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Selective antigen-specific CD4$^+$ T-cell, but not CD8$^+$ T- or B-cell, tolerance corrupts cancer immunotherapy

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Self-tolerance, presumably through lineage-unbiased elimination of self-antigen-specific lymphocytes (CD4$^+$ T, CD8$^+$ T, and B cells), creates a formidable barrier to cancer immunotherapy. In contrast to this prevailing paradigm, we demonstrate that for some antigens, self-tolerance reflects selective elimination of antigen-specific CD4$^+$ T cells, but preservation of CD8$^+$ T- and B-cell populations. In mice, antigen-specific CD4$^+$ T-cell tolerance restricted CD8$^+$ T- and B-cell responses targeting the endogenous self-antigen guanylyl cyclase c (GUCY2C) in colorectal cancer. Although selective CD4$^+$ T-cell tolerance blocked GUCY2C-specific antitumor immunity and memory responses, it offered a unique solution to the inefficacy of GUCY2C vaccines through recruitment of self-antigen-independent CD4$^+$ T-cell help. Incorporating CD4$^+$ T-cell epitopes from foreign antigens into vaccines against GUCY2C reconstituted CD4$^+$ T-cell help, revealing the latent functional capacity of GUCY2C-specific CD8$^+$ T- and B-cell pools, producing durable antitumor immunity without autoimmunity. Incorporating CD4$^+$ T-cell epitopes from foreign antigens into vaccines targeting self-antigens in melanoma (Trp2) and breast cancer (Her2) produced similar results, suggesting selective CD4$^+$ T-cell tolerance underlies ineffective vaccination against many cancer antigens. Thus, identification of self-antigens characterized by selective CD4$^+$ T-cell tolerance and abrogation of such tolerance through self-antigen-independent T-cell help is essential for future immunotherapeutics.

Keywords: Immunotherapy · T helper (Th) cells · Tolerance · Tumor immunology · Vaccination


See accompanying commentary by Anderson


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Introduction

Despite recent Food and Drug Administration (FDA) approval of the first cancer vaccine (sipuleucel-T), immunotherapy remains a suboptimal therapeutic approach available only for a limited subset of tumors [1]. Identification of mechanisms limiting cancer vaccine efficacy, and development of methods to overcome these limitations, will expand the breadth of treatable tumors and improve patient outcomes [2]. One potential mechanism limiting vaccine efficacy is self-tolerance, which affects the success of cancer vaccines by eliminating effector T and B cells through central and peripheral tolerance mechanisms. Although the critical role of tolerance in preventing autoimmunity is established [3], its impact on cancer vaccines remains undefined. Currently, it is unknown if elimination of self-antigen-specific T and B cells from the naïve lymphocyte pool restricts vaccine success in patients. More importantly, it is presumed, but unverified, that tolerance limits autoimmunity and tumor immunity through lineage-unbiased elimination of self-antigen-specific cells, eliminating CD4$^+$ T cells, CD8$^+$ T cells, and B cells [4].

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Guanylyl cyclase C (GUCY2C) is the index example of cancer mucosa antigens [5] and a potential target for colorectal cancer vaccines [6–8]. GUCY2C is a membrane protein expressed primarily in intestinal epithelium of humans and mice [9–11]. Further, GUCY2C is universally expressed in human colorectal cancers, serving as a biomarker for occult metastatic disease [9, 10, 12, 13]. Selective expression in intestinal epithelium, associated with immunological privilege limiting tolerance [5, 6], and its expression in gastrointestinal malignancies, suggest that GUCY2C may be an ideal target that overcomes limitations in current cancer vaccines.

Immunization of wild-type ($GUCY2C^{+/+}$) mice with replication-deficient, recombinant adenovirus expressing the extracellular domain of mouse GUCY2C (adenovirus serotype 5 (Ad5)-GUCY2C) produces GUCY2C-specific $CD8^+$ T cells that mediate modest antitumor immunity without autoimmunity [6–8]. Interestingly, $GUCY2C^{+/+}$ mice failed to produce GUCY2C-specific $CD4^+$ T-cell or antibody responses, although these responses were produced in GUCY2C-deficient ($GUCY2C^{-/-}$) mice [6, 7]. Here, we reveal that suboptimal antibody responses, along with suboptimal primary and memory $CD8^+$ T-cell responses and antitumor immunity targeting GUCY2C, reflect selective antigen-specific $CD4^+$ T-cell, but not $CD8^+$ T- or B-cell, tolerance. Incorporating a foreign (influenza) $CD4^+$ T helper (Th)-cell epitope into the vaccine revealed the latent activity of antigen-specific $CD8^+$ T- and B-cell pools, reconstituting maximum primary and memory $CD8^+$ T-cell and antibody responses producing optimum antitumor immunity without autoimmunity. Beyond GUCY2C in colorectal cancer, the efficacy of vaccines targeting self-antigens in melanoma (tyrosinase-related protein 2, Trp2) and breast cancer (human epidermal growth factor receptor 2, Her2) is also restricted by selective Th-cell tolerance. Thus, selective $CD4^+$ T-cell tolerance restricts cancer vaccine efficacy across self-antigens, tissues, MHC haplotypes, and cancers, but preserves functional $CD8^+$ T- and B-cell pools. Identification of self-antigens producing selective $CD4^+$ T-cell tolerance may be one strategy to establish ideal immunotherapeutic target antigens, allowing activation of latent $CD8^+$ T and B cells through self-antigen-independent $CD4^+$ T-cell help to produce effective antitumor immunity.

Results

GUCY2C-specific antibody responses are $CD4^+$ T-cell dependent

$CD4^+$ T cells play a critical role in helping B cells mount antibody responses [14] and helping $CD8^+$ T cells mount cytotoxic T lymphocyte (CTL) responses (particularly memory responses) against foreign antigens [15–17]. Induction of $CD8^+$ T-cell, but not $CD4^+$ T-cell or antibody, responses by Ad5-GUCY2C in $GUCY2C^{+/+}$ mice, across multiple mouse strains and haplotypes, suggested that absent antibody responses could reflect $CD4^+$ T-cell tolerance [6–8]. The $CD4^+$ T-cell dependence of antibody responses in $GUCY2C^{-/-}$ mice was explored using a $CD4^+$ T-cell-depleting antibody (Fig. 1). Indeed, anti-CD4 treatment eliminated $CD4^+$ T cells, whereas B and $CD8^+$ T cells were

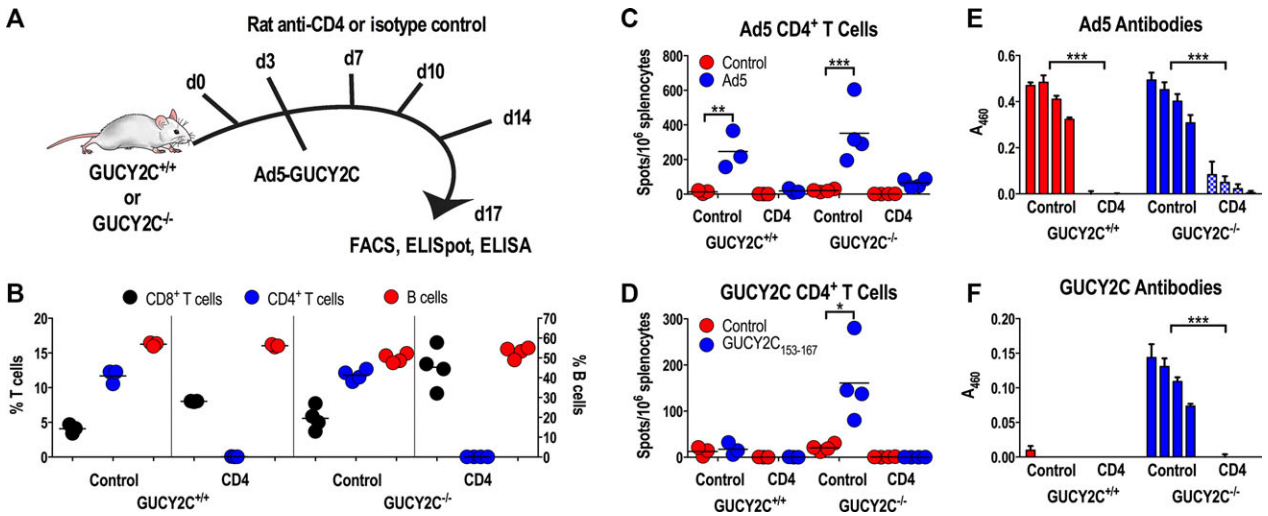

Figure 1. GUCY2C-specific antibody responses are $CD4^+$ T-cell dependent. (A) $CD4^+$ T cells were depleted in $GUCY2C^{+/+}$ or $GUCY2C^{-/-}$ BALB/c mice by i.p. injection of 300 μg of anti-CD4 (CD4) antibody every 3–4 days for 14 days. Control mice received isotype-matched antibody (Control). Three days after initiating depletion, mice were immunized i.m. with 10^8 IFU of Ad5-GUCY2C. Flow cytometry, ELISA, and ELISpot analyses were carried out 14 days after immunization. (B) Flow cytometry analysis was performed on splenic lymphocyte populations to determine depletion efficiency and specificity following anti-CD4 administration in $GUCY2C^{+/+}$ and $GUCY2C^{-/-}$ mice (gating shown in Supporting Information Fig. 5). (C) Ad5-specific $CD4^+$ T-cell responses and (E) antibody responses were assessed by IFN-γ ELISpot and ELISA, respectively, in isotype control and anti-CD4 antibody-treated $GUCY2C^{+/+}$ and $GUCY2C^{-/-}$ mice $^{**}p < 0.01$, $^{***}p < 0.001$ (Two-way ANOVA). (D) GUCY2C-specific $CD4^+$ T-cell and (F) antibody responses were assessed by IFN-γ ELISpot and ELISA, respectively, in $GUCY2C^{+/+}$ and $GUCY2C^{-/-}$ mice following isotype control or anti-CD4 antibody treatment $^{*}p < 0.05$, $^{***}p < 0.001$ (Two-way ANOVA). (B–D) each point represents one mouse from a single experiment and means are shown with a horizontal bar. (E and F) Mean A_{460} values + SEM are shown for 3–4 mice/group from a single experiment.

