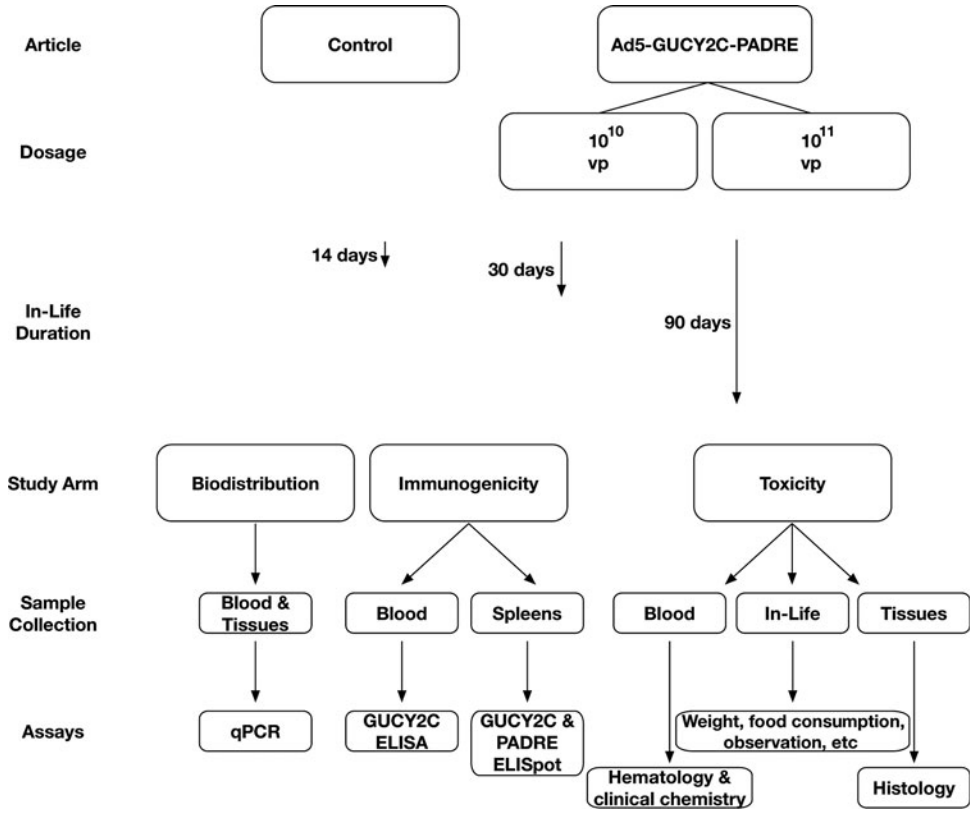

Figure 2. Ninety-day Ad5-GUCY2C-PADRE biodistribution, toxicity, and immunogenicity study design. C57BL/6 mice received vehicle control or a single intramuscular injection of Ad5-GUCY2C-PADRE at 10^{10} or 10^{11} vp. Mice were observed and euthanized after 14, 30, or 90 day to measure the endpoints. Each arm of the study (biodistribution, immunogenicity, and toxicity) employed separate but identically treated animals, reflecting the different processing requirements for the assays performed in each arm.

and feed consumption measurements (weekly) were performed for the duration of the study. Animals were fasted 1–3 h prior to blood collection and necropsy. Blood was collected by cardiac puncture (CO_2 anesthesia) for hematology and clinical chemistry analyses (see Supplementary Table S1 for the parameters). Gross necropsy was performed, organs were weighed, and tissues fixed in 10% neutral buffered formalin, except (1) one femur/animal was used to prepare a bone marrow smear, which was preserved in methanol; (2) eyes were fixed in Davidson's Solution; and (3) testes were fixed in Modified Davidson's Solution. Tissues collected from control and 10^{11} vp Ad5-GUCY2C-PADRE were embedded in paraffin, sectioned, stained with hematoxylin and eosin, and examined by a veterinary pathologist experienced in rodent toxicological pathology. In the absence of detectable toxicity at 10^{11} vp, tissue sections from mice receiving 10^{10} vp Ad5-GUCY2C-PADRE were not examined.

Immunogenicity

Sample collection and processing. Blood was collected by cardiac puncture (CO_2 anesthesia), processed to serum, and frozen. Spleens were collected, processed to a single cell suspension, and cryopreserved using a CTL-CryoABC Kit (Cellular Technology Ltd.) according to the manufacturer's instructions.

Enzyme-linked immunosorbent assay. GUCY2C antibody quantification employed a fixed dilution (1:20) of serum and samples were compared to a standard curve of mouse monoclonal antibody specific for GUCY2C (Supplementary Fig. S2). Absorbent plates were coated with mouse $GUCY2C_{ECD}$ protein[11–13] at 5 μg/mL in coating buffer. Plates were washed and free binding sites were blocked with 10% non-fat dry milk. Serum samples were thawed and diluted 1:20 in blocking buffer, and a standard curve of MS20[3,10,16] mouse anti-GUCY2C monoclonal antibody was prepared in blocking buffer. The plates were washed after blocking, and samples and standards were added to the coated plated and incubated. The plates were washed, and bound mouse antibody was detected with HRP-conjugated goat anti-mouse antibody (Jackson Immuno Research Laboratories). Following

