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Tumor radiation therapy creates therapeutic vaccine responses to the colorectal cancer antigen GUCY2C.

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Tumor Radiotherapy Creates Therapeutic Vaccine Responses to the Colorectal Cancer Antigen GUCY2C

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Key words: GUCY2C, vaccine, radiation therapy, immunotherapy, colorectal cancer.

Abbreviations: BED, biologically equivalent doses; GUCY2C, guanylyl cyclase C; Gy, Gray; IFU, infectious units; IM, intramuscular; IT, immunotherapy; RT, radiotherapy.

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ABSTRACT

PURPOSE: Radiotherapy is thought to produce clinical responses in cancer patients, through not only direct toxicity to cancer cells and supporting tumor stroma cells, but also through activation of immunological effectors. More recently, radiotherapy (RT) has potentiated local and systemic effects of cancer immunotherapy (IT). However, combination regimens that maximize immunologic and clinical efficacy remain undefined.

METHODS and MATERIALS: We evaluated the impact of local RT on adenoviral-mediated vaccination against the colorectal cancer antigen GUCY2C (Ad5-GUCY2C) in a murine subcutaneous tumor model using mouse CT26 colon cancer cells (CT26-GUCY2C). Immune responses were assessed by ELISpot and clinical responses were assessed by tumor size and incidence.

RESULTS: The specific sequence of tumor-directed RT preceding Ad5-GUCY2C IT transformed inactive therapeutic Ad5-GUCY2C vaccination into a curative vaccine. GUCY2C-specific T cell responses were amplified ($p < 0.05$), tumor eradication maximized ($p < 0.01$), and tumor volumes minimized ($p < 0.001$) in mice whose tumors were irradiated prior to, compared to following, Ad5-GUCY2C vaccination. The immunologic and

antitumor efficacy of Ad5-GUCY2C was amplified comparably by unfractionated (8 Gy x 1), or biologically equivalent doses of fractionated (3.5 Gy x 3), RT. Antitumor effects of sequential RT and IT (RT-IT) depended on expression of GUCY2C by tumor cells and the adenoviral vaccine vector, and tumor volumes were inversely related to the magnitude of GUCY2C-specific T cell responses. Moreover, mice cured of CT26-GUCY2C tumors by RT-IT exhibited long-lasting antigen-dependent protection, resisting tumors formed by GUCY2C-expressing 4T1 breast cancer cells inoculated 50 days after CT26 cells.

CONCLUSIONS: Optimal sequencing of RT and IT amplifies antigen-specific local and systemic immune responses, revealing novel acute and long-term therapeutic antitumor protection. These observations underscore the importance of modality sequence optimization prior to initiating clinical trials of RT and IT to maximize immune and antitumor responses.

Introduction

Radiotherapy (RT) plays a central role in the management of most malignancies. Historically, clinical responses following RT were attributed to radiation-induced mitotic catastrophe and apoptosis within cancer cells and to destruction of the tumor microenvironment [1,2]. Beyond these toxicities, the therapeutic efficacy of RT may reflect, in part,

immunologic mechanisms. The efficacy of RT in murine fibrosarcoma is impaired in mice that lack a normal T cell repertoire [3]. Similarly, patients with compromised immune systems exhibit markedly higher rates of local failures following RT compared to matched immunocompetent patients [4]. Further, tumor-directed RT in patients produces systemic immune responses associated with regression of non-irradiated metastases [5,6]. Moreover, prospective clinical trials revealed that tumor-directed RT adjuvanted systemic responses to immunotherapy (IT) [7,8].

In contrast to the established benefit of RT, the clinical efficacy of cancer vaccines generally has been unremarkable, particularly in patients with substantial volumes of disease. For example, while colorectal cancer patients with minimal residual disease enjoy modest improvements in disease-free (HR = 0.76, p = 0.03) and overall survival (HR = 0.76, p = 0.007) following vaccine therapy, clinical response rates in patients with advanced disease only approaches 1-2% [9]. This inefficacy reflects inhibitory immunologic mechanisms, which co-evolve during tumorigenesis, and quantitative tumor burden, which becomes immunologically insurmountable [10,11].

An emerging paradigm at the intersection of radiation-induced immunologic mechanisms and immune-based therapies suggests that RT can be systematically exploited to amplify local and systemic IT responses that overcome challenges in eradicating established tumors [8,12].