unaffected (Fig. 1B). Ad5-specific CD4$^+$ T-cell responses, which were similar in GUCY2C$^{+/+}$ and GUCY2C$^{-/-}$ mice, were eliminated by anti-CD4 treatment (Fig. 1C). In contrast, GUCY2C-specific CD4$^+$ T-cell responses were detected only in GUCY2C$^{-/-}$, but not GUCY2C$^{+/+}$, mice and these were also eliminated with anti-CD4 treatment (Fig. 1D). Further, anti-CD4 treatment eliminated Ad5-specific antibodies in GUCY2C$^{+/+}$ and GUCY2C$^{-/-}$ mice (Fig. 1E). Moreover, anti-CD4 treatment eliminated GUCY2C-specific antibody responses in GUCY2C$^{-/-}$ mice, recapitulating immunological responses observed in GUCY2C$^{+/+}$ mice (Fig. 1F). Thus, GUCY2C-specific antibody responses in GUCY2C$^{-/-}$ mice require CD4$^+$ T-cell help, and their absence in wild-type mice reflects, at least in part, the absence of GUCY2C-specific CD4$^+$ Th cells.

GUCY2C-specific antibody and CD8$^+$ T-cell responses are limited by CD4$^+$ T-cell tolerance

The absence of Th-cell responses in GUCY2C$^{+/+}$ mice, and the Th-cell dependence of GUCY2C antibody responses in GUCY2C$^{-/-}$ mice, suggests that provision of T-cell help could reveal functional GUCY2C-specific B cells and restore antibody responses in wild-type mice. To provide CD4$^+$ T-cell help for GUCY2C-specific antibody responses, we produced a recombinant adenovirus possessing GUCY2C incorporating a C-terminal influenza $HA_{107–119}$ epitope (known as Site 1 or S1). S1 is an I-E^d-restricted epitope from influenza virus A/Puerto Rico/8/34 [18]. Immunization of GUCY2C$^{+/+}$ mice with control Ad5, Ad5-GUCY2C, or Ad5-GUCY2C-S1 failed to produce GUCY2C-specific CD4$^+$ T-cell responses (Fig. 2A). In contrast, Ad5-GUCY2C-S1 immunization produced S1-specific CD4$^+$ T-cell responses (Fig. 2A). Importantly, immunization with Ad5-GUCY2C-S1, but not control Ad5 or Ad5-GUCY2C, produced GUCY2C-specific antibody responses in GUCY2C$^{+/+}$ mice (Fig. 2C), similarly to those of GUCY2C$^{-/-}$ mice immunized with Ad5-GUCY2C (Fig. 1F). Thus, in GUCY2C$^{+/+}$ mice, GUCY2C-specific B cells are present and competent to respond and produce antibodies following immunization (Fig. 2C) when provided with adequate CD4$^+$ T-cell help (Fig. 2A). Moreover, although primary GUCY2C-specific CD8$^+$ T-cell responses were not completely dependent on GUCY2C-specific CD4$^+$ T cells in GUCY2C$^{+/+}$ mice, GUCY2C-specific CD8$^+$ T-cell responses were augmented approximately tenfold following immunization with Ad5-GUCY2C-S1, compared with Ad5-GUCY2C (Fig. 2E). Thus, CD4$^+$ T-cell help is indispensable for GUCY2C-specific antibody responses and is required for optimal CD8$^+$ T-cell responses, and its absence in GUCY2C$^{+/+}$ mice restricts those responses. It is noteworthy that help provided by S1-specific CD4$^+$ T cells did not impact Ad5-specific antibody or CD8$^+$ T-cell responses in the context of adequate Ad5-specific CD4$^+$ T cells (Fig. 2B, D, and F). Antibody-mediated CD4$^+$ T-cell depletion confirmed that Ad5-GUCY2C-S1-induced reconstitution of antibody and CD8$^+$ T-cell responses to GUCY2C is CD4$^+$ T-cell dependent (Fig. 2G and H). Immunization of GUCY2C$^{+/+}$ mice

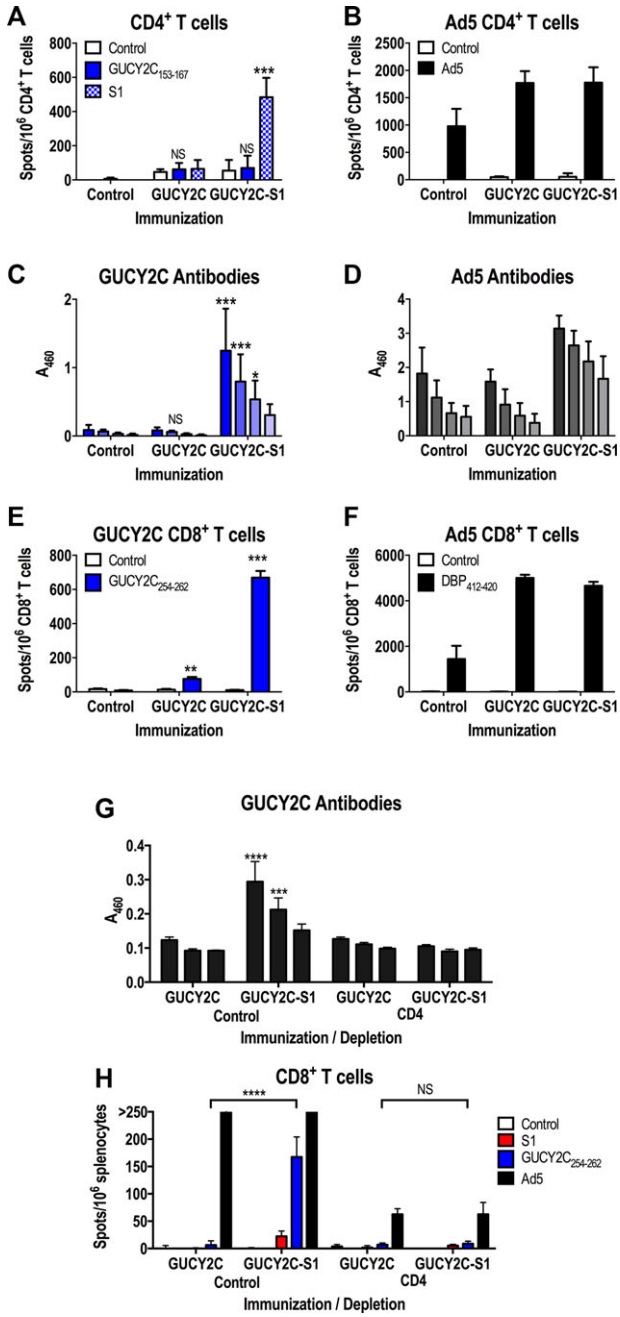

Figure 2. GUCY2C-specific antibody responses and CD8$^+$ T-cell responses are limited by CD4$^+$ T- cell tolerance. (A–F) wild-type (GUCY2C$^{+/+}$) BALB/c mice were immunized with control Ad5, Ad5-GUCY2C, or Ad5-GUCY2C-S1 and splenocytes and sera were collected 14 days later for (A and B) CD4$^+$ T-cell IFN-γ ELISpot, (C and D) ELISA, and (E and F) CD8$^+$ T-cell IFN-γ ELISpot assays for GUCY2C, S1 and Ad5-specific responses. (G and H) Antibody-mediated depletion of CD4$^+$ T cells was performed every 3–4 days with 300 μg anti-CD4 (CD4) or isotype control antibody (Control) beginning 3 days before immunization to confirm the CD4$^+$ T-cell dependence of GUCY2C-specific (G) antibody and (H) CD8$^+$ T-cell responses in Ad5-GUCY2C-S1-immunized mice. (A–H) Data are shown as mean + SEM of $n = 3$ mice/group and are representative of (A–F) at least three or (G, H) two independent experiments. (A and E) $^{**}p < 0.01$, $^{***}p < 0.001$ (two-way ANOVA versus control stimulation); (C and G) $^{*}p < 0.05$, $^{***}p < 0.001$, $^{****}p < 0.0001$ (two-way ANOVA versus (C) control Ad5 or (G) GUCY2C, respectively); (H) $^{****}p < 0.0001$ (two-way ANOVA). NS = not statistically significant.