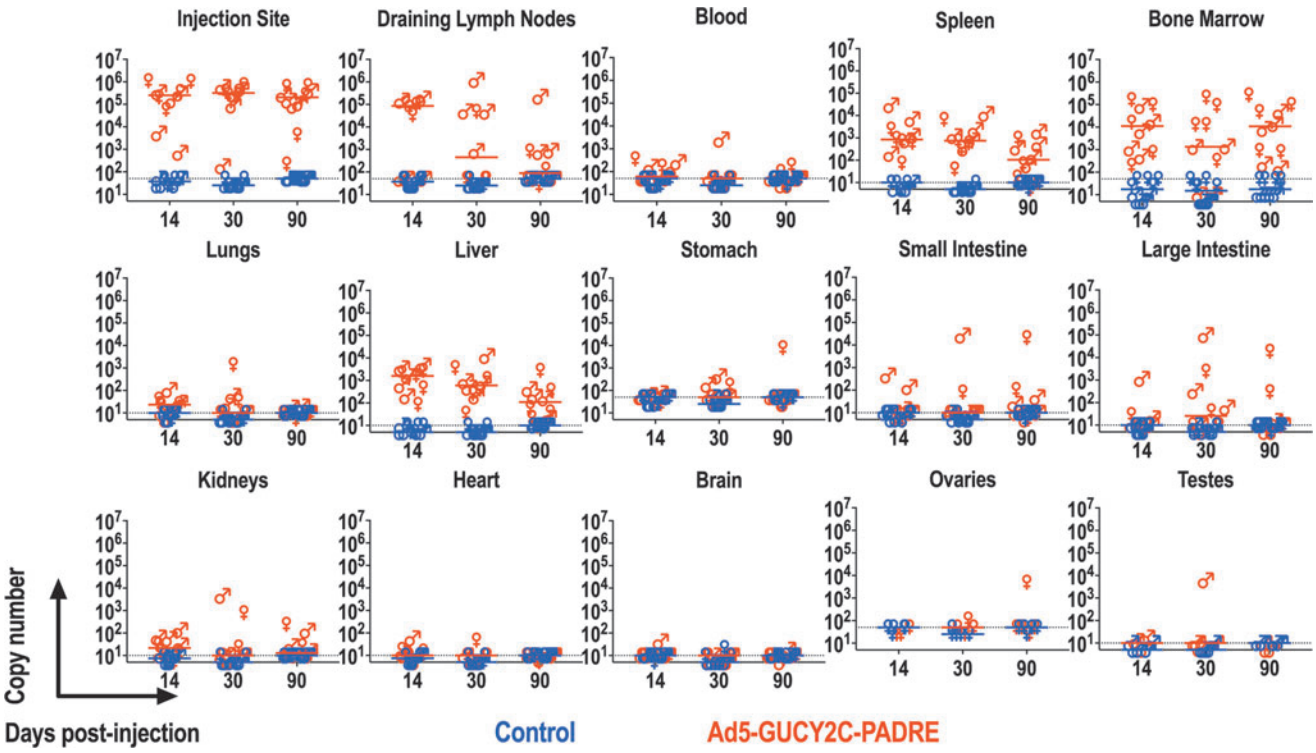

Figure 3. Ad5-GUCY2C-PADRE biodistribution. Blood and tissues were collected 14, 30, or 90 days after administration of 10^{11} vp of Ad5-GUCY2C-PADRE or control vehicle and subjected to quantitative PCR to quantify Ad5-GUCY2C-PADRE DNA copy number per μg of tissue. Male (♂) and female (♀) C57BL/6 mice are indicated by symbols and lines indicate the group median (males and females combined). Dashed lines indicate the limit of detection for each tissue.

a final wash, Turbo TMB substrate (Pierce) was added and the plates incubated for color development, followed by determination of optical absorbance.

Enzyme-linked immunosorbent assay sample data interpolation. The mean baseline absorbance values from each plate determined from the reagent (negative) control were subtracted from the absorbance values of all standards and samples on the respective plate. Standard curve values for each plate were plotted on a sigmoidal dose–response curve (variable slope) using GraphPad Prism v6. The quantity of anti-GUCY2C antibodies present in test samples was determined by interpolating values from the MS20 standard curve and are presented as ng/mL MS20 equivalents. Any mice with responses on the standard curve were considered responders.

Enzyme-linked immunospot. Blinded interferon gamma (IFN-γ) enzyme-linked immunospot (ELISpot) analysis was carried out by Cellular Technology Ltd. for the 90-day study. Mean spot numbers were obtained for each mouse tested against

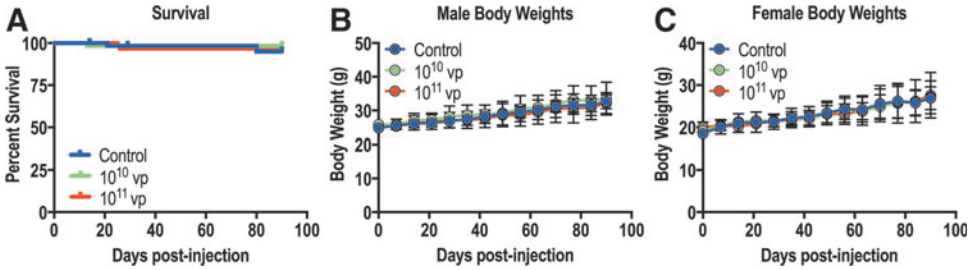

Figure 4. Ad5-GUCY2C-PADRE toxicity. C57BL/6 mice received vehicle control or a single intramuscular injection of Ad5-GUCY2C-PADRE at 10^{10} or 10^{11} vp. Mice were observed for survival (**A**) and body weight (**B** and **C**) for 90 days. Tissues were also collected at 14, 30, and 90 days and subjected to histopathologic evaluation. Ad5-GUCY2C-PADRE produced no toxicity compared with vehicle control, even in tissues robustly expressing GUCY2C (small and large intestine) or exhibiting Ad5-GUCY2C-PADRE vector persistence (bone marrow). No statistical difference was observed for any endpoint between the treatment groups.