However, effective combinations of RT and IT, and their precise sequencing to maximize immune responses and tumor eradication have not been defined [13-15]. Here, we combined an adenoviral-based vaccine against the colorectal cancer antigen guanylyl cyclase C (GUCY2C) with different schedules of tumor-directed RT to augment local and systemic GUCY2C-specific immune responses, which produced acute and long-term antitumor protection.

Methods and Materials

Mice and immunizations. Balb/c mice were obtained from the NCI Animal Production Program (Frederick, MD). Animal protocols were approved by our Institutional Animal Care and Use Committee. Vaccines included a second-generation adenovirus (Ad5) vector expressing the extracellular domain of mouse GUCY2C fused to the S1 CD4⁺ T helper epitope (Ad5-GUCY2C) [16] and an Ad5-Her2 negative control vector. Mice received 1×10^8 IFU of adenovirus by IM injection of the anterior tibialis.

Tumor models. GUCY2C-deficient CT26-WT cells were from ATCC® (CRL-2638). Generation of mouse CT26-GUCY2C colorectal cancer cells was previously described [16]. 4T1 mouse metastatic breast cancer cells (ATCC® CRL-2539) were transduced with pMSCV-Puro (Clontech) expressing a truncated GUCY2C-construct (GUCY2C₁₋₄₆₁). Subcutaneous tumors were established by injection of 5×10^5 cells in the flanks or in the

left hind leg as indicated. Tumor volumes were calculated by measuring three orthogonal diameters and calculating volumes using: $\frac{4}{3}\pi \times r_1 \times r_2 \times r_3$.

Radiotherapy. Mice were anesthetized and irradiated using X-rays generated by a PanTak, 310 kV_e X-ray machine. Each mouse was confined in a lead casing with its tumor-bearing left hind leg extended through an opening on the side to allow local tumor irradiation. Biologically equivalent doses (BED) of radiation, calculated using an α/β of 10 and the formula $BED = [(nd) + (nd^2 \div \alpha/\beta)]$, were administered as a single 8 Gy fraction (BED = 14.4 Gy) or as three fractions of 3.5 Gy delivered over one week (BED = 14.2 Gy).

ELISpot. IFN γ ELISpot assays were described previously [16]. Briefly, splenocytes (1×10^6) were stimulated with 10 μ g/mL of peptide [GUCY2C₂₅₄₋₂₆₂] [17] or adenovirus DBP₄₁₂₋₄₂₀ [18]] for 24 hours prior to spot development.

Statistics. Statistical differences between groups were analyzed with ANOVA and correlations between T cell responses and tumor volumes were analyzed with Pearson's Coefficient using GraphPad Prism (GraphPad Software). A p-value of <0.05 was considered statistically significant.

Results

Ad5-GUCY2C IT or 8 Gy RT, alone, have limited efficacy against CT26-GUCY2C tumors. Balb/c mice bearing established GUCY2C-expressing CT26 (CT26-GUCY2C) subcutaneous leg tumors were immunized with Ad5-GUCY2C, or irradiated with 8 Gy 7 days after implantation (Fig. 1A). All inoculated mice developed subcutaneous tumors (incidence=100%). By day 28, the volumes of tumors from mice vaccinated with Ad5-GUCY2C ($2,856.4 \text{ mm}^3 \pm 229.2$) were similar, while those from irradiated mice were smaller ($1,385.4 \text{ mm}^3 \pm 291.4$ [8 Gy]; $p < 0.05$), than tumor volumes from untreated control mice ($2,519.6 \text{ mm}^3 \pm 283.6$; Fig. 1B).

Radiotherapy prior to Ad5-GUCY2C amplified GUCY2C-specific T cell responses creating novel antitumor efficacy. Balb/c mice bearing CT26-GUCY2C subcutaneous leg tumors were treated with Ad5-GUCY2C immunization on day 7 after tumor inoculation, followed by 8 Gy 7 days later (Ad5-GUCY2C→RT; Fig. 2A). Alternatively, tumor-bearing mice were treated with 8 Gy on day 7 after tumor inoculation, followed by Ad5-GUCY2C immunization 7 days later (RT→Ad5-GUCY2C; Fig. 2A). On day 28 after tumor inoculation, T cell responses to GUCY2C were amplified about 6-fold in the RT→Ad5-GUCY2C group compared to Ad5-GUCY2C→RT (151.7 ± 49.09 vs 23.9 ± 6.7 spots; $p < 0.05$; Fig. 2B). In contrast, Ad5-specific T cell responses were similar between treatment groups (356.3 ± 47.2 vs 423.7 ± 65.1 spots; $p = \text{NS}$; Fig. 2B). Importantly, amplified immunologic responses produced by combining RT and IT created novel antitumor efficacy, eradicating some tumors (Fig. 2C).