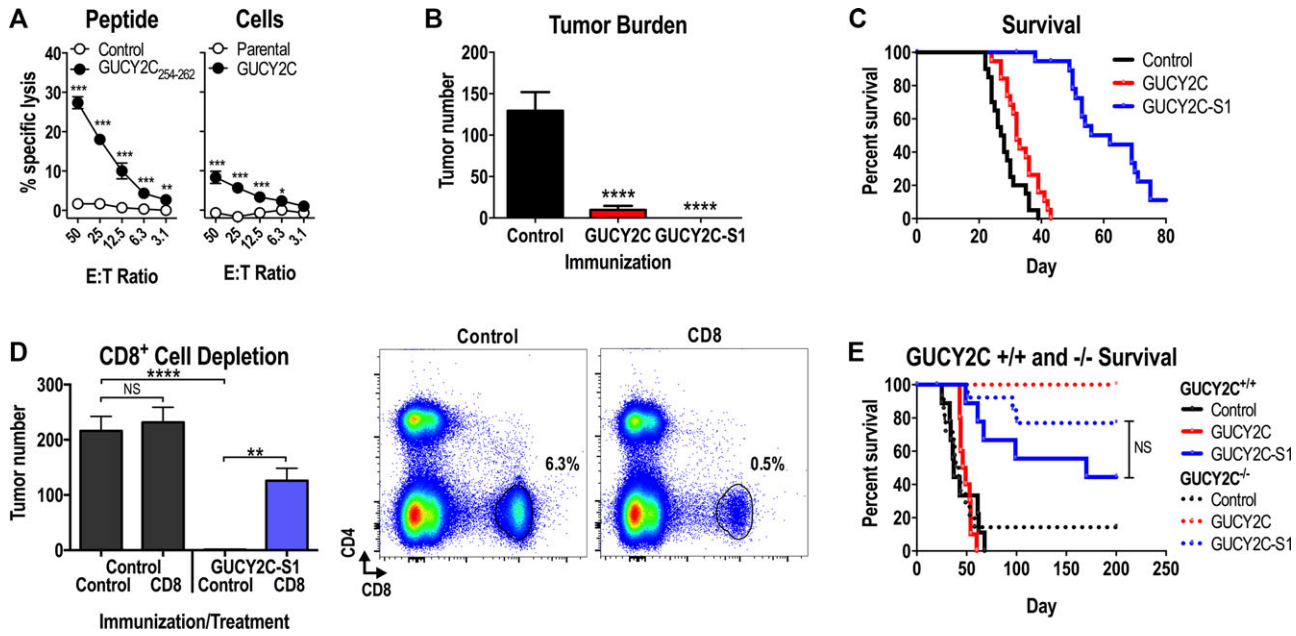

Figure 3. GUCY2C-specific antitumor responses are limited by CD4$^+$ T-cell tolerance. (A) CTL cultures were produced two weeks after immunizing BALB/c mice with Ad5-GUCY2C-S1 and were tested at the indicated E:T ratios for their ability to lyse GUCY2C$_{254–262}$ peptide-pulsed targets (left) or those expressing full-length GUCY2C (right) by β-galactosidase release. CTL data are representative of two experiments using pooled splenocytes from five immunized mice $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.0001$ (two-way ANOVA versus control). (B and C) BALB/c mice were immunized with control Ad5, Ad5-GUCY2C, or Ad5-GUCY2C-S1 and challenged intravenously with 5×10^5 CT26-GUCY2C cells 7 days later. (B) Lungs were collected 17 days postchallenge, stained to reveal tumors, and tumor multiplicity was quantified by gross inspection ($n = 29–34$ mice/group). Data are shown as mean + SEM. $^{****}p < 0.0001$, (one-way ANOVA versus control). (C) Survival was measured longitudinally for the indicated groups of mice ($n = 19–20$ mice/group pooled from four independent experiments). $p < 0.01$ control Ad5 versus Ad5-GUCY2C, $p < 0.0001$ Ad5-GUCY2C-S1 versus control Ad5, or Ad5-GUCY2C (Mantel–Cox log-rank test). (D) Mice immunized with control Ad5 or Ad5-GUCY2C-S1 were treated with a CD8$^+$ T-cell-depleting antibody beginning on day 3 after immunization and then challenged intravenously with 5×10^5 CT26-GUCY2C cells 4 days later. On day 17 postchallenge, lungs were stained to reveal tumors and tumor multiplicity was quantified by gross inspection, and splenic lymphocyte populations were pooled within groups, stained for CD4, CD8, and CD19 and analyzed by flow cytometry (values indicate percentages of live splenocytes; gating in Supporting Information Fig. 6). Tumor number data show mean + SEM of 7–8 mice/group. $^{**}p < 0.01$, $^{****}p < 0.0001$ (one-way ANOVA). (E) GUCY2C$^{+/+}$ or GUCY2C$^{-/-}$ BALB/c (N3) mice were immunized with control Ad5, Ad5-GUCY2C, or Ad5-GUCY2C-S1 ($n = 9–14$ mice/group pooled from two independent experiments), challenged intravenously with 5×10^5 CT26-GUCY2C cells 7 days later, and survival was measured longitudinally $p < 0.0001$ Ad5-GUCY2C in GUCY2C$^{+/+}$ versus GUCY2C$^{-/-}$ mice, $p = 0.1197$ Ad5-GUCY2C-S1 in GUCY2C$^{+/+}$ versus GUCY2C$^{-/-}$ mice (Mantel–Cox log-rank test). NS = not statistically significant.

with Ad5-GUCY2C-S1 in the presence of isotype control antibody produced GUCY2C-specific antibodies (Fig. 2G) and enhanced CD8$^+$ T-cell responses (Fig. 2H) compared with Ad5-GUCY2C immunization. However, these responses were eliminated in the presence of a CD4$^+$ T-cell-depleting antibody (Fig. 2G and H).

GUCY2C-specific antitumor responses are limited by CD4$^+$ T-cell tolerance

Reviving primary antibody and CD8$^+$ T-cell responses by incorporating S1 into the Ad5-GUC2YC vaccine suggests that this CD4$^+$ T-cell epitope could reconstitute complete vaccine-induced antitumor responses. Indeed, Ad5-GUCY2C-S1 immunization produced cytotoxic T lymphocytes (CTLs) that recognized and killed CT26 colorectal cancer cells pulsed with GUCY2C$_{254–262}$ CD8$^+$ T-cell epitope peptide or expressing GUCY2C protein in vitro (Fig. 3A). As previously demonstrated [6–8], Ad5-GUCY2C immunization reduced lung metastasis multiplicity by >90% (Fig. 3B) and was associated with improved survival (Fig. 3C) in mice with GUCY2C-expressing colorectal cancer metastases in lung (CT26-GUCY2C). However, Ad5-GUCY2C-S1 immunization was more effective ($p < 0.001$), producing near complete elimination of metastases (Fig. 3B), with macroscopic metastases in only 3% of mice. More importantly, Ad5-GUCY2C-S1 immunization enhanced survival >750% (34.5 versus 4.5 days beyond control Ad5) following immunization (Fig. 3C). The CD8$^+$ T-cell dependence of this effect was revealed by treating mice with CD8$^+$ T-cell-depleting monoclonal antibody, reducing Ad5-GUCY2C-S1 antitumor efficacy approximately 60% (Fig. 3D). Residual antitumor immunity reflected the incomplete (about 90%) elimination of CD8$^+$ T cells with antibody treatment (Fig. 3D).

The antitumor efficacy of Ad5-GUCY2C-S1 vaccination was quantitatively similar to that produced by targeting the foreign antigen β-galactosidase (Supporting Information Fig. 1), suggesting that responses seen with Ad5-GUCY2C-S1 may be maximal and unhindered by tolerance. To directly determine if Ad5-GUCY2C-S1 immunization fully overcomes tolerance, we compared antitumor efficacy in tolerant GUCY2C$^{+/+}$ and nontolerant GUCY2C$^{-/-}$ mice following control Ad5, Ad5-GUCY2C, or

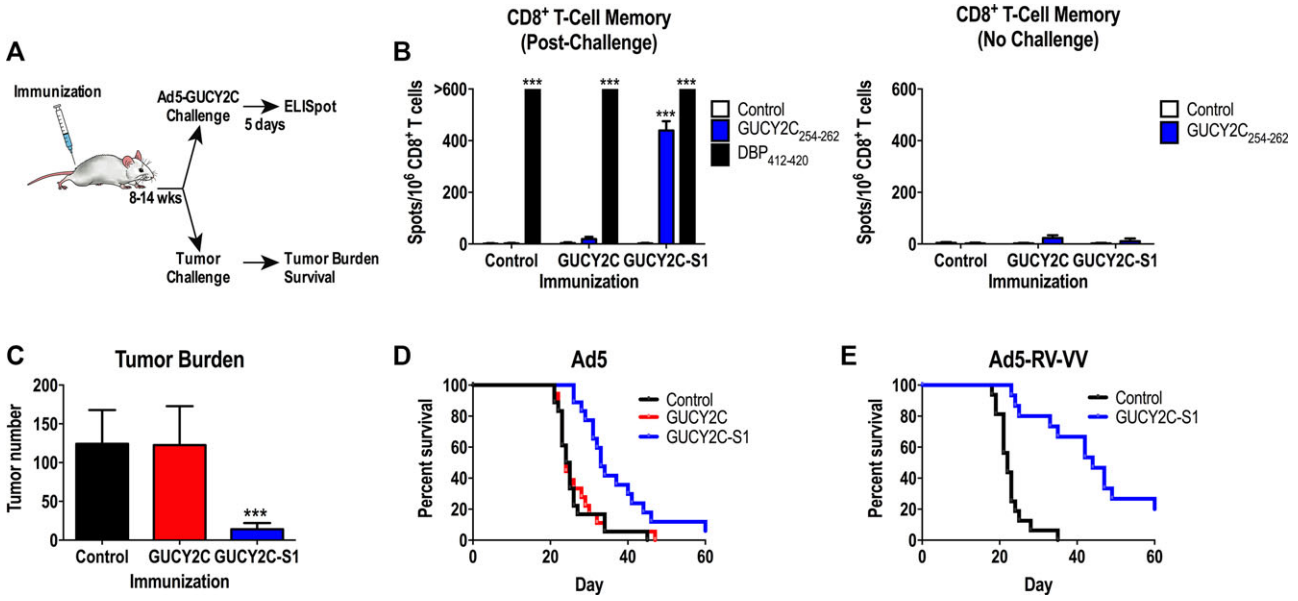

Figure 4. GUCY2C-specific CD8$^+$ T-cell memory responses are eliminated by CD4$^+$ T-cell tolerance. (A) Experimental design for (B–D). (B) BALB/c mice were immunized with control Ad5, Ad5-GUCY2C, or Ad5-GUCY2C-S1 and were challenged with Ad5-GUCY2C 13–14 weeks later. Recall CD8$^+$ T-cell responses to Ad5 and GUCY2C were assessed by IFN-γ ELISpot following Ad5-GUCY2C challenge (left) or no challenge (right). Data show mean + SEM of 2–3 mice/group and are representative of two independent experiments. ***$p < 0.001$ (Two-way ANOVA versus control stimulation). (C and D) BALB/c mice were immunized with control Ad5, Ad5-GUCY2C, or Ad5-GUCY2C-S1 and were challenged with 5×10^5 CT26-GUCY2C cells 8–11 weeks after immunization. Memory antitumor immunity was measured by (C) tumor enumeration and (D) survival. In (C), data are shown as mean + SEM of 10 mice from a single experiment. ***$p < 0.001$ (one-way ANOVA versus control). In (D), $n = 18$ mice/group from a single experiment. $p < 0.001$ (Mantel–Cox log-rank test Ad5-GUCY2C-S1 versus control Ad5 or Ad5-GUCY2C). (E) BALB/c mice were primed with control or Ad5-GUCY2C-S1. Twenty-eight days later, mice were boosted with control or GUCY2C-S1-expressing rabies virus (RV) followed by a subsequent boost 28 days later with control or GUCY2C-S1-expressing vaccinia virus (VV). Eleven weeks after the final boost, mice were challenged intravenously with 5×10^5 CT26-GUCY2C cells, and survival was monitored ($n = 13$–16 mice/group from a single experiment). $p < 0.0001$ (Mantel–Cox log-rank test).