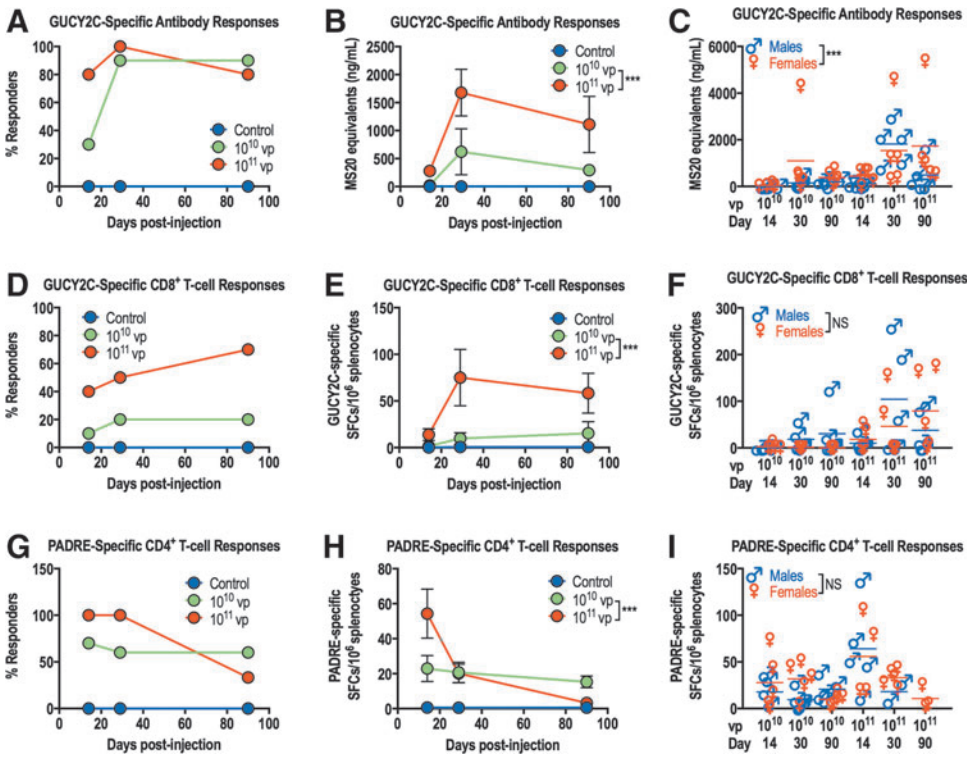

Figure 5. Ad5-GUCY2C-PADRE immunogenicity. C57BL/6 mice were immunized intramuscularly with 10^{10} or 10^{11} vp Ad5-GUCY2C-PADRE or vehicle control. Serum and spleens were collected 14, 30, or 90 days later and subjected to GUCY2C enzyme-linked immunosorbent assay (ELISA) and IFN-γ ELISpot, respectively. The response rate (**A**) and magnitude (**B** and **C**) of GUCY2C-specific antibody responses were quantified by fixed-dilution ELISA. The response rate (**D**) and magnitude (**E** and **F**) of GUCY2C-specific T-cell responses were quantified by IFN-γ ELISpot. The response rate (**G**) and magnitude (**H** and **I**) of PADRE-specific T-cell responses were quantified by IFN-γ ELISpot. ***$p < 0.001$.

each antigen. Tested antigens included negative (medium) and positive (anti-CD3) controls and GUCY2C and PADRE. For GUCY2C responses, two H-2^b-restricted GUCY2C-derived peptides ($GUCY2C_{217–225}$ and $GUCY2C_{377–386}$) were tested separately. The mean background (medium) spot numbers were subtracted from mean values for $GUCY2C_{217–225}$, $GUCY2C_{377–386}$, and PADRE to calculate epitope-specific responses in each animal. Total GUCY2C-specific responses were calculated by summing the epitope-specific responses to $GUCY2C_{217–225}$ and $GUCY2C_{377–386}$. ELISpot responders for GUCY2C and for PADRE were defined as those producing more than five spots per 10^6 splenocytes and more than a fourfold increase over medium control.

CB6F1/J studies

CB6F1/J studies (Fig. 2) employed 8- to 12-week-old male CB6F1/J mice from Jackson Immuno Research Laboratory. Mice were immunized as above with 10^{11} vp Ad5-GUCY2C-PADRE or Ad5-Control-PADRE,[12] and splenocytes were frozen (CTL-CryoABC; Cellular Technology Ltd.), thawed (CTL-Anti-Aggregate; Cellular Technology Ltd.), and stimulated with $GUCY2C_{217–225}$, $GUCY2C_{377–386}$, and $GUCY2C_{254–262}$ in an IFN-γ ELISpot plate, as previously described.[11–13,16] To quantify antitumor immunity, immunized mice were challenged with CT26 cells expressing mouse GUCY2C and K^b, lungs were collected and stained with India ink 16–17 days later, and tumors were enumerated (tumor burden).[12,13,16]

Statistical methods

Biodistribution. The following validity criteria were required for each assay: (1) LOQ <10 copies; (2) reagent control less than the LOQ; (3) process control less than the LOQ; (4) the quality control of an appropriate dilution greater than or equal to the LOQ; (4) R^2 of the standard line ≥0.95; and (5) slope of the assay between –3.0 and –4.0. For any sample producing a signal greater than the LOQ in at least two of the three replicates, the average result of the positive replicates was reported. For any sample producing a signal less than the LOQ in at least two of the three replicates, the sample was considered negative. The mean and standard deviation (*SD*) of the replicates for all samples greater than the LOQ

were calculated. No mean and *SD* values were calculated for samples below the LOQ. The mean value for each animal is reported in Fig. 3.

