<SUPPLEMENTARY INFORMATION FILE>

Prime-Boost Immunization with Chimeric Adenoviral (Ad5.F35) and *Listeria* Vectors is a Safe and Effective Strategy for Cancer Immunotherapy

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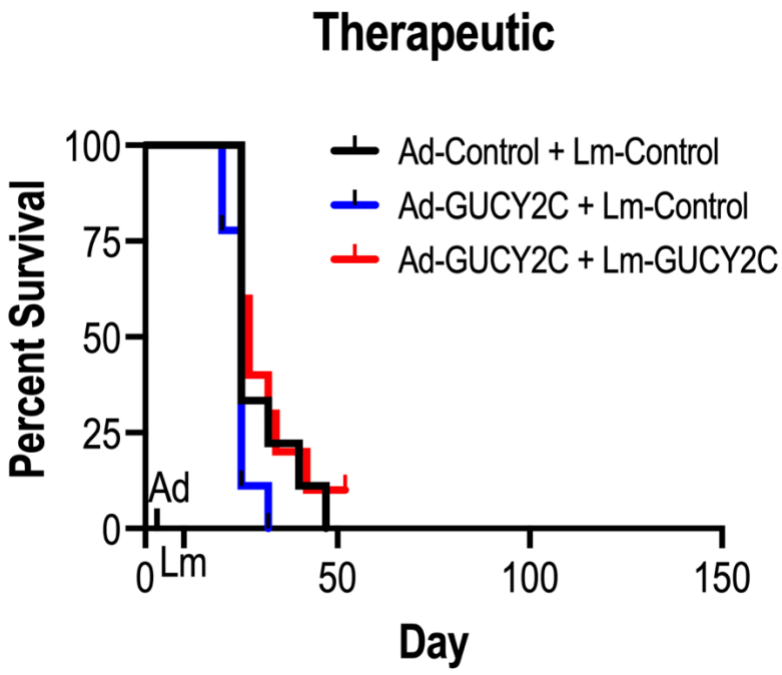
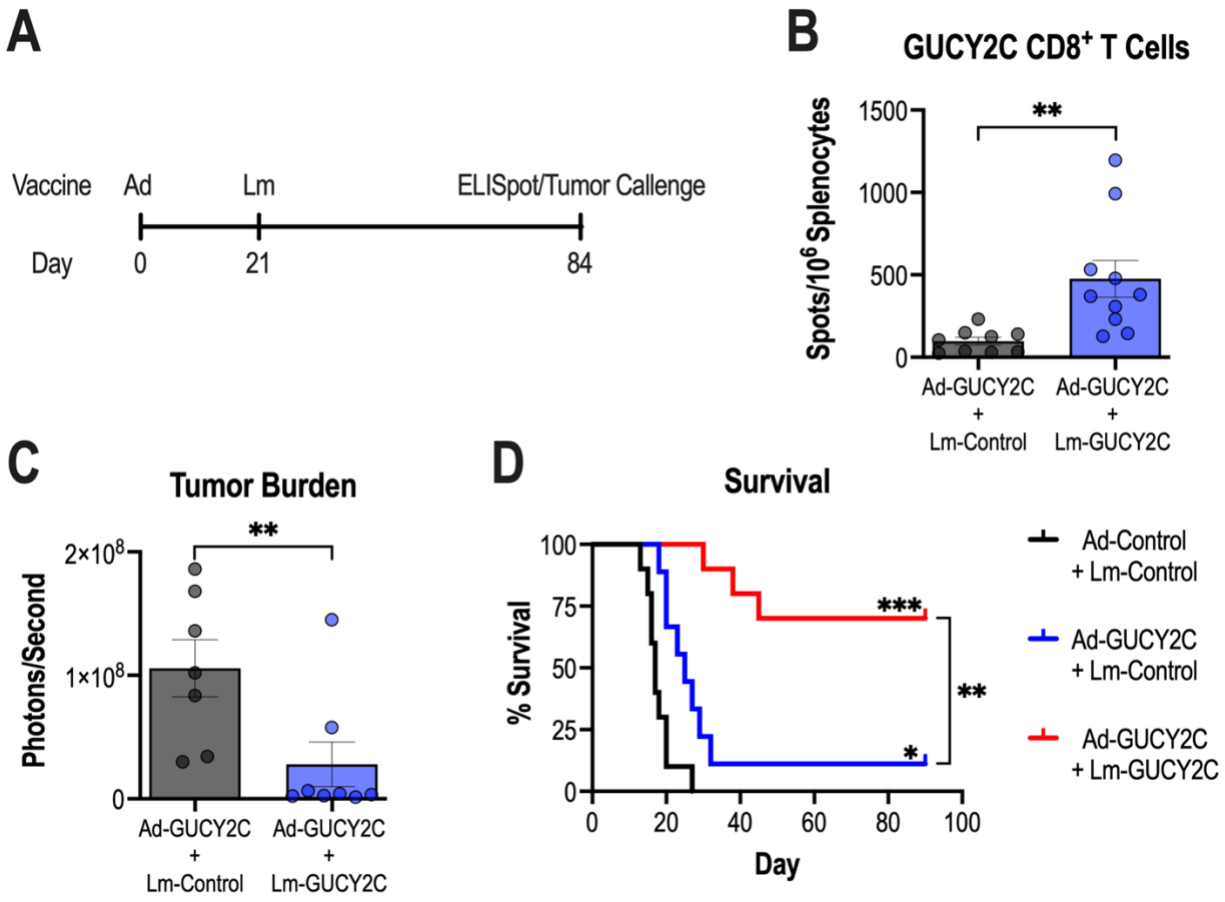
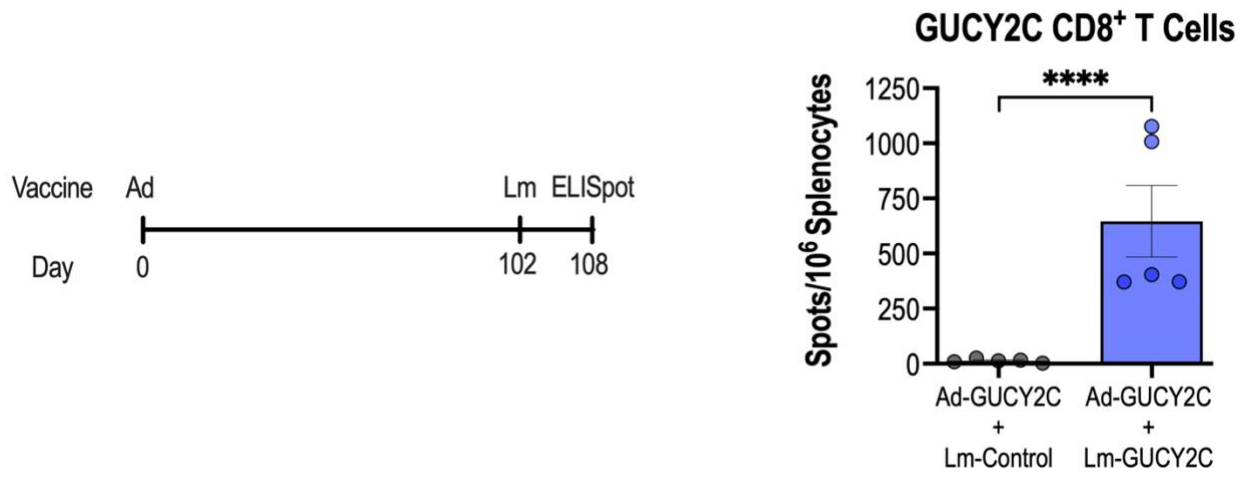
TABLE OF CONTENTS



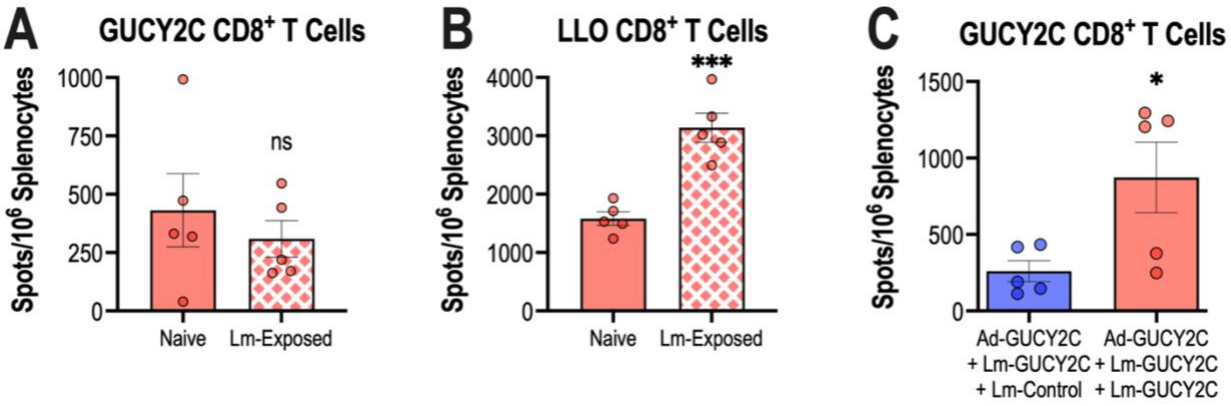
Supplementary Figure 1. Ad-GUCY2C + Lm-GUCY2C is ineffective in a therapeutic model of metastatic colorectal cancer. Metastatic colorectal cancer was established by i.v. administration of $1x10^5$ CT26 colorectal cancer cells to BALB/cJ mice (n = 9-10/group). Mice were immunized as in **Fig. 3**, except an abbreviated scheduled was used: primed on day 3 with Ad5.F35 (Ad) vaccines and boosted 7 days later with Lm vaccines. Survival was monitored throughout the experiment and analyzed by the Mantel-Cox log rank test with all immunized groups compared to control immunization using the Bonferroni method to correct for multiple comparisons. No significant differences were observed.