Ad5-GUCY2C-S1 immunization (Fig. 3E). As expected, Ad5-GUCY2C efficacy was restricted by tolerance in wild-type mice producing median survival of only about 50 days following establishment of lung metastases. In contrast, all GUCY2C$^{-/-}$ mice immunized with Ad5-GUCY2C were alive beyond 200 days following tumor challenge ($p < 0.0001$). Importantly, Ad5-GUCY2C-S1 immunization was similarly efficacious in GUCY2C$^{+/+}$ and GUCY2C$^{-/-}$ mice, and each produced median survival times >150 days that were not statistically different ($p = 0.1197$). Similar Ad5-GUCY2C-S1 antitumor efficacy in the absence (GUCY2C$^{-/-}$) and presence (GUCY2C$^{+/+}$) of tolerance suggests that selective elimination of CD4$^+$ T-cell help is the dominant mechanism of GUCY2C-specific tolerance, which can be abrogated by CD4$^+$ T-cell help targeting foreign antigens. Importantly, producing maximal antibody and CD8$^+$ T-cell responses and antitumor immunity through GUCY2C-independent CD4$^+$ T-cell help did not produce acute or chronic autoimmunity targeting gastrointestinal or other tissues (Supporting Information Fig. 2).

GUCY2C-specific CD8$^+$ T-cell memory responses are eliminated by CD4$^+$ T-cell tolerance

Although CD4$^+$ T-cell help is dispensable for primary CD8$^+$ T-cell responses to some antigens, including GUCY2C [6–8], it is typically required for the production of memory CD8$^+$ T cells [15–17]. To determine if GUCY2C-specific CD8$^+$ T-cell memory was eliminated by the absence of CD4$^+$ T-cell help, we immunized GUCY2C$^{+/+}$ mice with control Ad5 or Ad5-GUCY2C and, after 13–14 weeks, challenged them with Ad5-GUCY2C to measure recall responses 5 days later (Fig. 4A and B). GUCY2C-specific memory responses were absent in Ad5 and Ad5-GUCY2C-immunized mice (Fig. 4B). However, Ad5-GUCY2C-S1 immunization restored CD8$^+$ T-cell memory responses that persisted for at least 14 weeks (Fig. 4B). Absence of responses in control Ad5-primed mice, or without Ad5-GUCY2C challenge (Fig. 4B), confirmed that measured responses were memory, rather than primary, which require approximately 10 days to achieve detection. Consistent with the direct measurement of CD8$^+$ T-cell memory responses, mice challenged with GUCY2C-expressing CT26 cells 8–11 weeks following immunization with Ad5-GUCY2C were not protected, whereas Ad5-GUCY2C-S1 immunization produced protection that persisted for months, decreasing tumor number (Fig. 4C) and increasing survival (Fig. 4D). Moreover, heterologous prime-boost immunization at 4 week intervals with adenovirus (Ad5), rabies virus (RV), and vaccinia virus (VV) incorporating GUCY2C-S1 followed by tumor challenge 11 weeks later enhanced protective memory responses, extending survival (Fig. 4E) beyond that obtained with a single immunization (Fig. 4D).

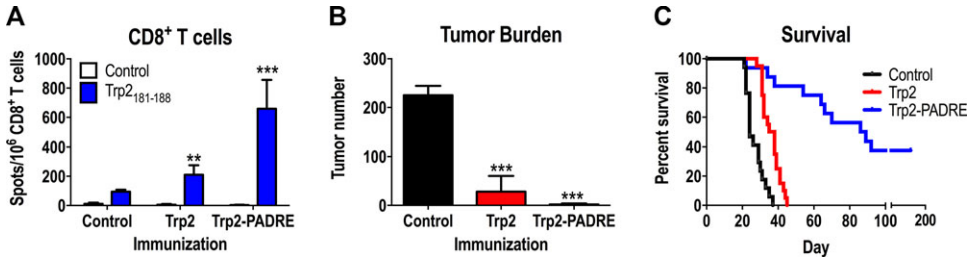

Figure 5. Melanoma antigen-specific CD8+ T-cell and antitumor responses are limited by CD4+ T-cell tolerance. (A) C57BL/6 mice were immunized with control Ad5, Ad5-Trp2, or Ad5-Trp2-PADRE, and Trp2-specific CD8+ T-cell responses were measured 10 days later by IFN-γ ELISpot ($n = 3$ mice/group, representative of two independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$, versus control stimulation (two-way ANOVA). (B and C) C57BL/6 mice were immunized with control Ad5, Ad5-Trp2, or Ad5-Trp2-PADRE and challenged intravenously with 5×10^5 B16-F10 cells 7 days later. (B) Lungs were collected 17 days postchallenge from some mice and tumor multiplicity was quantified ($n = 8$–10 mice/group from a single experiment). $^{***}p < 0.001$ (one-way ANOVA versus control). (C) Survival was measured longitudinally in other mice ($n = 16$–20 mice/group from a single experiment. $p < 0.0001$, control Ad5 versus Ad5-Trp2 or Ad5-Trp2-PADRE and Ad5-Trp2 versus Ad5-Trp2-PADRE, (Mantel–Cox log-rank test).

Anti-melanoma CD8+ T-cell and antitumor responses are limited by CD4+ T-cell tolerance

To explore the generalizability of selective CD4+ T-cell tolerance to other self-antigens, we examined responses to the mouse melanosomal antigen Trp2. Like GUCY2C, mouse Trp2-specific immunization produced weak CD8+ T-cell responses, and no CD4+ T-cell responses in wild-type mice, whereas full CD4+ and CD8+ T-cell responses are produced in Trp2-deficient mice [19]. In agreement with that observation, immunization of wild-type C57BL/6 mice with Ad5-expressing mouse Trp2 (Ad5-Trp2) produced modest CD8+ T-cell responses (Fig. 5A) and antitumor immunity following i.v. challenge with Trp2-expressing B16-F10 melanoma cells, reducing lung metastases (Fig. 5B) and extending survival (Fig. 5C). Here, to reveal latent Trp2-specific CD8+ T-cell responses, Trp2-independent CD4+ T-cell help was provided using the synthetic pan DR epitope (PADRE), reflecting the inactivity of S1 in C57BL/6 mice. This epitope binds to a wide range of human DR molecules as well as murine I-A^b [20]. Immunization of wild-type C57BL/6 mice with Ad5-Trp2-PADRE reconstituted Trp2-specific CD8+ T-cell responses ($p < 0.001$; Fig. 5A) and antitumor immunity, decreasing tumor number ($p < 0.001$; Fig. 5B) compared with Ad5-Trp2 immunization. Like Ad5-GUCY2C-S1 immunization, Ad5-Trp2-PADRE increased survival approximately 400% beyond that induced by Ad5-Trp2 immunization (63.5 versus 12.5 days beyond control Ad5; $p < 0.0001$; Fig. 5C).

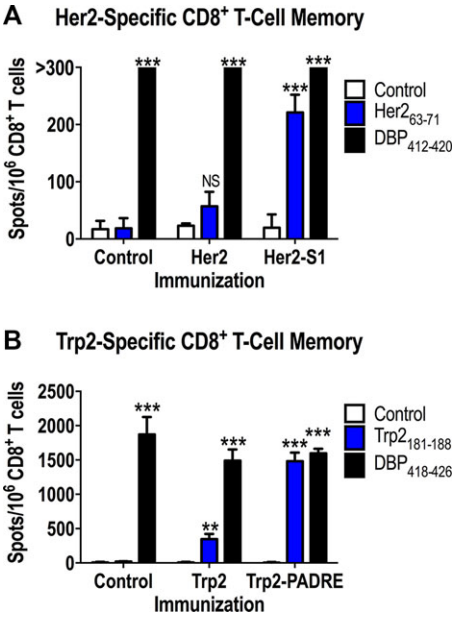

Figure 6. CD8+ T-cell memory to melanoma and breast cancer antigens is limited by CD4+ T-cell tolerance. (A) BALB/c mice were immunized with control Ad5, Ad5-Her2, or Ad5-Her2-S1 and challenged >100 days later with Ad5-Her2. IFN-γ ELISpot responses were measured 5 days postchallenge. (B) C57BL/6 mice were immunized with control Ad5, Ad5-Trp2, or Ad5-Trp2-PADRE and challenged 8 weeks later with Ad5-Trp2. IFN-γ ELISpot responses were measured 5 days postchallenge. (A, B) Data show mean + SEM of five mice/group pooled from two independent experiments. $^{**}p < 0.01$, $^{***}p < 0.001$ (two-way ANOVA versus control stimulation); NS = not statistically significant.