Toxicity. Statistical analysis was conducted on the hematology, clinical chemistry, organ weight, weekly body weight, and feed consumption data to compare the treatment groups (Ad5-GUCY2C-PADRE vs. control), and the data were analyzed separately for males and females. Parametric data were analyzed using a one-way analysis of variance (ANOVA) while non-parametric data were analyzed using the Kruskal–Wallis test.

Immune responses. Multiple imputation methods were used to impute values for GUCY2C antibody response when the response was at the lower limit of detection. This approach uses the known lower limit to estimate a truncated normal distribution, allowing estimation of population means and variances for imputation of low responses in the tail of the distribution. The purpose of this is to account properly for the variance in hypothesis testing rather than assigning a fixed value to all of these data elements. Antibody response analysis was log-transformed prior to imputation to have a symmetric distribution. As recommended, 10 imputations were used in analyses, with summary over 10 imputations for final results reporting. Linear mixed models were then fit to GUCY2C-specific antibody and T-cell responses and PADRE-specific T-cell responses. Several hierarchical models of variance components were assessed to determine the best model, including dose, day, and sex, with model selection based on the Akaike Information Criteria. Least squares means and post-hoc comparisons were then completed to assess potential differences due to dose, time, sex, and the interaction of these factors. Correlations between the magnitude of GUCY2C-specific antibody, GUCY2C-specific T-cell, and PADRE-specific T-cell responses were perform using GraphPad Prism v6 software. Analyses in CB6F1/J mice employed two-way ANOVA (ELISpot) and *t*-test (tumor burden).

RESULTS

Ad5-GUCY2C-PADRE vector

Examination of the sequence homology between murine and human GUCY2C revealed only 82% identity overall and just 70% within the extracellular domain (Supplementary Fig. S1). Thus, investigating Ad5-GUCY2C-PADRE expressing human GUCY2C in mice does not sufficiently model the human situation. Immune responses produced with human GUCY2C in mice would likely target regions of dissimilarity between mouse and human GUCY2C, producing immune responses that do not recognize mouse GUCY2C and do not pose an autoimmunity risk. In that context, mouse GUCY2C-expressing vector was identified as the appropriate test vector in pre-IND meetings with the Food and Drug Administration (FDA) and was employed for studies of safety and efficacy in mice. This vector is limited by tolerance mechanisms operating against the self-antigen mouse GUCY2C,[11–13] and produces immune responses that are quantitatively and qualitatively representative of those expected in human subjects immunized with a human GUCY2C-expressing vaccine. Thus, mouse GUCY2C-expressing Ad5-GUCY2C-PADRE was used in all preclinical pharmacology (immunogenicity), pharmacokinetics (biodistribution), and toxicology studies, producing results that are representative of those that could be expected when immunizing subjects.

Codon-optimized cDNA encoding mouse GUCY2C extracellular domain ($GUCY2C_{1–429}$) fused on the C-terminus to PADRE (Fig. 1A) was cloned into the E1 region of E1/E3-deleted Ad5 under the control of a CMV promoter (Fig. 1B). Replication-deficient Ad5-GUCY2C-PADRE vector was then produced in HEK293 cells, purified by CsCl ultracentrifugation, and subjected to quality control testing. *In vitro* transduction studies confirmed dose- (Fig. 1C) and time-dependent (Fig. 1D) expression of GUCY2C extracellular domain protein quantified by Western blot on supernatants.

Acute maximum tolerated dose study

C57BL/6 mice possess the appropriate MHC molecule for PADRE reactivity[18] and are responsive to GUCY2C vaccination.[13] Thus, C57BL/6 mice were selected as the appropriate preclinical model for testing, and an initial toxicity assessment was performed by exploring the acute maximum tolerated dose. Ad5-GUCY2C-PADRE was administered to C57BL/6 mice (five mice/sex/group) by the intramuscular route once on day 0 at 4×10^8 vp, 10^{10} vp, and 10^{11} vp. A vehicle control group was also included, and all doses were prepared by dilution in formulation buffer. In-life toxicological endpoints included: clinical observations, body weight, feed consumption, hematology, and clinical chemistry. Mice were sacrificed on day 14, and gross pathology was evaluated. All animals survived to study termination and appeared normal throughout the study. There were no meaningful changes or effects on body weight, feed consumption, hematology, clinical chemistry, or necropsy findings. While there were no meaningful toxicological findings for

hematology and clinical chemistry, some statistical differences were noted in some female hematology parameters between control and Ad5-GUCY2C-PADRE groups (Supplementary Table S2). Those results are consistent with previous studies demonstrating that GUCY2C vaccination produces GUCY2C-specific immunity and antitumor efficacy in the absence of autoimmunity.[11–13]

Given the absence of acute toxicity of Ad5-GUCY2C-PADRE in the initial study, a large 90-day study examining the biodistribution, toxicity, and immunogenicity of Ad5-GUCY2C-PADRE was performed (Fig. 4). Each arm of the study (biodistribution, toxicity, and immunogenicity) employed separate but identically treated animals, reflecting the different processing requirements for the assays performed in each arm. For this study, mice received a single intramuscular administration of 10^{10} or 10^{11} vp of Ad5-GUCY2C-PADRE or vehicle control on day 0, followed by longitudinal in-life measurements (food consumption, body weight, observation, etc.) and terminal blood and tissue collections on days 14, 30, and 90 for qPCR analysis (biodistribution); hematology, clinical chemistry, and histology (toxicity); and enzyme-linked immunosorbent assay (ELISA) and ELISpot (immunogenicity).