Importantly, tumor eradication by RT→Ad5-GUCY2C was augmented 6-fold compared to Ad5-GUCY2C→RT (60% vs 10% disease-free; $p < 0.01$; Fig. 2C). Moreover, tumor volumes for RT→Ad5-GUCY2C were reduced about 5-fold compared to Ad5-GUCY2C→RT ($436 \text{ mm}^3 \pm 234.9$ vs $2,006.0 \text{ mm}^3 \pm 321.9$; $p < 0.001$; Fig. 2C).

RT amplification of IT responses is independent of fractionation. To determine if RT amplification of Ad5-GUCY2C vaccination was impacted by RT fractionation, cohorts were evaluated using a single dose of 8 Gy or a fractionated schedule of 3.5 Gy x 3 that had similar predicted biologic effects. Indeed, 8 Gy and 3.5 Gy x 3 regimens comparably amplified GUCY2C-specific T cell responses (121.0 ± 29.3 vs 91.2 ± 25.2 spots; $p = \text{NS}$; Fig. 3A) and similarly improved tumor eradication (67% vs 60%; $p = \text{NS}$, Fig. 3B) and volumes ($367.4 \text{ mm}^3 \pm 196.8$ vs $549.5 \text{ mm}^3 \pm 275.2$; $p = \text{NS}$; Fig. 3C).

RT amplification of IT immunologic and tumor responses is GUCY2C-specific. Mice bearing CT26-GUCY2C tumors received 8 Gy radiation to the tumor on day 7 following tumor inoculation, and vaccination 7 days later with either Ad5-Her2 (control) or Ad5-GUCY2C. On day 28 following tumor inoculation, GUCY2C-specific T cell responses were amplified (121.8 ± 35.1 vs 2.1 ± 1.6 spots; $p < 0.05$), while Ad5-specific T cell responses were comparable (231.1 ± 19.6 vs 224.7 ± 7.7 spots; $p = \text{NS}$), in mice vaccinated with Ad5-GUCY2C compared to Ad5-Her2 (Fig. 4A).

Similarly, tumor eradication (70% vs 20%; $p < 0.05$; Fig. 4B) and volumes ($1,324.8 \text{ mm}^3 \pm 424.2$ vs $131.9 \text{ mm}^3 \pm 88.0$; $p < 0.01$; Fig. 4C) were improved in mice vaccinated with Ad5-GUCY2C compared to Ad5-Her2. Conversely, mice bearing GUCY2C-deficient (CT26-WT) or GUCY2C-expressing (CT26-GUCY2C) tumors received 8 Gy radiation to the tumor on day 7 following tumor inoculation, and received vaccination 7 days later with Ad5-GUCY2C. On day 28 following tumor inoculation, GUCY2C-specific T cell responses were amplified (161.7 ± 46.6 vs 50.3 ± 15.9 spots; $p < 0.05$), while Ad5-specific T cell responses were comparable (353.4 ± 75.5 vs 508.6 ± 88.3 spots; $p = \text{NS}$), in mice bearing CT26-GUCY2C, compared to those bearing CT26-WT, tumors (Fig. 4D). Amplification of T cell responses in mice inoculated with CT26-GUCY2C, compared to CT26-WT, cells reflects adjuvation of immune responses by tumor cells undergoing radiation-induced immunogenic cell death. Similarly, tumor eradication (60% vs 20%; $p < 0.01$; Fig. 4E) and volumes ($415.5 \text{ mm}^3 \pm 199.2$ vs $802.3 \text{ mm}^3 \pm 210.3$; $p = 0.08$; Fig. 4F) in mice bearing CT26-GUCY2C tumors were improved compared to those bearing CT26-WT tumors. Moreover, analysis of *all* CT26-GUCY2C-bearing mice across experimental regimens in Fig. 2-4 revealed an inverse relationship between GUCY2C ($p = 0.0016$; Fig. 5A), but not Ad5 ($p = \text{NS}$; Fig. 5B), -specific T cell responses and tumor volume.

