SUPPLEMENTAL MATERIAL

Radiotherapy-exposed CD8$^+$ and CD4$^+$ neoantigens enhance tumor control

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SUPPLEMENTAL METHODS

Liquid Chromatography Mass Spectrometry (LC-MS) Analysis

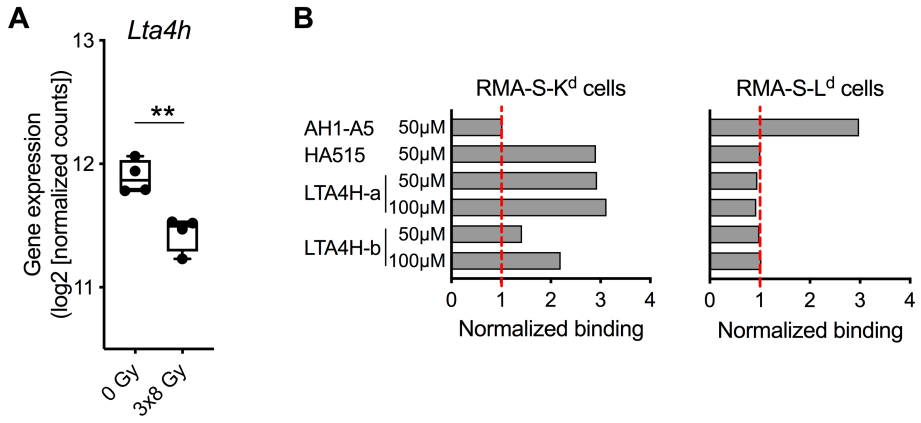
Peptidome samples were fractionated through a Thermo Scientific™ EASY-nLC 1000™ HPLC system with analytical nanoflow column system including a 2 cm trap column and a 75µm x 20 cm analytical column both packed with SuperB C18 HIFI, 5µm, 100Å (Omics Technologies Inc., Maryland). Eluted samples were ionized through Thermo Scientific EASYSpray ™ source analyzed on a Thermo Scientific™ Q Exactive HF-X Mass Spectrometer using FT HCD MS2 fragmentation mode with Orbitrap mass analyzer operated at 45,000 mass resolution. Peptides were electrosprayed through a 15µm emitter (Omics Technologies Inc., Maryland) at a voltage of 2.0kV spray voltage. Reversed-phase solvent gradient consisted of 0.1% formic acid with increasing levels of 90% acetonitrile in 0.1% formic acid over a period of 130 minutes. The Q Exactive HF-X instrument was operated to automatically switch between full scan MS and MS/MS acquisition. Survey full scan MS spectra (m/z 350−1800) was acquired in the Orbitrap with 45,000 resolution after accumulation of ions to a target value based on predictive AGC from the previous full scan. Intense multiply-charged ions ($z \geq 2$) were sequentially isolated and fragmented in the Axial Higher energy Collision-induced Dissociation (HCD) cell using normalized HCD collision energy at 30% with an AGC target 1e5 and a maximal injection time of 400 ms at 45,000 resolution.

Mass spectrometry raw files were automatically processed through MyProt-ID pipeline (Omics Technologies Inc., Maryland) developed for Peptide Identification services. Search parameter included oxidation on methionine, deamidation on residues N and Q as different variable modifications, and heavy isotope labeled amino acids. Mass tolerances on precursor and fragment masses were set to 15 ppm and 0.03 Da, respectively. Peptide validator node was used for peptide validation with stringent cutoff of 0.01 and relaxed cutoff of 0.005 (False Discover Rate).