Biodistribution

Overall, vector DNA was detectable in each tissue type of at least one animal receiving a fixed dose of 10^{11} vp of Ad5-GUCY2C-PADRE (Fig. 3). At day 14, vector was detected at the injection site, bone marrow, spleen, and liver of all animals. Vector was detected in the lymph node, kidneys, lungs, blood, and heart of at least 50% of the animals. Copy number was greatest at the injection site, followed by the lymph nodes and bone marrow, and was >10,000 at those sites in at least 50% of the animals. At day 30, vector levels were present at decreased frequency in the bone marrow, lymph node, kidneys, lungs, blood, and heart of animals receiving Ad5-GUCY2C-PADRE. At this time point, vector remained detectable at the injection site, spleen, and liver of all animals. High copy number, defined as >10,000 copies, remained at the injection site and in the bone marrow and decreased in the lymph node. At day 90, vector DNA was still detectable in each tissue type of at least one animal, except the testes. The overall pattern of expression, however, was decreasing in frequency and magnitude. The frequencies of high copy numbers at the injection site and bone marrow remained essentially unchanged. By day 90, vector remained at >10,000 copies at the injection site (8/10 animals), bone marrow (5/10 animals), lymph nodes (1/10 animals), large intestine (1/10 animals), and small intestine (1/10 animals).

There was no clear trend between male and female mice. Sex differences were evaluated based on high copy number. The vector biodistribution was similar among male and female animals when evaluating tissues consistently having high copy numbers (>10,000). Values at the injection site, lymph nodes, and bone marrow were similar among male and female animals. Blood, stomach, brain, kidney, lungs, heart, and testes/ovaries consistently expressed <10,000 copies in both male and female animals at all time points. In the remaining tissues, no consistent patterns related to animal sex were identified. Low frequency responses (1/5 animals, 20%) were scattered among the remaining tissues over all time points. Based on five animals per time point, sex differences in vector biodistribution are not likely to be clinically significant.

Toxicology

Ad5-GUCY2C-PADRE was administered intramuscularly to C57BL/6 mice once at 10^{10} vp and 10^{11} vp. A control group was also included and received vehicle only. Animals were terminated on day 14, 30, or 90. Blood was collected for hematology and clinical chemistry assessment prior to necropsy. Tissues were collected for histology. The 90-day toxicity study was conducted in conjunction with the biodistribution (above) and immunogenicity (below) studies. Thus, some analyses (such as mortality, clinical observations, body weights, etc.) were performed on animals in the biodistribution, immunogenicity, and toxicology arms to increase the number of mice analyzed.

Clinical observations for all animals prior to dosing, within 5 min, 1 h, and 3 h post dose, were normal. Of 240 animals, 235 (97.9%) survived to study termination across the biodistribution, immunogenicity, and toxicology arms of the study, with no effect by Ad5-GUCY2C-PADRE (Fig. 5A). One vehicle control mouse experienced lethargy with labored respiration on day 20 and early death on day 21. Four animals were humanely euthanized early due to ulcerative dermatitis (one mouse at 0 vp, one mouse at 10^{10} vp, and two mice at 10^{11} vp). Spare animals were available to replace the five animals that did not survive until the scheduled termination date.

The most frequent abnormal clinical observation was alopecia and/or idiopathic ulcerative dermatitis, which were observed in 16 mice (four of which were humanely euthanized) spread across the

safety, biodistribution, and immunogenicity studies (five mice at 0 vp, two mice at 10^{10} vp, and nine mice at 10^{11} vp). Other abnormal clinical observations included a cloudy eye (one mouse at 10^{11} vp, days 77–90). Collectively, mortality and clinical observations were not affected by Ad5-GUCY2C-PADRE treatments at either dose. Similarly, no statistically significant differences in body weight or body weight change were found among treatment groups (Fig. 5B and C), and no treatment-related or toxicologically significant effects on feed consumption were observed.

Statistically significant differences in a few hematology parameters and clinical chemistry parameters were noted in the day 30 and day 90 cohorts (Supplementary Table S3). However, these findings were not considered toxicologically significant and were not related to the test article because a dose–response was not observed, and trends were not apparent among the different study arms, the different time points, or the sexes. These differences were likely attributed to individual variability.

Necropsy was performed on the animals in the toxicology arm of study, and tissues were subjected to histopathology for the vehicle and high-dose treated mice (0 vp or 10^{11} vp cohorts). Tissues from the lower dose (10^{10} vp) cohort were archived for future analyses if warranted. No specific patterns of Ad5-GUCY2C-PADRE-related findings were present in the tissues of mice treated with 0 or 4.5×10^{11} vp Ad5-GUCY2C-PADRE, including GUCY2C-rich small and large intestine.