Sequential RT-IT induces long-term antitumor protection. Naïve mice, or those cured of subcutaneous CT26-GUCY2C leg tumors by RT→Ad5-

GUCY2C, were inoculated in the left flank with the Balb/c metastatic breast cancer cells (4T1-WT) and in the right flank with 4T1 cells expressing GUCY2C (4T1-GUCY2C) (Fig. 6A). On day 17 after tumor inoculation, 4T1-WT and 4T1-GUCY2C tumor volumes were identical in naïve mice ($519.9 \text{ mm}^3 \pm 54.6$ vs $517.3 \text{ mm}^3 \pm 66.9$; $p=\text{NS}$; Fig. 6B). However, 4T1-GUCY2C tumor volumes were reduced, compared to 4T1-WT tumors, in mice previously cured of CT26-GUCY2C tumors ($575.7 \text{ mm}^3 \pm 121.2$ vs $288.6 \text{ mm}^3 \pm 98.39$; $p<0.001$; Fig. 6C).

Discussion

RT is thought to induce immunogenic cancer cell death that, in part, activates tumor antigen-specific immune responses [19]. Combining RT with immunotherapies amplifies local and systemic antitumor activity beyond that of either modality alone [8,12]. Although molecular mechanisms underlying the synergy of combination RT and IT have been described [20], optimal sequencing of these treatments remains to be defined [13-15]. To our knowledge, this is the first study evaluating the impact of sequencing of combination RT and IT on immunologic responses and tumor indices. Here, we demonstrate that immunologic responses to the colorectal cancer antigen GUCY2C are amplified, and associated with the creation of novel therapeutic tumor responses by irradiating tumors before, rather than after, vaccination.

In addition to the optimal scheduling of RT and IT, the most effective RT dose and fractionation remains to be defined. Here, a dose of 8 Gy was selected because it caused a transient tumor growth delay that potentially could be amplified by an immune response. In that context, moderate RT doses (~8 Gy) optimize tumor control and immunity [21]. Although large fraction sizes delivered to bone metastases or small visceral lesions is considered safe in clinical practice, 8 Gy delivered to large intra-abdominal or intra-thoracic lesions, such as those encountered in patients with GI malignancies, may produce unacceptable side effects, since long term toxicity increases with fraction size. Here, we evaluated whether fractionated RT with a similar biologically-equivalent dose, and potentially a more favorable side effect profile, could produce antitumor and immune responses that were comparable to a large single fraction of radiotherapy. Indeed, 3.5 Gy x 3 produced similar increases in GUCY2C-specific T cell responses and improvement in tumor eradication and volumes, compared to a single 8 Gy dose (Fig. 3). Thus, the temporal relationship between RT and IT may be more important than RT dose or fractionation. Indeed, clinical responses in a recent phase I trial evaluating three stereotactic RT dose cohorts prior to interleukin-2 administration in patients with metastatic melanoma or renal cell carcinoma did not support an RT dose response [7].

Ideally, tumor antigens would be expressed specifically by neoplastic tissues, thereby limiting on-target, off-site effects of immunotherapeutic

responses on normal tissues. A variation on this theme exploits the structural and functional compartmentalization of central and mucosal immune systems by employing vaccines against antigens that are normally confined to intestinal epithelial cells and their derivative malignancies that would be considered foreign following systemic dissemination [22]. GUCY2C is normally expressed by enterocytes lining the small and large intestines [23-25] and is retained by nearly all colorectal tumors and their associated metastases [26-28], underscoring its utility as a marker for colorectal cancer staging [29] and making it an attractive vaccine target [22]. Thus, the dependence on GUCY2C expression for achieving synergy between RT and Ad5-GUCY2C in our therapeutic model was evaluated (Fig. 4). Indeed, in mice challenged with cancer cells or receiving vaccine in which GUCY2C was absent, GUCY2C-specific T cell responses and antitumor efficacy were reduced compared to mice challenged with cancer cells and vaccinated with adenovirus expressing GUCY2C. Thus, GUCY2C antigen expression by tumors and the vaccine is critical to produce maximum T cell and antitumor responses. The highly selective expression of GUCY2C within intestinal epithelia [23,24,30] and the near universal over-expression of GUCY2C by intestinal malignancies, combined with the safety and efficacy of GUCY2C-targeted immunotherapies [16,17,31,32], make GUCY2C an ideal target for combined RT-IT therapy in patients.