CD8+ T-cell memory to melanoma and breast cancer antigens is limited by CD4+ T-cell tolerance

Having established that CD4+ T-cell help is essential to produce GUCY2C-specific CD8+ T-cell memory responses and enduring antitumor immunity (Fig. 4), we examined the ability of self-antigen-independent CD4+ T-cell help to rescue CD8+ T-cell memory targeting the melanoma antigen, Trp2, and the breast cancer antigen, mouse Her2. Like Trp2 [19], tolerance blocks the generation of antigen-specific CD8+ T-cell responses to mouse Her2 in wild-type mice [21]. Moreover, Ad5-Her2-S1 enhanced primary antitumor responses compared with Ad5-Her2 (Supporting Information Fig. 3). Like GUCY2C, Her2-specific CD8+ T-cell memory responses were absent (Fig. 6A) and Trp2-specific CD8+ T-cell memory was low (Fig. 6B) in the absence of T-cell help. In contrast, CD4+ T-cell help through S1 or PADRE rescued mouse Her2 (Fig. 6A) and Trp2 (Fig. 6B)-specific CD8+ T-cell memory responses, respectively.

Discussion

Limitations in the identification and availability of tumor-specific antigens have focused attention primarily on self-antigens as vaccine targets to prevent and treat cancer. In turn, immunotherapeutic approaches targeting self-antigens are burdened by the reciprocal liabilities of tolerance and autoimmunity. On the one hand, tolerance mechanisms attenuate immune responses to self-antigens to preserve tissue homeostasis, but restrict antitumor efficacy. On the other hand, abrogating tolerance to create robust immune responses to self-antigens enhances therapeutic antitumor efficacy, but at the risk of autoimmunity. Indeed, tolerance mechanisms attenuating immune responses to self-antigens principally contribute to therapeutic failures of cancer vaccines [2]. Defining the repertoire of molecular and cellular programs mediating immunological tolerance to self-antigens is required to create mechanism-based solutions that maximize the therapeutic impact of cancer vaccines while minimizing autoimmune tissue destruction, to optimize patient outcomes.

Here, we reveal that bona fide self-antigens are associated with a previously unappreciated mechanism involving lineage-specific split tolerance in which antigen-specific $CD4^+$ T cells are selectively eliminated. Elimination of antigen-specific Th cells is specific, and functional pools of antigen-specific $CD8^+$ T and B cells remain intact. In the absence of antigen-specific $CD4^+$ T cells, primary and memory antigen-specific $CD8^+$ T- and B-cell responses are attenuated when stimulated by vaccines targeting self-antigens. Further, attenuated immune responses are associated with blunted and transient therapeutic antitumor responses. In contrast, incorporating a single foreign antigen MHC class II epitope into the vaccine engaged self-antigen-independent $CD4^+$ T cells. In turn, these $CD4^+$ T cells provided the necessary help that revealed the nascent functional capacity of self-antigen-specific $CD8^+$ T- and B-cell pools in tolerant wild-type mice. Remarkably, the resulting primary and memory $CD8^+$ T-cell, antibody, and antitumor responses targeting self-antigen were maximal. Indeed, these responses were quantitatively similar to those achieved in nontolerant models, including responses in a self-antigen-deleted ($GUCY2C^{-/-}$ mice) system and responses in a native system to a foreign (bacterial β-galactosidase) antigen. These observations suggest that rather than eliminating self- antigen-specific reactivity across lymphocyte populations, tolerance to some self-antigens preferentially impacts $CD4^+$ Th cells, thereby preserving functional $CD8^+$ T- and B-cell pools. This challenges the current paradigm suggesting that only functionally inept lymphocyte pools escape tolerance, establishing a fixed limitation in the available antitumor effectors [22]. Rather, preservation of highly functional $CD8^+$ T- and B-cell pools permits their full engagement through self-antigen-independent $CD4^+$ T-cell help to maximize antitumor immunity.

Moreover, split tolerance provides a unique mechanism-based strategy to abrogate tolerance and maximize antitumor responses without the associated liability of autoimmunity. Although examples of $CD8^+$ T-cell-mediated autoimmune disease in mice and humans exist, animal models and epidemiological data suggest that autoreactive $CD4^+$ T cells are principal effectors in autoimmunity. $CD4^+$ T cells targeted to a gastric self-antigen [23] or commensal bacterial antigen [24] induce gastrointestinal autoimmunity in mice. Further, experimental autoimmune diseases such as autoimmune encephalomyelitis [25], thyroiditis [26], colitis [27], and oophritis [28] are $CD4^+$ T-cell-mediated. Importantly, MHC class II genes are the most significant susceptibility genes for autoimmunity, with HLA-DQ2/DR3, -DQ6/DR2, and -DQ8/DR4 haplotypes accounting for 90% of disease [29]. These observations support the suggestion that preferential elimination of $CD4^+$ T, but not $CD8^+$ T or B, cells may be a common tolerance strategy for select self-antigens, which silences cells most associated with autoimmunity, but preserves a broad immune repertoire. Indeed, removal of T cells recognizing a single self-antigen epitope may eliminate reactivity against >1 million different peptide-MHC complexes due to the high promiscuity of T-cell receptors [30]. Thus, by allowing more $CD8^+$ T and B cells to escape tolerance, a significantly broader immune repertoire is produced, conferring recognition of $>10^{15}$ different antigens [30].

Examples of split tolerance are limited and have been previously observed only with experimental systems employing model foreign antigens expressed as self-antigen transgenes, producing $CD4^+$ T-cell, but not B-cell, tolerance [31, 32]. It is noteworthy that $CD8^+$ T-cell tolerance was not examined in those systems. In another study, skin-specific transgenic expression of the human papilloma virus E7 protein produced not only E7-specific tolerance in the CTL repertoire, but also paradoxical expansion of the Th-cell repertoire [33]. Thus, transgenic systems have produced heterogeneous, and largely uninformative, results with respect to $CD8^+$ T cells. Here, we demonstrate that split tolerance selectively affects $CD4^+$ T cells specific for some endogenous self-antigens. Although $CD4^+$ T-cell responses can naturally occur [34], or be produced by immunization [35], against some tumor-associated antigens, our results suggest that selective $CD4^+$ T-cell tolerance may be common to many self-antigens, and defining tolerance mechanisms for individual tumor antigens is necessary to tailor immunotherapeutic approaches that overcome the relevant tolerance mechanisms.

Addition of foreign proteins, particularly tetanus toxoid, to whole self-antigens [36] or self-antigen peptides [37] was demonstrated previously to enhance immune responses and antitumor immunity. In the case of vaccines employing only immunodominant $CD8^+$ T-cell epitopes, which lack endogenous $CD4^+$ T-cell epitopes, conjugation to a tetanus toxin fragment restored $CD4^+$ T-cell responses, amplifying vaccine efficacy [37–40]. In contrast, the precise mechanisms mediating the immunoadjuvant effects of appending foreign proteins to whole self-antigens remain undefined. The present observations suggest that rather than "breaking tolerance," those approaches, at least in part, restored absent $CD4^+$ T-cell help, engaging functional $CD8^+$ T- and B-cells that amplified clinical efficacy. In that context, it is significant that in $GUCY2C^{-/-}$ mice, which have active pools of GUCY2C-specific $CD4^+$ T cells, Ad5-GUCY2C-S1 immunization was less efficacious (75% survival), compared with Ad5-GUCY2C immunization (100% survival; $p < 0.05$; Fig. 3E). Here, incorporating the S1

helper epitope diminished vaccine efficacy, demonstrating that exogenous $CD4^+$ T-cell help in the absence of selective $CD4^+$ T-cell tolerance can restrict, rather than amplify, immune responses. These considerations underscore the importance of defining the contribution of selective $CD4^+$ T-cell tolerance for individual antigens, to direct mechanism-based application of self-antigen-independent $CD4^+$ T-cell help that reconstitutes the latent anti-tumor potential of vaccine regimens.

Self-antigen-independent $CD4^+$ T-cell epitopes likely amplify $CD8^+$ T-cell responses through dendritic cell (DC) licensing mediated by CD40–CD40L interactions [41–44]. Alternatively, helper cells may amplify $CD8^+$ T-cell responses through passive "bystander" effects, such as cytokines acting directly on $CD8^+$ T cells [17, 45]. Here, robust adenovirus-specific $CD4^+$ T-cell responses were produced, yet adenovirus-specific Th cells were inadequate to help self-antigen-specific responses, reducing the likelihood of a contribution by bystander mechanisms in our system. Rather, DCs presenting both self MHC class I and foreign MHC class II helper epitopes are licensed by foreign antigen-specific Th cells to activate self-antigen-specific CTLs. In that context, Ad5-GUCY2C is nonreplicating due to deletion of the immediate early E1 genes, and viral protein expression is limited in vivo. Thus, peak adenovirus protein processing and presentation occurs immediately after immunization wherein injected viral particles, but not de novo synthesized adenovirus proteins, serve as the antigen source. In contrast, GUCY2C protein is absent in the viral particle and in vivo transduction and de novo GUCY2C protein synthesis is required to produce material for processing and presentation to T cells. In the context of peak GUCY2C expression occurring >96 hours after transduction and bolus delivery of viral particles without replication, GUCY2C epitope presentation is delayed and protracted, while adenovirus epitope presentation is immediate and short-lived. This produces temporal dysynchrony in processing and presentation and an absence of GUCY2C-presenting DC licensing by Ad5-specific $CD4^+$ T cells. Thus, S1-specific Th cells succeed, whereas Ad5-specific Th cells fail, to help GUCY2C-specific $CD8^+$ T-cell responses due to overlap in antigen expression kinetics and co-presentation of MHC I and II epitopes necessary for DC licensing.