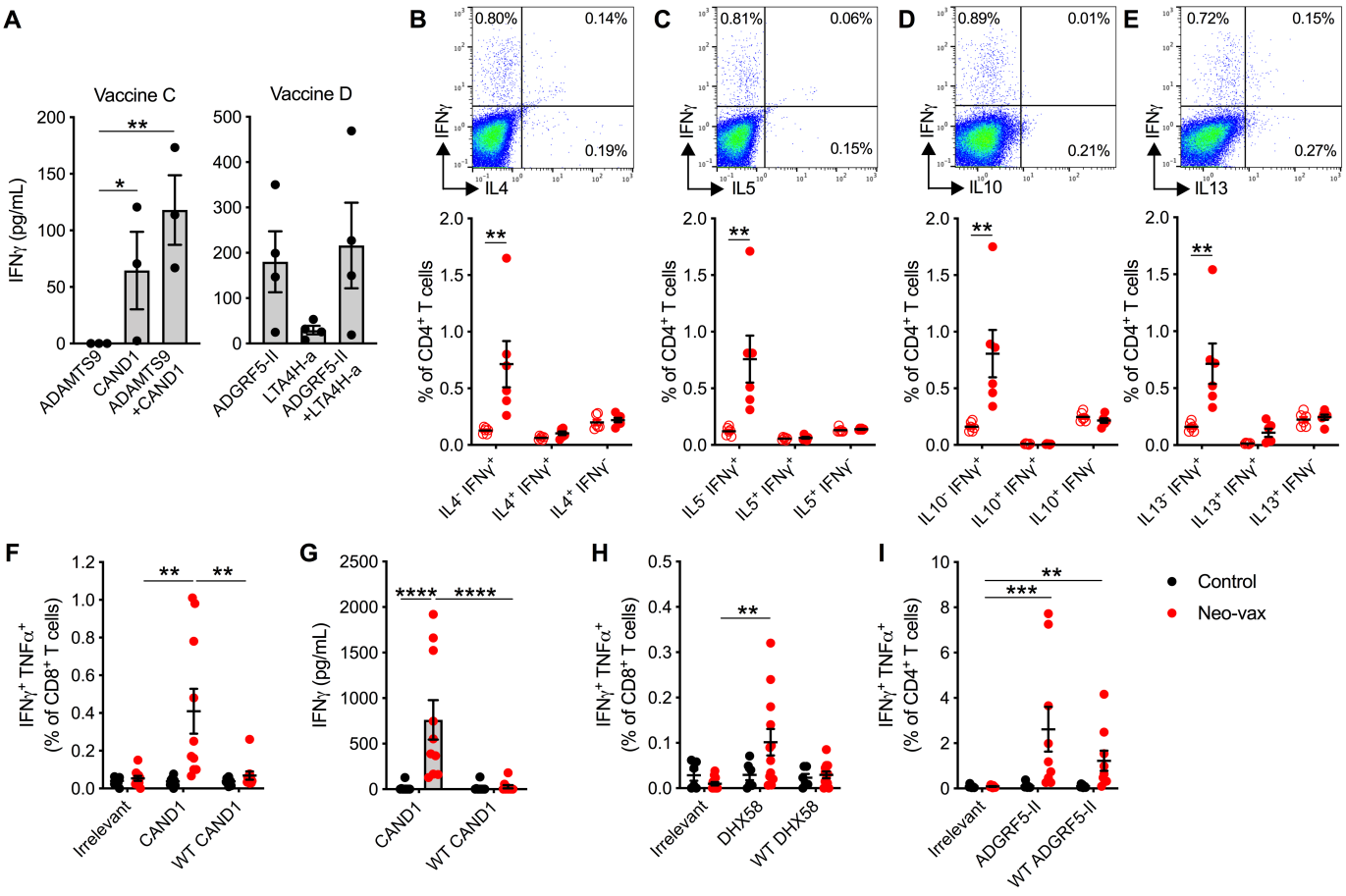
Flow cytometry analysis of MHC-II expression

4T1 cells were irradiated with 8 Gy radiation dose on three consecutive days (3x8 Gy), or were treated with IFNγ (2 ng/mL, R&D systems) for 24h, or left untreated. Cells were harvested and stained with the Fixable Viability Dye (eFluor® 450, eBioscience) followed by staining with Alexa Fluor® 700-conjugated MHC-II (IA/IE) (clone M5/114, eBioscience). Samples were acquired on the MACSQuant (Miltenyi) flow cytometer and data were analyzed using FlowJo v.10 software.