Immunogenicity

The purpose of these assessments was to examine both the humoral (antibody) and T-cell responses to the GUCY2C and PADRE epitopes in the context of a 90-day GLP biodistribution and safety study in immunocompetent C57BL/6 mice. As mentioned above, C57BL/6 mice are responsive to the PADRE MHC class II epitope and were anticipated to generate immune responses to PADRE *in vivo*.[12] Ad5-GUCY2C-PADRE was administered intramuscularly to C57BL/6 mice once at 0 vp, 10^{10} vp, and 10^{11} vp. Animals were terminated on day 14, 30, or 90. Blood was collected, processed into serum, and analyzed by ELISA; spleens were collected, processed into a single-cell suspension, and analyzed by IFN-γ ELISpot.

GUCY2C-specific antibody responses were quantified at a fixed dilution (1:20) of serum and compared to a standard curve of mouse monoclonal antibody specific for GUCY2C (Supplementary Fig. S2). The LOQ for the assay was equivalent to 20 ng/mL monoclonal antibody, reflecting the 1:20 dilution of samples and the lowest standard curve quantity of 1 ng/mL. All standard curves produced an R^2 value >0.99, confirming the accuracy of curve fitting. Anti-GUCY2C antibody concentrations were interpolated from duplicate measurements (Fig. 6A–C). Across all time points and both sexes, 0/30 control mice exhibited GUCY2C-specific antibodies above the LOQ (Fig. 6A). In comparison, GUCY2C-specific antibody responses were detected in both the 10^{10} vp and 10^{11} vp Ad5-GUCY2C-PADRE-immunized mice, producing 70% and 86.7% overall response rates, respectively (Fig. 6A). There were highly significant differences in response magnitude based on dose, sex, and time. The magnitude of GUCY2C-specific responses increased with dose ($p<0.001$; Fig. 6B). Furthermore, responses increased from day 14 to day 30 ($p<0.001$), and remained

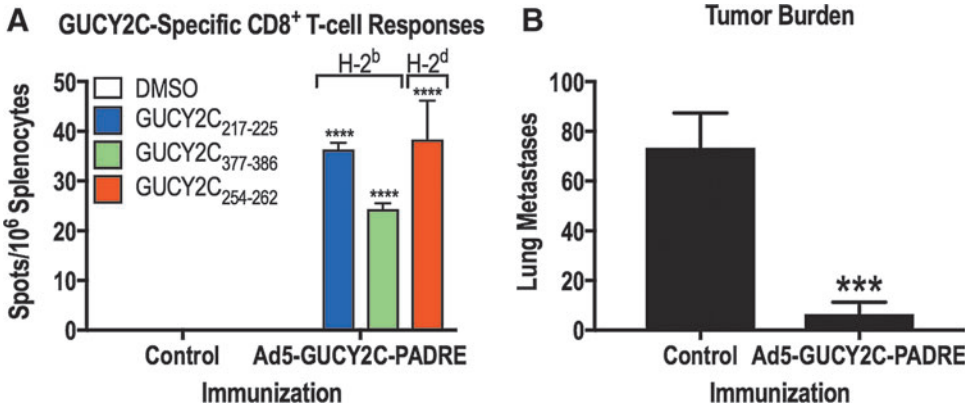

Figure 6. Ad5-GUCY2C-PADRE antitumor efficacy. CB6F1/J mice were immunized with 10^{11} vp of Ad5-GUCY2C-PADRE intramuscularly or with Ad5-Control-PADRE. T-cell responses were quantified 14 days later by interferon gamma (IFN-γ) enzyme-linked immunspot (ELISpot) (**A**), or mice were challenged with 5×10^5 CT26-K^b-GUCY2C cells intravenously 7 days after immunization, followed by quantification of tumor number 17 days later (**B**). ****$p<0.0001$, two-way analysis of variance (ANOVA) compared to dimethyl sulfoxide (DMSO) stimulation; ***$p<0.01$, t test.

at approximately day 30 levels on day 90 ($p = 0.32$; Fig. 6B). In general, responses were also greater in females than they were in males ($p < 0.001$; Fig. 6C).

Viable splenocyte yields were sufficient to test T-cell responses specific for GUCY2C, PADRE, and the positive and negative controls in 78/90 samples. Thus, 12/90 samples were tested against only select antigens. No control animal produced GUCY2C- or PADRE-specific responses at any time point or dose or in either sex (Fig. 6D–I). GUCY2C-specific responses were observed in some mice immunized with 10^{10} vp Ad5-GUCY2C-PADRE and most mice immunized with 10^{11} vp Ad5-GUCY2C-PADRE, producing 16.7% and 53.3% overall response rates, respectively (Fig. 6D). The magnitude of responses increased over time ($p = 0.011$; Fig. 6E) and with dose ($p < 0.001$; Fig. 6E) and were unaffected by sex ($p > 0.05$; Fig. 6F).

PADRE-specific responses were observed in most mice immunized with 10^{10} and 10^{11} vp Ad5-GUCY2C-PADRE, producing 63.3% and 89.5% overall response rates, respectively (Fig. 6G). The dose of Ad5-GUCY2C-PADRE had a significant impact on the magnitude of responses ($p < 0.001$; Fig. 6H). However, time impacted responses only marginally ($p = 0.072$; Fig. 6H), and responses were unaffected by mouse sex ($p = 0.67$; Fig. 6I).

Comparison of the three immune responses measured in each mouse revealed that GUCY2C-specific T-cell responses were a slightly better predictor of GUCY2C-specific antibody responses than PADRE-specific T-cell responses. Comparing antibody response rates in GUCY2C-specific T-cell responders and non-responders revealed that 90.5% of T-cell responders were also antibody responders. However, 40.6% of T-cell non-responders were also antibody responders. In contrast, 77.8% of PADRE responders were also antibody responders, while 23.8% of T-cell non-responders were also antibody responders. Thus, neither measure was a particularly accurate predictor. Examination of response magnitudes revealed a positive correlation between magnitude of antibody responses and GUCY2C-specific T-cell responses ($R^2 = 0.22$; $p < 0.0001$) and between antibody responses and PADRE-specific T-cell responses ($R^2 = 0.20$; $p < 0.0001$), but not between GUCY2C- and PADRE-specific T-cell responses ($R^2 = 0.02$; $p = 0.17$).