Beyond restoring self-antigen-specific $CD8^+$ T- and B-cell responses through self-antigen-independent $CD4^+$ T-cell help, defining mechanisms mediating selective $CD4^+$ T-cell tolerance may offer substantial utility in cancer immunotherapy. In that context, Ad5-GUCY2C was superior in $GUCY2C^{-/-}$ (nontolerant) mice (100% survival) compared with Ad5-GUCY2C-S1 in $GUCY2C^{+/+}$ (tolerant) mice (~50% survival, $p = 0.0014$; Fig. 3E). These observations suggest that GUCY2C-specific $CD4^+$ T cells may exhibit antitumor activity beyond helping $CD8^+$ T and B cells in $GUCY2C^{-/-}$ mice. Alternatively, exogenous $CD4^+$ T-cell help may be inferior to that provided by endogenous $CD4^+$ T-cell help in the context of certain vaccines [46]. $CD4^+$ T cells coordinate antitumor responses through a broad range of mediators that include Th1-mediated activation of macrophages to produce reactive oxygen species and Th2-mediated eosinophil activation [47]. Thus, the full spectrum of $CD4^+$ T-cell antitumor effector mechanisms may be required to maximize vaccine efficacy, and may be achievable only by reversing $CD4^+$ T-cell tolerance. In that context, the present results do not define the mechanisms mediating GUCY2C-specific $CD4^+$ T-cell tolerance. Rather, they demonstrate only that tolerance prevents the generation of GUCY2C-specific $CD4^+$ Th1 cells in $GUCY2C^{+/+}$ mice. In turn, these cells may be anergic, deleted, converted to Treg cells, or eliminated by another mechanism or combination of mechanisms. Ultimately, the precise contribution of these mechanisms to GUCY2C-specific $CD4^+$ T-cell tolerance will be defined using sophisticated transgenic models.

In summary, lineage-specific tolerance, in which $CD4^+$ T cells are eliminated but functional pools of $CD8^+$ T cells and B cells are preserved, characterizes self-antigens across mouse strains, antigens, and tumor types. Split tolerance involving $CD4^+$ T cells defends normal tissue integrity against autoimmune damage at the expense of an attenuated immunological and antitumor efficacy that characterizes most cancer vaccines targeting self-antigens. However, selective $CD4^+$ T-cell tolerance provides a unique therapeutic opportunity to maximize self-antigen-targeted immune and antitumor responses without inducing autoimmunity by incorporating self-antigen-independent $CD4^+$ T-cell epitopes into cancer vaccines.

Materials and methods

Mice and immunizations

BALB/c and C57BL/6 mice were obtained from the NCI Animal Production Program (Frederick, MD). $GUCY2C^{-/-}$ C57BL/6 mice were described previously [6, 8]. $GUCY2C^{-/-}$ BALB/c mice were produced by backcrossing >5 generations for all experiments, unless indicated. Animal protocols were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. Adenovirus expressing the extracellular domain of mouse GUCY2C (Ad5-GUCY2C) and Ad5-LacZ (control Ad5) were described previously [6]. Rabies and VV expressing GUCY2C-S1 and adenoviruses expressing GUCY2C-S1, Trp2, Trp2-PADRE, Her2, and Her2-S1 were produced as previously described [6]. For immunizations, mice received 1×10^8 infectious units (IFU) of adenovirus, 1×10^7 foci-forming units (FFU) of RV or 1×10^7 plaque-forming units (PFU) of VV by i.m. injection of the anterior tibialis.

Trp2 and Her2

All Trp2 and Her2 constructs employed the endogenous mouse antigens. The mouse Trp2 extracellular domain ($Trp2_{1–472}$) was cloned from the Mammalian Gene Collection full-length collection (Invitrogen) to produce Trp2 and Trp2-PADRE adenoviruses. The mouse Her2 extracellular domain ($Her2_{1–651}$) was cloned by RT-PCR from mouse Her2-expressing CT26 cells [21] kindly provided by Chang-Yuil Kang (Seoul National University, Seoul, Korea) to produce Her2 and Her2-S1 adenoviruses.

ELISpot

ELISpot assays were described previously [6]. Briefly, multiscreen filtration plates (Millipore) were coated with anti-mouse IFN-γ-capture antibody (BD Biosciences). $CD8^+$ T cells or $CD4^+$ T cells were MACS-purified (Miltenyi Biotec) from immunized mice and plated with naïve splenocytes serving as antigen-presenting cells and 5–10 μg/mL peptide. In some experiments, bulk splenocytes, rather than sorted T-cell populations were analyzed. After about 24 hours of peptide stimulation, spots were developed with biotinylated anti-IFN-γ detection antibody (BD Biosciences) and alkaline phosphatase-conjugated streptavidin (Pierce), followed by NBT/BCIP substrate (Pierce). Spot-forming cells were enumerated using computer-assisted video imaging analysis (ImmunoSpot v5, Cellular Technology). GUCY2C-specific peptides were $GUCY2C_{254–262}$ for $CD8^+$ T cells [8] and $GUCY2C_{153–167}$ for $CD4^+$ T cells (Supporting Information Fig. 4). Trp2- and Her2-specific $CD8^+$ T-cell peptides were $Trp2_{181–188}$ [19] and $Her2_{63–71}$ [21], respectively. Adenovirus-specific $CD8^+$ T-cell peptides were the H-2b-restricted $DBP_{418–426}$ or H-2d-restricted $DBP_{412–420}$ [48].

$CD4^+$ T-cell epitope mapping

A library of 105 $GUCY2C_{ECD}$-derived peptides 15 amino acids in length, with 11-amino acid overlap with adjacent peptides was synthesized (JPT Peptide Technologies). Aliquots of individual peptides or pools of 10–11 peptides were dissolved in DMSO and used in ELISpot assays at a final concentration of 5–10 μg/mL of each peptide with ≤1% DMSO. Splenocytes were collected from $GUCY2C^{-/-}$ BALB/c mice 10 days after immunization with Ad5-GUCY2C, and $CD4^+$ T cells were MACS purified (Miltenyi Biotec) and mixed with naïve splenocytes as APCs and peptides for ELISpot analysis.

ELISA

GUCY2C and Ad5-specific ELISAs were described previously [6]. Briefly, immunosorbent plates (Nunc) were coated with purified GUCY2C-6xHis at 10 μg/mL or with irrelevant adenoviral particles at 1×10^7 IFU/ml. Coated plates were incubated with titrated serum collected from immunized mice, and specific antibodies were detected with HRP-conjugated goat anti-mouse immunoglobulin (Jackson Laboratories) and Turbo TMB substrate (Pierce).

$CD4^+$ and $CD8^+$ T-cell depletion

Rat anti-CD4 clone GK1.5 and anti-CD8 clone 2.43 antibodies were purified from hybridoma supernatant, and 300 μg was administered i.p. every 3–4 days for the duration of the experiment. Control mice received 300 μg of rat isotype control IgG (Abcam).

$CD4^+$ T-cell depletion

Ad5-GUCY2C or Ad5-GUCY2C-S1 immunization was administered 3 days after initiating $CD4^+$ T-cell depletion as indicated. Sera and splenocytes were collected 17 days after initiating depletion for ELISA, ELISpot, and FACS analysis.

$CD8^+$ T-cell depletion

Control Ad5 or Ad5-GUCY2C-S1 were administered 3 days after initiating $CD8^+$ T-cell depletion. Mice were challenged i.v. 4 days later with 5×10^5 CT26-GUCY2C cells. Lungs were collected for tumor enumeration and spleens were collected for FACS 17 days later. For both $CD4^+$ and $CD8^+$ cell depletion experiments, lymphocytes were stained in splenocyte samples using anti-CD4 (clone RM4–4), anti-CD8b (clone H35–17.2), and anti-CD19 (clone 1D3), all from BD Biosciences. Full gating strategies for $CD4^+$ and $CD8^+$ cell depletion FACS analysis are shown in Supporting Information Figure 5 and 6, respectively.

Metastatic tumor model

BALB/c-derived CT26 colorectal cancer cells and C57BL/6-derived B16-F10 cells were from ATCC. β-galactosidase-expressing CT26 cells (CL25) were also from ATCC [49]. The GUCY2C-expressing CT26 cell line (CT26-$GUCY2C_{TM}$) and Her2-expressing CT26 cell line (CT26-Her2) were described previously [6, 21]. BALB/c or C57BL/6 mice were immunized 7 days prior to administration of 5×10^5 CT26 or B16-F10 cells, respectively, via tail vein injection to establish lung metastases. Some mice were euthanized and metastases enumerated 17 days after challenge [50]. Survival was measured longitudinally in other mice.

β-gal-release cytotoxic T-cell (CTL) assay

Splenocytes were collected from mice 2 weeks after immunization with Ad5-GUCY2C-S1. Cells were stimulated in upright T25 flasks for 1 week with 10 U/mL recombinant human IL-2 (NCI-Frederick Cancer Research and Development Center, Biological Resources Branch) and 10 μg/mL $GUCY2C_{254–262}$ peptide. Target cells stably expressing β-galactosidase, CT26-CL25 were pulsed with 10 μg/mL $GUCY2C_{254–262}$ or control peptide for 1 hour at 37°C and washed. CT26-CL25 cells expressing GUCY2C were previously described [8]. Effector CTLs (E) were incubated at 37°C with target cells (T) for 4 hours. Released β-galactosidase was measured in the media [51] using the Galacto-Light Plus System (Applied Biosystems). Maximum release was determined from supernatants of cells that were lysed by the addition of supplied lysis buffer. Spontaneous release was determined from target cells incubated without effector cells. The following equation was used to calculate % specific lysis:

$$\%\, specific\ lysis = \left(\frac{experimental\ release - spontaneous\ release}{maximum\ release - spontaneous\ release} \right) \times 100$$

Autoimmune pathology

Tissues from immunized mice were collected, fixed in formalin, and embedded in paraffin. Sections were stained with hematoxylin and eosin and scored for pathology.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism Software v5. All data represent means ± SEM or individual mice with means indicated by a bar.