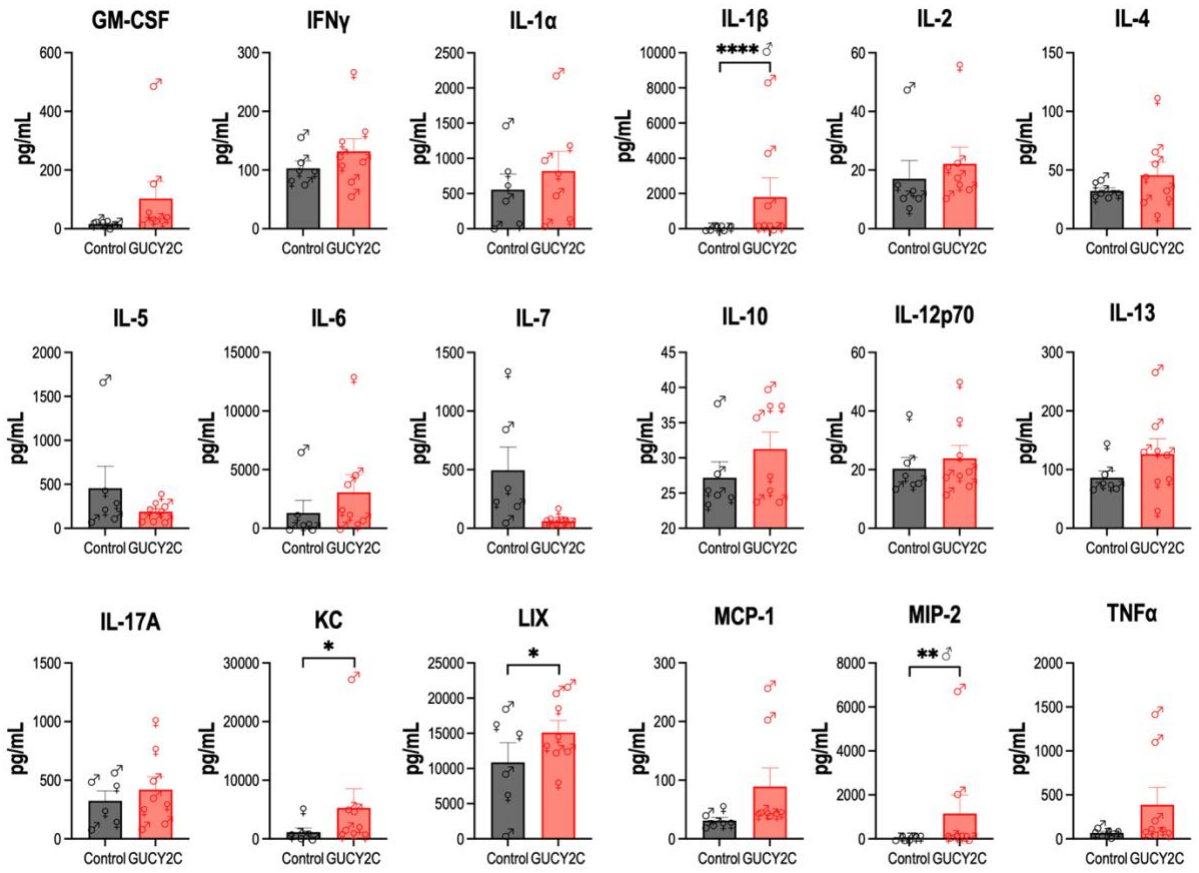
Supplementary Figure 2. Lm-GUCY2C boosting enhances GUCY2C-specific memory CD8$^+$ T-cell responses. A-D BALB/cJ mice were immunized with 10^{10} vp of control or GUCY2C adenovirus (Ad5) on day 0 followed by boosting with $5x10^6$ CFU of control or GUCY2C Lm on day 21. Sixty-three days later, mice were euthanized to quantify GUCY2C-specific CD8$^+$ T-cell responses (**A**) or challenged with $5x10^5$ CT26 colorectal cancer cells expressing GUCY2C and firefly luciferase (**C-D**). Tumor burden was quantified on day 20 (**C**) and survival was monitored throughout the experiment (**D**). GUCY2C-specific CD8$^+$ T-cell counts (**B**) and tumor-burden (**C**) were analyzed by T-test and survival (**D**) was analyzed by the Mantel-Cox log rank test (multiple comparisons were corrected with the Bonferroni method). In **D**, significance compared to control is indicated on the line and compared between GUCY2C vaccine groups as indicated. Error bars indicate mean +/- SEM.