SUPPLEMENTAL FIGURES

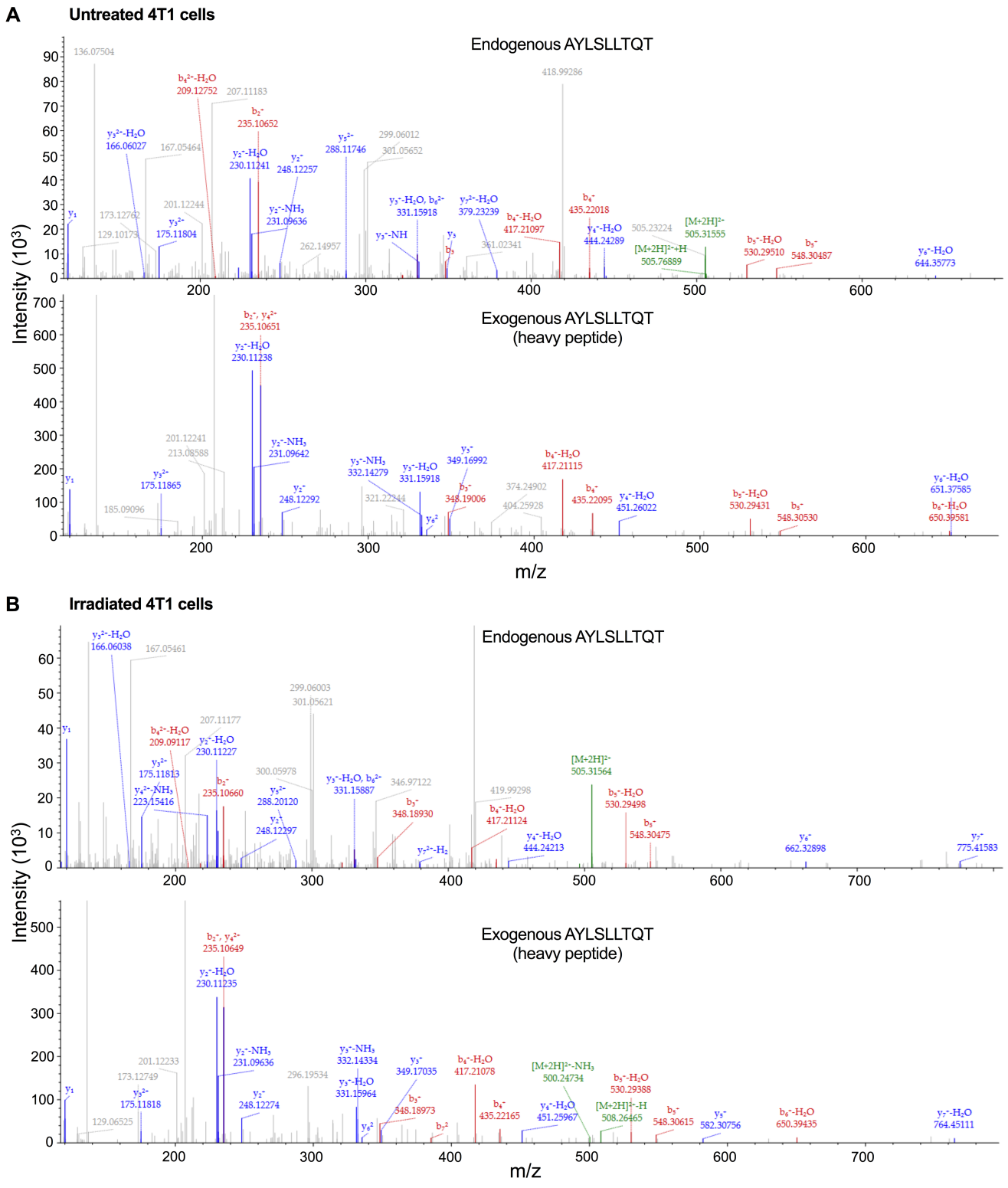


Supplemental Figure 1. MHC-I binding of neoepitopes arising from *Lta4h* gene. (**A**) *Lta4* gene expression determined by RNA sequencing of 4T1 cells irradiated (3x8 Gy) or untreated (0 Gy) in vitro (n=4 independent experiments). ***p*<0.01, with unpaired two-tailed Welch's t-test. Data are expressed as mean ± SEM. (**B**) In vitro MHC-I binding assay. RMA-S cells expressing either H2-K^d (left panel) or H2-L^d (right panel) were incubated with candidate peptides (50 or 100 μM) for 2h and then tested for MHC-I expression by flow cytometry. The binding capability of these peptides to MHC-I was calculated by normalizing the mean fluorescence intensity (MFI) of H2-K^d or H2-L^d in presence of peptides compared to the MFI in absence of peptides. HA515 peptide was used as positive control for binding to H2-K^d, whereas AH1-A5 was used as positive control for binding to H2-L^d.