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Abbreviations: **Ad5**: adenovirus serotype 5 · **GUCY2C**: guanylyl cyclase c · **HA**: hemagglutinin · **Her2**: human epidermal growth factor receptor 2 · **PADRE**: Pan DR epitope · **RV**: rabies virus · **S1**: influenza HA107-119 site 1 epitope · **Trp2**: tyrosinase-related protein 2 · **VV**: vaccinia virus

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Supporting Information

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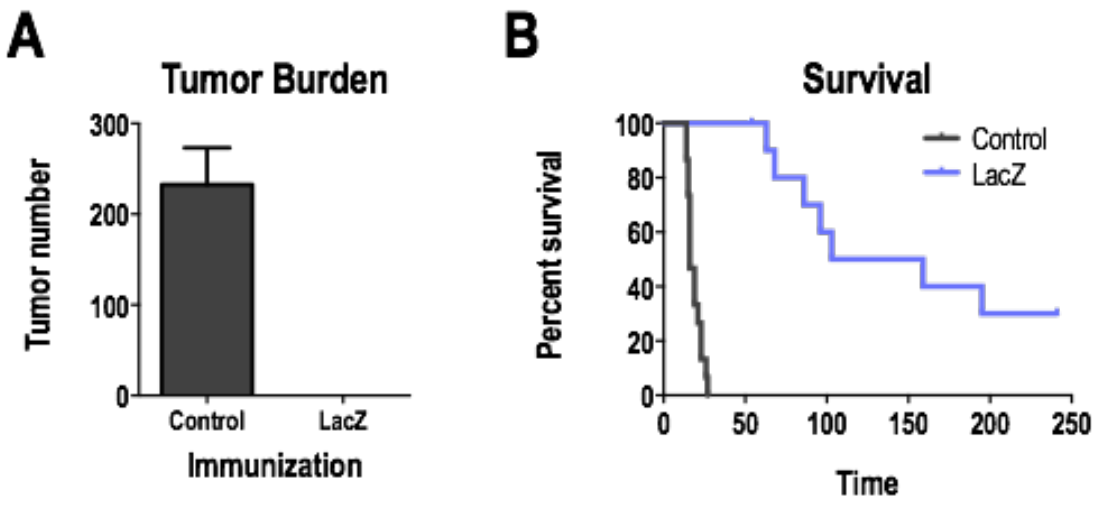
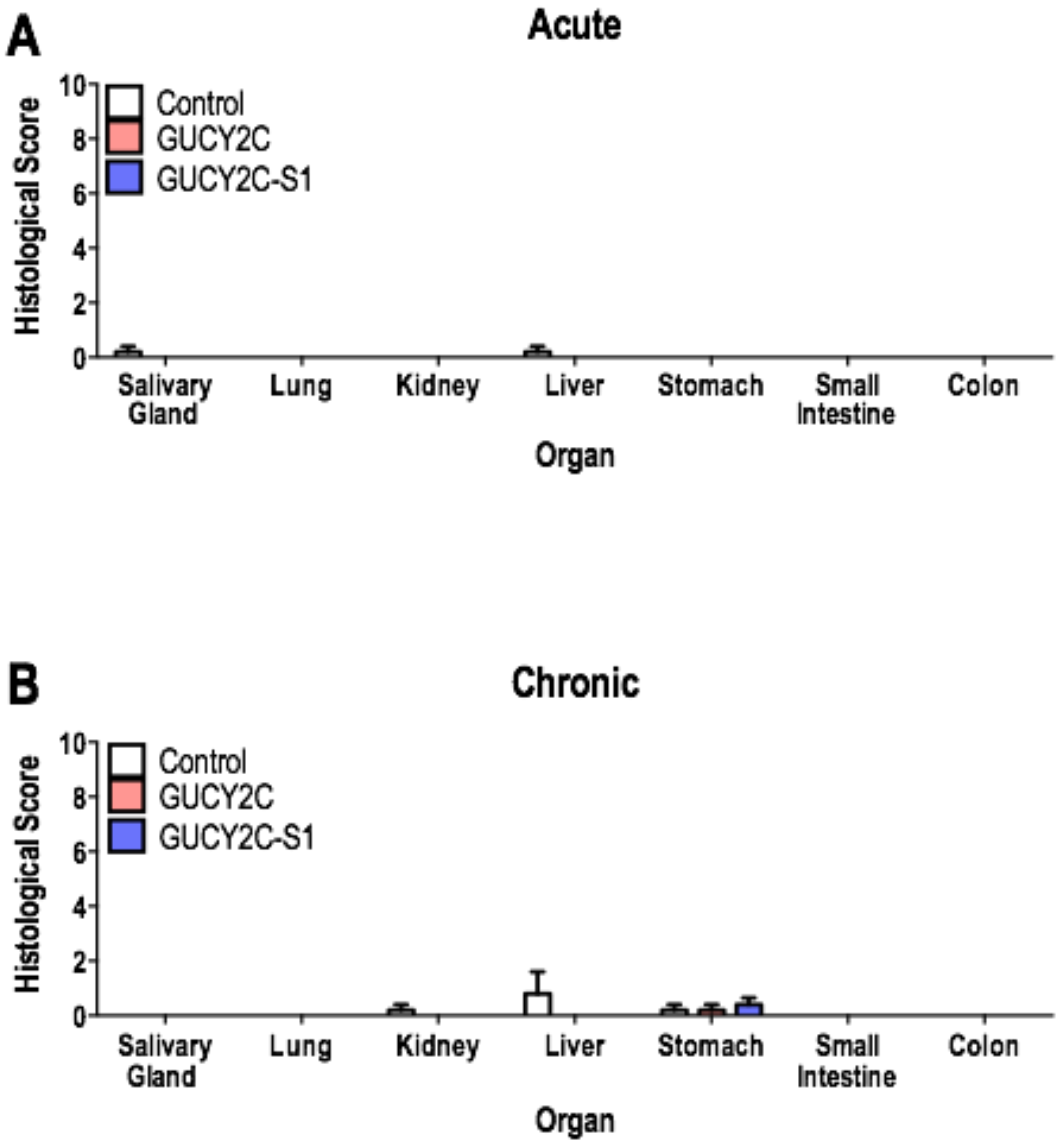
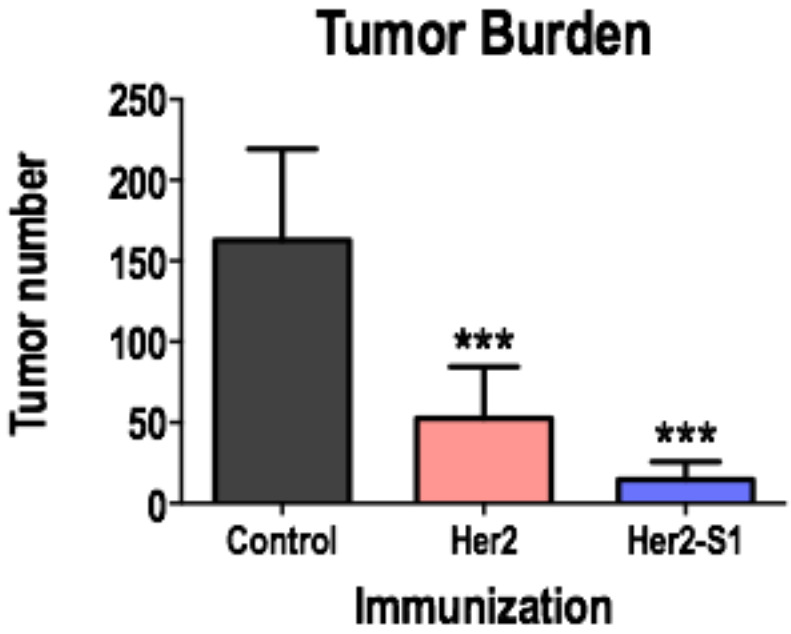
Selective antigen-specific CD4$^+$ T-cell, but not CD8$^+$ T- or B-cell, tolerance corrupts cancer immunotherapy



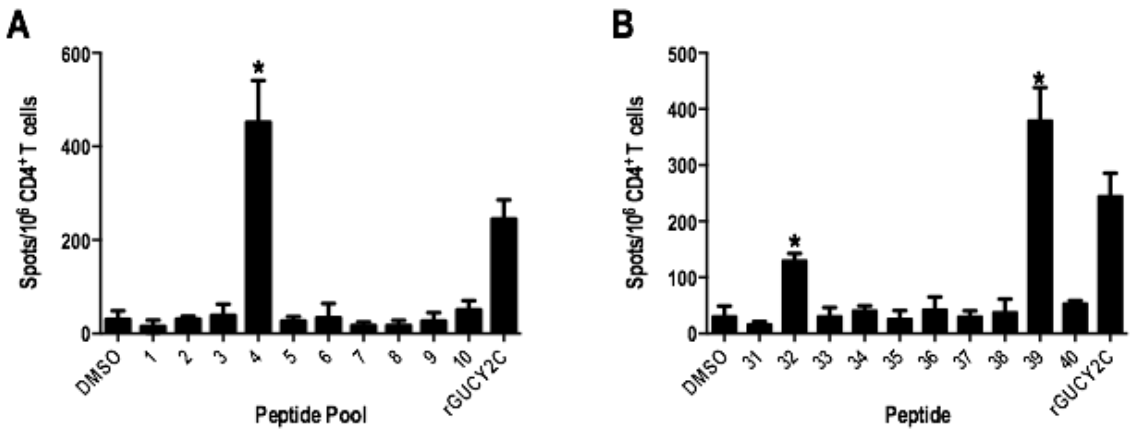
Supporting Information Figure 1. Ad5-LacZ immunization produces antitumor immunity comparable to GUCY2C-S1. Mice were immunized with control Ad5 or Ad5-LacZ and challenged intravenously with $5x10^5$ β-galactosidase-expressing CT26 cells (CL25) 7 days later. (A) Lungs were collected 17 days later from some mice, stained and tumor multiplicity was quantified (N=9-10 mice/group). (B) Survival was measured longitudinally in other mice ($P<0.0001$, Mantel-Cox log-rank test; N=15 mice/group).