Antitumor efficacy

The PADRE helper epitope in Ad5-GUCY2C-PADRE is active in C57BL/6 but not BALB/c mice.[19] However, the established GUCY2C-expressing metastatic mouse colon cancer model CT26 is syngeneic with BALB/c mice.[20] Therefore, to conduct an antitumor efficacy study with Ad5-GUCY2C-PADRE, BALB/c x C57BL/6 F1 mice (CB6F1/J) mice were used. CB6F1/J mice are an F1 cross between C57BL/6 mice (responsive to PADRE) and BALB/c mice (allow CT26 tumor engraftment). These mice were selected because they will respond to PADRE to produce GUCY2C-specific T-cell responses recognizing both H-2^b (C57BL/6) and H-2^d (BALB/c)–restricted epitopes. Moreover, these mice are tolerant to CT26 cells (BALB/c-origin), allowing their engraftment and formation of lung metastases. Indeed, CB6F1/J mice produced PADRE-specific responses as well as responses to the H-2^b-restricted ($GUCY2C_{217–225}$ and $GUCY2C_{377–386}$) and H-2^d-restricted ($GUCY2C_{254–262}$) GUCY2C epitopes (Fig. 2A). Importantly, these mice also produced antitumor immunity against GUCY2C-expressing CT26 cells following Ad5-GUCY2C-PADRE immunization (Fig. 2B), confirming the antitumor efficacy of the Ad5-GUCY2C-PADRE vector.

DISCUSSION

The purpose of this study was to define the biodistribution, toxicity, and immunogenicity of Ad5-GUCY2C-PADRE, a potential vaccine for GUCY2C-expressing malignancies in gastric, esophageal, pancreatic, and colorectal cancer patients. When administered at a dose of 10^{11} vp, Ad5-GUCY2C-PADRE was detected at variable levels in all tissue types at day 14. With the exceptions of the injection site and bone marrow, the magnitude or frequency of vector decreased over the 90-day in-life phase. No significant differences in vector biodistribution were apparent between male and female mice. At day 90, high copy number persisted only at the injection site and bone marrow of at least 50% of the treated animals.

Persistence of vector DNA in the injection site muscle is expected with replication-deficient Ad5 vectors injected intramuscularly.[21,22] Indeed, transgene expression may persist in the muscle for >365 days after administration, and this persistence positively contributes to transgene-specific immune responses.[21] Moreover, distribution to draining lymph nodes, liver, and spleen are consistent with other biodistribution studies of adenovirus.[23–25] In contrast, the magnitude and duration of persistence of Ad5-GUCY2C-PADRE DNA in the bone marrow is difficult to assess in the context of previous experience, since many studies did not analyze bone marrow.[26,27] An Ad5 or fiber-modified vector (Ad5/11, Ad5/35) administered intravenously (i.v.) to baboons yielded about 2,000 copies/μg gDNA in bone marrow mononuclear cells (BM-MNCs) 3 days after

administration.[23] BM-MNC levels were about two orders of magnitude lower than liver, which produced the highest level of distribution. Consistent with those results, transduction of BM-MNCs or peripheral blood leukocytes following Ad5 administration yielded transduction efficiencies of 0.0004% and 0%, respectively, suggesting that distribution of vector DNA to the bone marrow is not associated with gene expression within the bone marrow. In a separate study of an Ad2 vector in mice, administration of 5×10^8 or 5×10^9 vp/animal (20–200× less than our study) intradermally to C57BL/6 mice yielded detectable vector in bone marrow in <10% of animals 2 days after administration but not at later time points,[24] suggesting that vector persistence in the bone marrow may be dose and/or serotype specific. In other recent published studies, an oncolytic Ad5 (INGN 007) was administered i.v. in C57BL/6N mice for analysis of toxicity[28] and biodistribution.[29] INGN 007 was detectable in the bone marrow from days 2 to 92, but caused no bone marrow pathology (normal erythrocyte numbers, hematocrit, hemoglobin, and red cell distribution width), consistent with Ad5-GUCY2C-PADRE bone marrow distribution without toxicity.

Interestingly, Ad5 has been shown to interact with neutrophils via a novel mechanism, independent of the coxsackievirus adenovirus receptor (CAR) and the capsid RGD motifs.[30] Rather, interaction occurred via antibodies, Fc receptors, and complement receptors. Viral gene or transgene expression was not analyzed. These data suggest that bone marrow–derived cells can acquire Ad5 by mechanisms independent of conventional adenovirus transduction/infection. The absence of Ad5-GUCY2C-PADRE in blood and the 10–100× higher distribution in the bone marrow than in the liver strongly suggest that Ad5-GUCY2C-PADRE distribution to the bone marrow also does not reflect transduction of bone marrow cells with Ad5-GUCY2C-PADRE vector, consistent with those studies.[30] Rather, DNA is likely being delivered to the bone marrow in leukocytes migrating from the injection site to the bone marrow by mechanisms previously described for neutrophils,[30] by phagocytosis or by other mechanisms. Future studies are required to determine the cell types associated with Ad5 vector in the bone marrow, the level of transgene expression within the bone marrow, and the role of bone marrow biodistribution in immune responses to Ad5 vectors.