The abscopal effect, which occurs when RT induces immune-mediated regression of tumors beyond the irradiation field, has emerged as a key focus of intense study in model systems and patients [5,7,8]. Abscopal regression depends on the ability to generate local immunologic responses that translate systemically to tumors anatomically separated from, but coincident in time with, the irradiated tumor. While bridging this spatial continuum to treat coincident metastases, the ability of the abscopal effect to create memory responses that endure temporally to provide long-term antitumor protection over time has not yet been defined. Here, mice previously cured of subcutaneous CT26-GUCY2C tumors by RT→Ad5-GUCY2C were challenged 50 days after the first cancer cell inoculation with an unrelated (breast) tumor cell line engineered to express GUCY2C (4T1-GUCY2C). Mice cured of CT26-GUCY2C tumors by sequential RT-IT resisted 4T1-GUCY2C, but not 4T1-WT (control), tumor growth. These observations suggest that sequential RT-IT induces long-term GUCY2C-specific immune memory, supporting the use of RT-IT in the curative setting to provide systemic surveillance against metastatic recurrences.

Sequential RT-IT approaches ultimately will require surrogate biomarkers of efficacy that predict clinical responses, and recent guidelines focus on detection and quantification of antigen-specific T cells [33]. In that context, T cell responses to HPV E6 and E7 antigens were associated with a complete resolution of vulvar intraepithelial neoplasia in patients

vaccinated with those proteins [34]. However, it remains unknown if minimum T cell responses are required to produce clinical effects. Here, quantification of T cell responses in individual tumor-bearing mice across *all* treatment regimens enabled evaluation of the relationship between T cell and tumor responses. Indeed, GUCY2C, but not Ad5, -specific T cell responses strongly correlated with tumor responses (Fig. 5). In these analyses, independence of Ad5-specific T cell and tumor responses confirms that the observed relationship reflects treatment-induced tumor responses, rather than variations in immunocompetency of individual tumor-bearing animals. Moreover, these analyses suggest T cell response thresholds are required for tumor responses. Thus, the majority (73%) of mice that produced less than 60 GUCY2C-specific T cells/ 10^6 splenocytes experienced progressive disease. In contrast, the majority (77%) of mice that exceeded 60 GUCY2C-specific T cells/ 10^6 splenocytes were cured of their tumors. If confirmed in patients, T cell response thresholds as a surrogate marker for clinical responses to sequential RT-IT could accelerate the development of new therapeutic paradigms, offering patients an opportunity to receive additional treatments before disease progression.

While RT-IT produced favorable murine outcomes, correlating with immunological responses, limitations in translating animal studies to clinical practice suggest that clinical comparisons of RT-IT regimens are required to maximize patient outcomes. Spontaneously metastatic tumors

are rare in mice [35], limiting animal studies to orthotopic or ectopic models of metastasis. Xenogeneic systems are not feasible for these studies, because intact immunity is required to produce immunological responses following RT-IT. Avatars - mice expressing human GUCY2C, containing a human immune system and human colorectal cancer xenografts - would be ideal for testing GUCY2C immunotherapy [36], but these systems are not yet developed.

The present study reveals a relationship between the sequence of RT and IT and immunologic and antitumor efficacy. Unfractionated or fractionated RT prior to IT with a single dose of an adenoviral GUCY2C-based vaccine amplifies antigen-specific T cell responses creating novel antigen-dependent antitumor responses. Generally, these observations suggest the importance of careful assessment of sequencing modalities in clinical trials integrating RT and IT. More specifically, these studies highlight the translational potential for sequential RT-IT employing GUCY2C as an antigen target. Indeed, an ongoing phase I clinical trial is evaluating the immunogenicity of Ad5-GUCY2C in patients with colon cancer. Pending positive outcomes in that study, the results presented here can be directly translated to patients with esophageal, gastric and rectal cancer, which express GUCY2C as part of their pathophysiology [26,37] and which frequently include tumor-directed RT as standard of care [38-40].