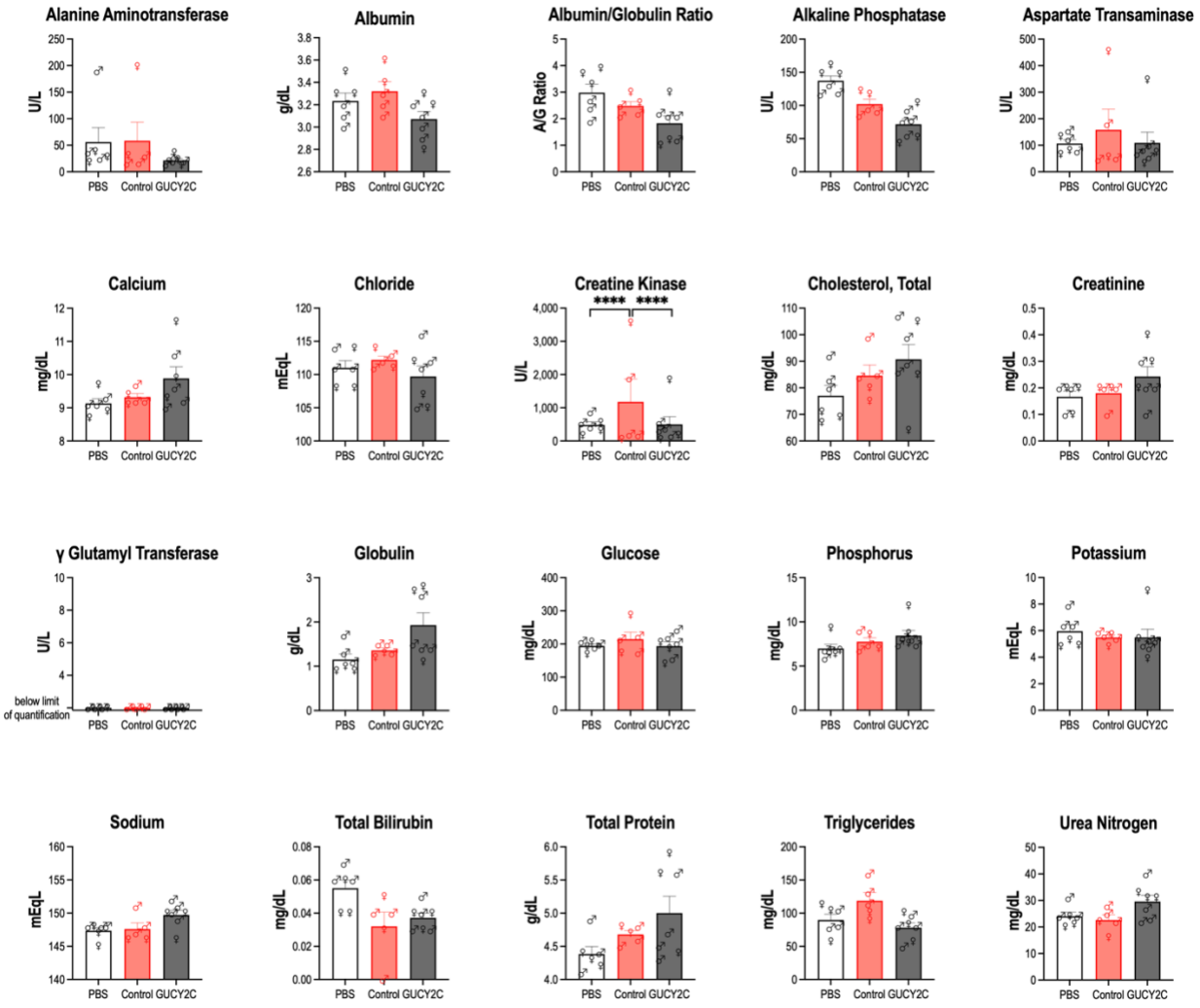
Supplementary Figure 3. Lm-GUCY2C boosts GUCY2C-specific CD8$^+$ T-cells generated long after initial priming vaccination. BALB/cJ mice were immunized intramuscularly with 10^{10} vp of Ad-GUCY2C, boosted 102 days later with $5x10^6$ CFU of control or GUCY2C Lm intravenously. Six days later, GUCY2C-specific CD8$^+$ T-cells counts were quantified by IFNγ-ELISpot. GUCY2C-specific CD8$^+$ T-cell counts were analyzed by T-test. Error bars indicate mean +/- SEM.