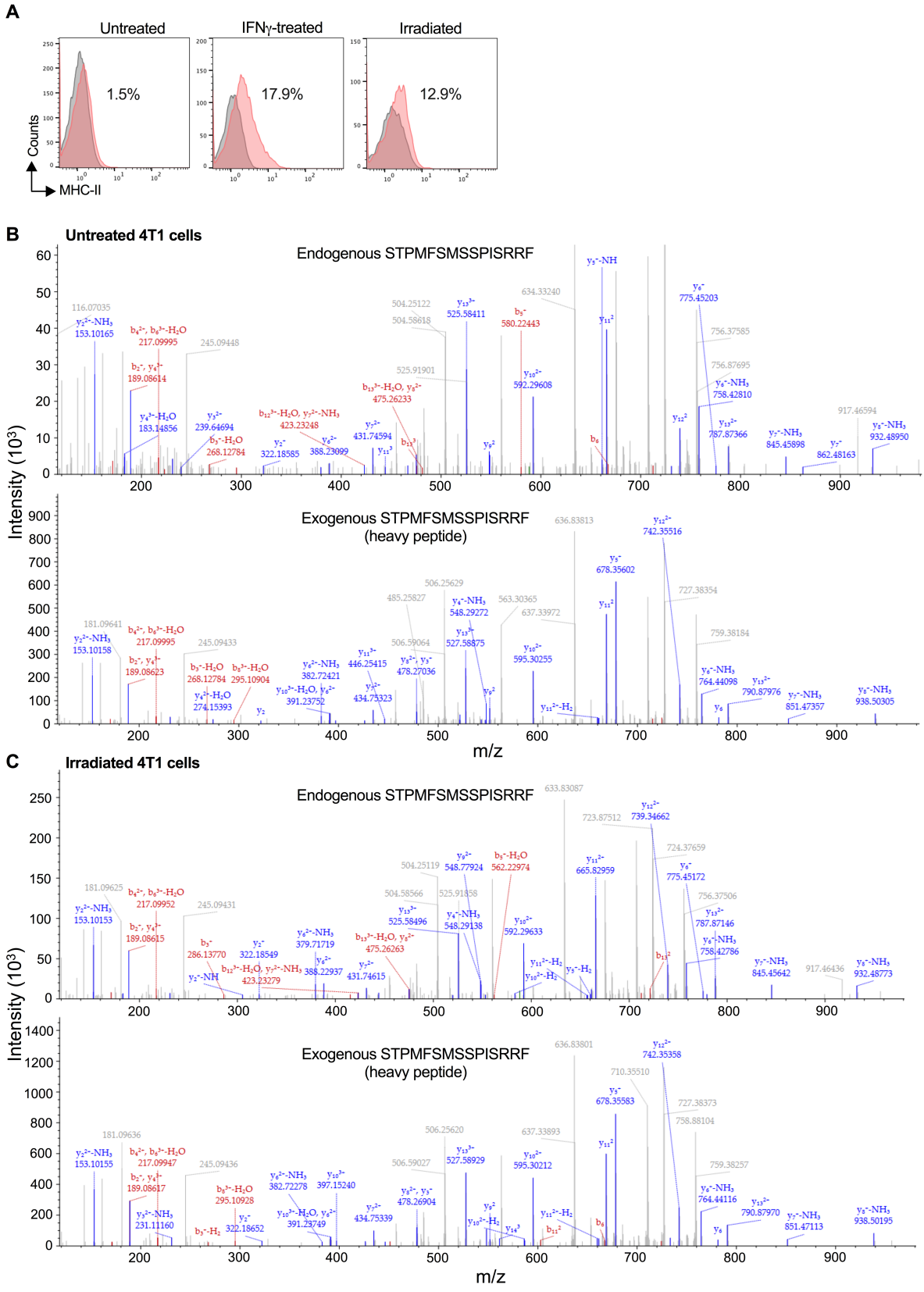


Supplemental Figure 2. CAND1, DHX58 and ADGRF5-II neoepitope-specific T cells produce IFNγ and TNFα. (**A**) Mice were vaccinated twice with a combination of two neoepitopes, as indicated in Figure 2A. One week after the second vaccination, vaccine-draining lymph node cells were stimulated ex vivo with indicated peptides for 48h and secreted IFNγ was measured in the cell supernatant. Background values obtained after restimulation with AH1-A5 (used as negative control) were subtracted. Data are expressed as mean ± SEM. *p<0.05, **p<0.01, with ordinary one-way ANOVA and Tukey's multiple comparisons test after log transformation of the data. (**B-E**) Splenocytes isolated from mice vaccinated twice with Neo-vax (DHX58, CAND1 and ADGRF5-II) were cultured for 5 days with ADGRF5-II, harvested and tested for cytokine production by intracellular staining for IL4 (**B**), IL5 (**C**), IL10 (**D**) and IL13 (**E**) and IFNγ after overnight restimulation with ADGRF5-II (filled red circles) or no peptide (empty circles). Representative flow cytometry plots (top panels) and associated percentages (bottom panels) are shown after gating on $CD4^+$ T cells (n=6). Comparisons between unstimulated and stimulated cells were done using unpaired two-tailed Welch's t-test; **p<0.01. (**F-I**) Vaccine-draining lymph node cells and/or splenocytes from mice vaccinated twice with Neo-vax or with adjuvant alone (control) were stimulated ex vivo with