Supporting Information Figure 2. GUCY2C-S1 immunization does not produce autoimmunity. Mice were immunized with control Ad5, Ad5-GUCY2C or Ad5-GUCY2C-S1. Tissues were collected 14 days (acute; A) or 13 weeks (chronic; B) later, fixed and sections were stained with H&E. Sections were scored for inflammation (0-4) and tissue damage (0-4). Scores were combined to yield acute (A) and chronic (B) autoimmunity scores. Two-way ANOVA revealed no statistical increase in autoimmunity in GUCY2C or GUCY2C-S1 -immunized mice in any tissue at either time point. N=5 mice/group.



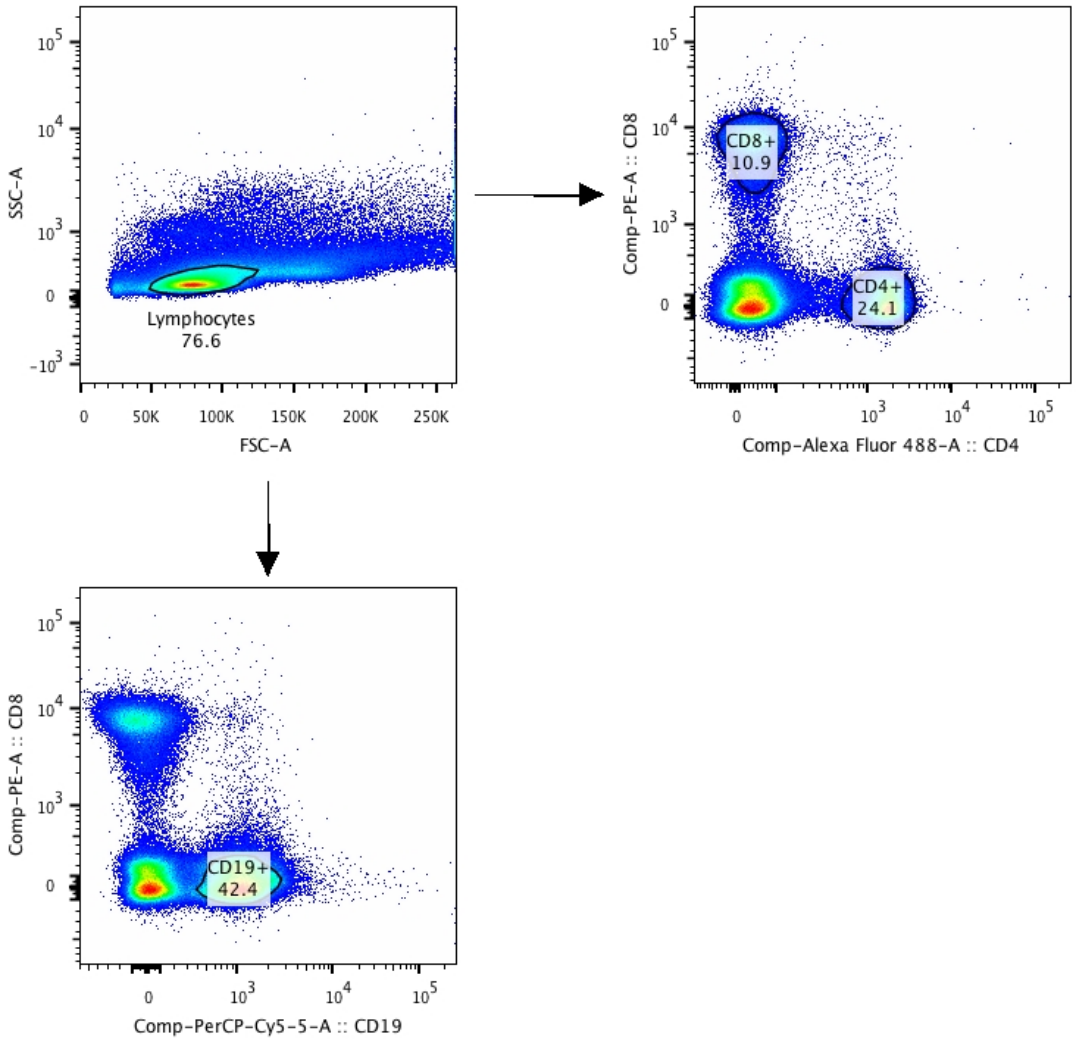
Supporting Information Figure 3. Optimization of Her2-specific antitumor immunity adding an influenza CD4$^+$ T cell epitope to the Ad5-Her2 vaccine. BALB/c mice were immunized with control Ad5, Ad5-Her2 or Ad5-Her2-S1 and challenged intravenously with 5x10^5 CT26-Her2 cells 7 days later. Lungs were collected 17 days later, stained and tumor multiplicity was quantified (*** P<0.001, One-way ANOVA vs. control; N=10 mice/group).



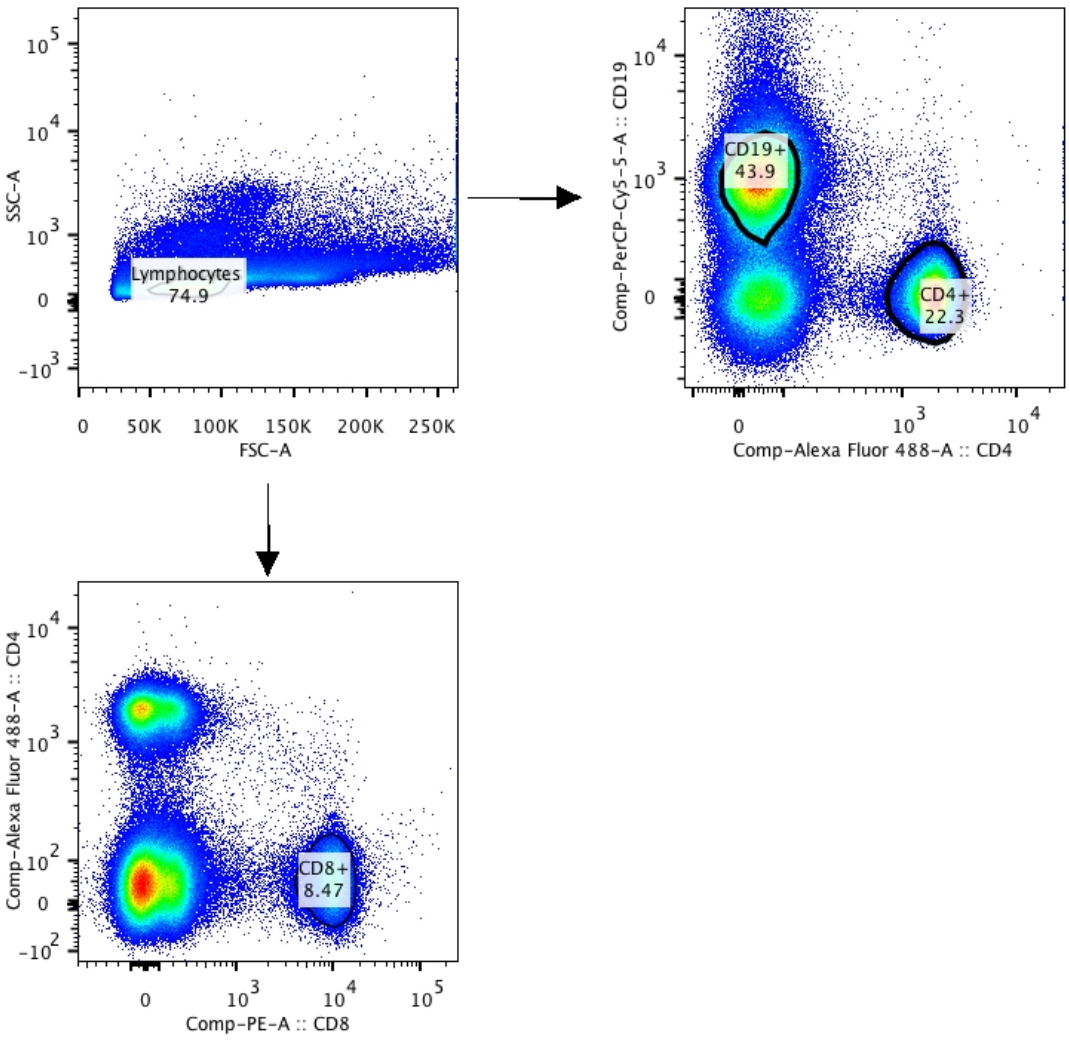
C

Peptide 32 = GUCY2C$_{125-139}$
Peptide 39 = GUCY2C$_{153-167}$

Supporting Information Figure 4. GUCY2C-specific CD4$^+$ T cell epitope mapping. GUCY2C$^{-/-}$ BALB/c mice were immunized with Ad5-GUCY2C, splenocytes were collected 10 days later and CD4$^+$ T cells were purified. (A) purified CD4$^+$ T cells were stimulated with pools of GUCY2C peptides containing 10-11 peptides 15 amino acids in length and overlapping adjacent peptides by 11 amino acids. Naïve splenocytes were added to serve as antigen presenting cells. Pool #4 was the only pool that stimulated GUCY2C-specific CD4$^+$ T cells and it recapitulated responses seen with recombinant GUCY2C protein (rGUCY2C). (B-C) analysis of single peptides within pool #4 revealed two peptides (#32 and #39) possessing GUCY2C-specific CD4$^+$ T cell epitopes. Data are representative of 2 experiments employing pooled splenocytes from 5 immunized mice.



Supporting Information Figure 5. Gating strategy for determining efficacy of $CD4^+$ T cell depletion.



Supplemental Figure 6. Gating strategy for determining efficacy of $CD8^+$ T cell depletion.