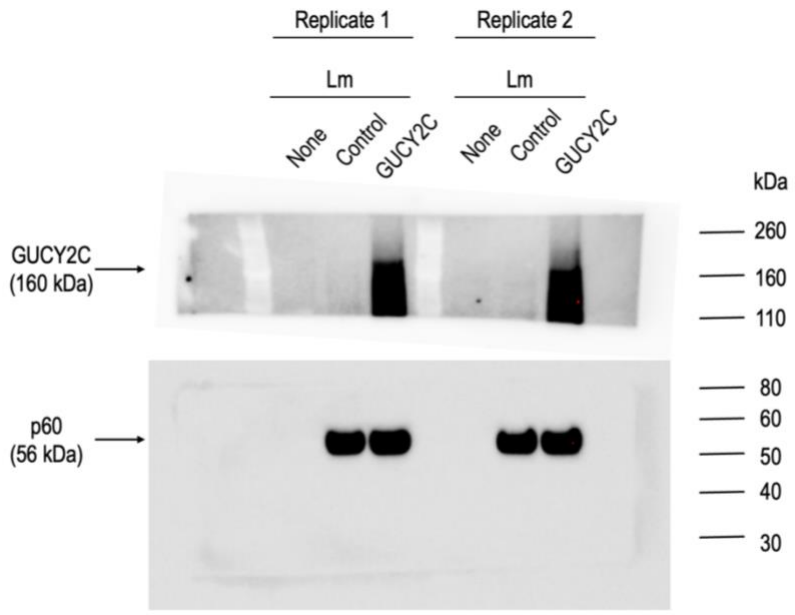
Supplementary Figure 4. Prior Lm exposure does not reduce GUCY2C-specific CD8$^+$ T-cell expansion by Lm-GUCY2C boosting. A-B BALB/cJ mice were exposed to control Lm by intravenous administration of 5x10^6 CFU or left naïve (vehicle exposure) on day 0. Mice were immunized on day 14 with 10^{10} vp of Ad-GUCY2C intramuscularly and boosted on day 35 with 5x10^6 CFU of Lm-GUCY2C intravenously. Six days later, mice were euthanized and GUCY2C-specific (**A**) and LLO-specific (**B**) CD8$^+$ T-cell counts were quantified by IFNγ-ELISpot. **C** BALB/cJ mice were immunized intramuscularly with 10^{10} vp of Ad-GUCY2C on day 0, boosted with 5x10^6 CFU of Lm-GUCY2C on day 21, and on day 42, mice were boosted a second time with 5x10^6 CFU of control or GUCY2C Lm. GUCY2C-specific CD8$^+$ T-cells were quantified by IFNγ-ELISpot 6 days after final immunization. T-cell counts were analyzed by T-test. Error bars indicate mean +/- SEM.