Of significance, the dose of Ad5-GUCY2C-PADRE employed in this biodistribution study was selected to match that employed in immunogenicity and toxicity studies to establish a potential relationship between biodistribution, immunogenicity, and toxicity. In that context, the 10^{11} vp dose is *3,600-fold greater* in mice (4.5×10^{12} vp/kg) than it is in an 80 kg human (1.25×10^9 vp/kg). Thus, the magnitude of bone marrow (or other organ) biodistribution in human subjects is expected to be several orders of magnitude lower than that observed here in mice.

While Ad5-GUCY2C-PADRE persisted in various tissues (Fig. 3) and produced GUCY2C-specific immune responses (Fig. 6) and antitumor immunity (Fig. 2), the vaccine was well tolerated, with no vaccine-related clinicopathologic findings (Fig. 5 and Supplementary Tables S2 and S3). Indeed, no abnormal Ad5-GUCY2C-PADRE-related signs were noted throughout the study. Ad5-GUCY2C-PADRE did not affect weekly body weight or feed consumption. There were no meaningful clinical findings for hematology and clinical chemistry, although some statistical differences were noted between Ad5-GUCY2C-PADRE and control groups (Supplementary Tables S2 and S3). These findings were not considered clinically significant because a dose–response was not observed, and trends were not apparent among the different study arms, the different time points, or the sexes. No abnormalities related to Ad5-GUCY2C-PADRE were observed macroscopically at necropsy or microscopically via histological evaluation. Thus, Ad5-GUCY2C-PADRE vaccination occurred without detectable pathology during a 90-day observation period in mice receiving doses up to and including the 10^{11} vp dose. In the absence of any Ad5-GUCY2C-PADRE toxicity in C57BL/6 animal models, including the bone marrow, spleen, liver, and injection site, the extensive body of animal and human experience with adenovirus as a vector for vaccine delivery, and the 3,600× lower dose to be applied to humans, Ad5-GUCY2C-PADRE is expected to have no toxicity in humans.

Serum and splenocyte samples collected from immunized mice were also evaluated for the presence of GUCY2C-specific antibody and T-cell, and PADRE-specific T-cell, responses (Fig. 6). In the control group, GUCY2C- and PADRE-specific responses were detected in 0/30 mice. In the two test groups receiving 10^{10} vp or 10^{11} vp of Ad5-GUCY2C-PADRE, specific responses were detectable. Overall, Ad5-GUCY2C-PADRE elicited both humoral and cell-mediated immune responses specific to GUCY2C. Ad5-GUCY2C-PADRE appeared to be more effective at eliciting GUCY2C-specific humoral response than GUCY2C-specific T-cell responses: GUCY2C-specific antibody responses were produced in ~78% of mice, while GUCY2C-specific T-cell responses were produced in only 35% of mice.

A clear Ad5-GUCY2C-PADRE dose relationship was observed with respect to GUCY2C-specific anti-

body and T-cell responses and PADRE-specific T-cell responses. Sex had only a marginal impact overall: GUCY2C-specific antibody responses were generally higher in females, but GUCY2C- and PADRE-specific T-cell responses were unaffected by sex. Both antibody and T-cell responses generally peaked at day 30 and either remained constant or diminished at day 90. Neither GUCY2C-specific nor PADRE-specific T-cell response rates were accurate predictors of antibody responders. However, there was a correlation between the magnitude of either GUCY2C- or PADRE-specific T-cell responses and GUCY2C-specific antibody responses, but not between GUCY2C- and PADRE-specific T-cell responses.

In summary, intramuscularly administered Ad5-GUCY2C-PADRE at a dose of 10^{11} vp/animal ($\sim 4.5\times10^{12}$ vp/kg) distributed primarily to the injection site, draining lymph nodes, liver, spleen, and bone marrow. Levels in the injection site and bone marrow remained relatively constant during the 90-day evaluation period. There were no treatment-related toxicities or adverse responses to the vector observed, and the vector was well tolerated at doses up to 10^{11} vp ($\sim 4.5\times10^{12}$ vp/kg). Based on the immunogenicity (antibody, T-cell, and antitumor responses) and lack of toxicity in mice, these data supported the initiation of an FDA-approved Phase I study of Ad5-GUCY2C-PADRE in colon cancer patients (ClincialTrials.gov identifier NCT01972737).

ACKNOWLEDGMENTS

Financial support was provided by: NIH (P30 CA56036, R01 CA170533 to S.A.W.); Targeted Diagnostic and Therapeutics, Inc. (to S.A.W.); PhRMA Foundation (to A.E.S.); and Margaret Q. Landenberger Research Foundation (to A.E.S.). S.A.W. is the Samuel M.V. Hamilton Professor of Thomas Jefferson University. This project was funded, in part, by a grant from the Pennsylvania Department of Health (SAP #4100051723). The Department specifically disclaims responsibility for any analyses, interpretations, or conclusions. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

AUTHOR DISCLOSURE

S.A.W. was the Chair of the Data Safety Monitoring Board for the CHART-1 Trial™ sponsored by Cardio3 Biosciences, and the Chair (uncompensated) of the Scientific Advisory Board to Targeted Diagnostics and Therapeutics, Inc., which provided research funding that, in part, supported this work and has a license to commercialize inventions related to this work. No competing financial interests exist for the remaining authors.

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Received for publication July 22, 2016;
accepted after revision November 26, 2016.

Published online: November 30, 2016