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Figure Legends

Figure 1. Individual Ad5-GUCY2C and 8 Gy modalities are therapeutically ineffective. (A) Mice challenged with CT26-GUCY2C cells were observed (n=10), vaccinated with Ad5-GUCY2C (n=10), or irradiated with 8 Gy (n=5) on day 7. (B) All mice developed tumors. Vaccinated mice exhibited growth rates similar to control. Tumors in irradiated mice were significantly smaller than controls.

Figure 2. Radiotherapy prior to Ad5-GUCY2C amplifies GUCY2C-specific T cells and reduces tumor growth. (A) Mice challenged with CT26-GUCY2C cells (day 0) were treated with Ad5-GUCY2C or 8 Gy on day 7 and then treated with the opposing modality on day 14, generating two cohorts: Ad5→RT (n=10) and RT→Ad5 (n=10). Tumor volumes and GUCY2C-specific T cell responses were quantified on day 28. (B) T cell responses to GUCY2C were increased in the RT→Ad5 cohort compared to Ad5→RT. There was no difference in Ad5-specific T cell responses. Tumor cure rates (C) were increased and volumes (D) were decreased following RT→Ad5 compared to Ad5→RT.

Figure 3. Unfractionated or fractionated RT preceding Ad5-GUCY2C produces similar immunologic and antitumor responses. (A) Mice challenged with CT26-GUCY2C cells (day 0) were observed (n=10), treated with 8 Gy on day 7 (n=10), or treated with 3.5 Gy on days 7, 10, and 13 (n=10). Irradiated mice received Ad5-GUCY2C on day 14. GUCY2C-

specific and Ad5-specific T cell responses (B), tumor cure rates (C), and volumes (D) were similar between unfractionated and fractionated RT treatment groups.

Figure 4. GUCY2C is required for RT-amplified therapeutic vaccination.

(A-C) Mice challenged with CT26-GUCY2C cells (day 0) were irradiated with 8 Gy on day 7 followed by Ad5-Her2 (n=5) or Ad5-GUCY2C (n=10) on day 14, and immune and antitumor responses quantified on day 28. (A) GUCY2C-specific, but not Ad5-specific, T cell responses were amplified in mice vaccinated with Ad5-GUCY2C compared to Ad5-Her2 vaccine. Tumor cure rates were increased (B) and volumes were decreased (C) in mice vaccinated with Ad5-GUCY2C, compared to Ad5-Her2. (D-F) Mice challenged with CT26-WT (n=10) or CT26-GUCY2C (n=10) cells (day 0) were irradiated with 8 Gy on day 7, vaccinated with Ad5-GUCY2C on day 14, and immune and antitumor responses quantified on day 28. (D) GUCY2C-specific, but not Ad5-specific, T cell responses were amplified in mice challenged with CT26-GUCY2C cells compared to mice challenged with CT26-WT cells. Tumor cure rates were increased (E) and volumes were decreased (F) in mice challenged with CT26-GUCY2C, compared to CT26-WT, cells.

Figure 5. Tumor volumes correlate with GUCY2C, but not Ad5, -specific T cell responses. Antigen-specific immune responses by individual mice were rank-ordered for GUCY2C (A) or Ad5 (B) from animals across all experiments employing CT26-GUCY2C tumors, regardless of treatment

regimen. There was a significant association between GUCY2C-specific (A), but not Ad5-specific (B), T cell responses and tumor volumes.

Figure 6. Mice cured of CT26-GUCY2C tumors by sequential RT-IT exhibit GUCY2C-dependent long-term antitumor protection. (A) Mice were challenged with CT26-GUCY2C cells and cured with 8 Gy followed by therapeutic Ad5-GUCY2C vaccination. Fifty days following initial tumor challenge, cured and naïve mice were challenged with 4T1-WT in the left flank and 4T1-GUCY2C in the right flank and tumor volumes were measured longitudinally. (B) 4T1-WT and 4T1-GUCY2C tumor growth was equivalent in naïve mice. (C) In contrast, 4T1-GUCY2C tumor growth was inhibited, compared to 4T1-WT tumors, in mice previously cured of CT26-GUCY2C tumors.