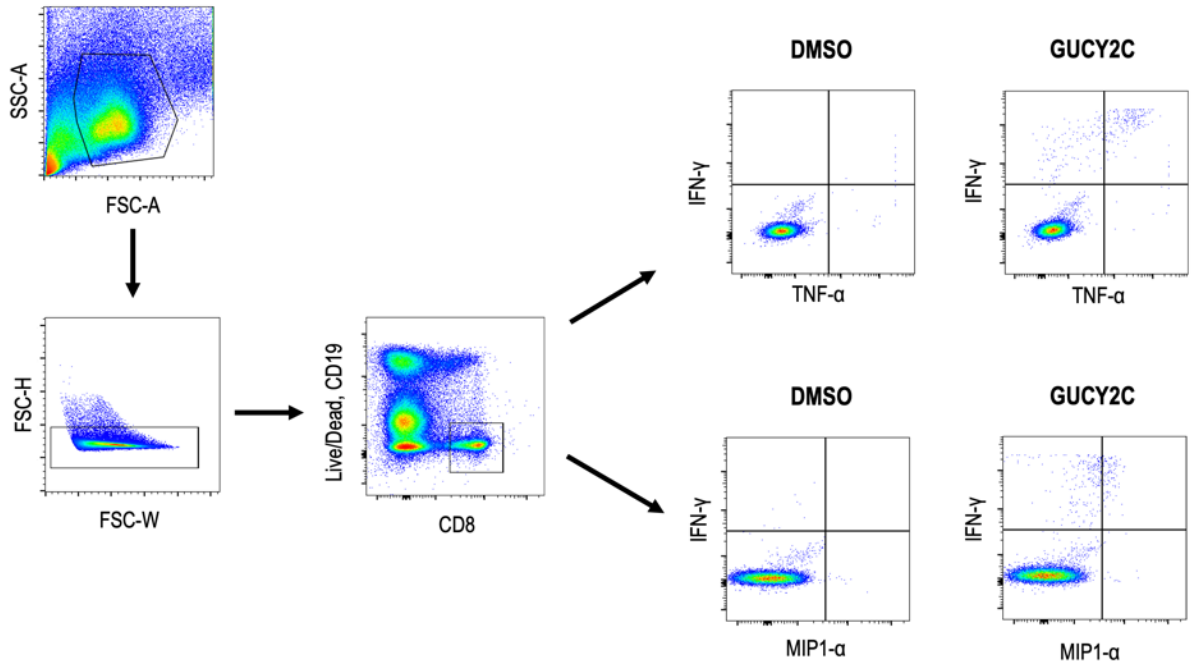
Supplementary Figure 5. Blood cytokine responses to Ad-GUCY2C + Lm-GUCY2C immunization. BALB/cJ mice were primed with 10^{10} vp of control or GUCY2C Ad5.F35 i.m. and boosted with $5x10^6$ CFU of control or GUCY2C Lm i.v. at a 21-day interval. Plasma was collected 6 days later, and cytokines were quantified (Charles River). Statistics indicate comparisons by two-way ANOVA, with both sexes analyzed together. IL1-β and MIP-2 were significantly different only in males. Symbols indicate male (♂) and female (♀) mice. Error bars indicate mean +/- SEM.



Supplementary Figure 6. Clinical chemistry profiles following Ad-GUCY2C + Lm-GUCY2C immunization. BALB/cJ mice received only PBS or were primed with 10^{10} vp of control or GUCY2C Ad5.F35 i.m. and boosted with $5x10^{6}$ CFU of control or GUCY2C Lm i.v. at a 21-day interval. Plasma was collected 6 days later, and analytes were quantified (Eve Technologies). Statistics indicate comparisons by two-way ANOVA, with both sexes analyzed together. Symbols indicate male (♂) and female (♀) mice. Error bars indicate mean +/- SEM.



Supplementary Figure 7. Uncropped western blot images. Uncropped western blot images of replicate samples pertaining to cropped images shown in **Fig. 1C**, as indicated above.



Supplementary Figure 8. Representative intracellular cytokine staining for polyfunctionality analysis. Lymphocytes were gated based on SSC-A and FSC-A profiles with doublets excluded based on FSC-H vs. FSC-W profiles. Dead cells and $CD19^+$ B cells were excluded by staining with LIVE/DEAD Aqua Blue and anti-CD19-BV510 antibody. Live/$CD19^-CD8^+$ cells were gated and assessed for IFNγ, TNFα, and/or MIP1α staining using FlowJo Boolean gates ($IFN\gamma^+TNF\alpha^-MIP1a^-$, $IFN\gamma^+TNF\alpha^+MIP1a^-$, etc). A representative immunized mouse stimulated with DMSO or $GUCY2C_{254-262}$ peptide is shown, demonstrating specific detection of GUCY2C-reactive $CD8^+$ T cells. Single, double, and triple cytokine population frequencies are depicted in **Fig. 4B-D**.