indicated peptides. $H2K^d$-binding HA515 or $H2L^d$-binding pMCMV were used as irrelevant peptides as appropriate. (**F**) Intracellular staining for IFNγ and TNFα of lymph node $CD8^+$ T cells stimulated with irrelevant peptide, CAND1 or WT CAND1. Data are expressed as mean ± SEM of two combined independent experiments (n=10 per group). **p<0.01, with Kruskall-Wallis and Dunn's multiple comparisons test. (**G**) Secreted IFNγ was measured in the supernatant of cells from (F) (n=10 per group). Background values obtained after stimulation with HA515 were subtracted. Data are expressed as mean ± SEM. Mann-Whitney test was used to compare the reactivity towards CAND1 and WT CAND1 within the Neo-vax group and to compare the reactivity towards CAND1 between the control (adjuvant alone) and Neo-vax-treated groups; ****p<0.0001. (**H**) Intracellular staining for IFNγ and

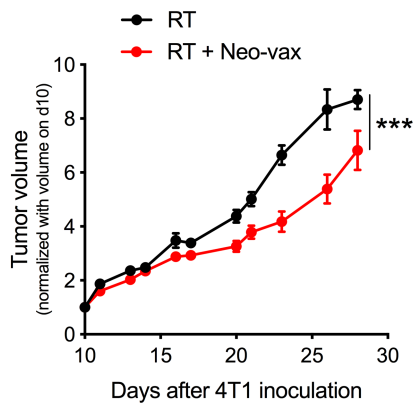
TNFα of lymph node $CD8^+$ T cells stimulated with irrelevant peptide, DHX58 or WT DHX58. Data are expressed as mean ± SEM of two combined independent experiments (n=6-12 per group). $^{**}p<0.01$, with Kruskall-Wallis and Dunn's multiple comparisons test. (**I**) Intracellular staining for IFNγ and TNFα of splenic $CD4^+$ T cells stimulated with irrelevant peptide, ADGRF-II or WT ADGRF5-II. Data are expressed as mean ± SEM of two combined independent experiments (n=10 per group). $^{**}p<0.01$, $^{***}p<0.001$ with Kruskall-Wallis and Dunn's multiple comparisons test.



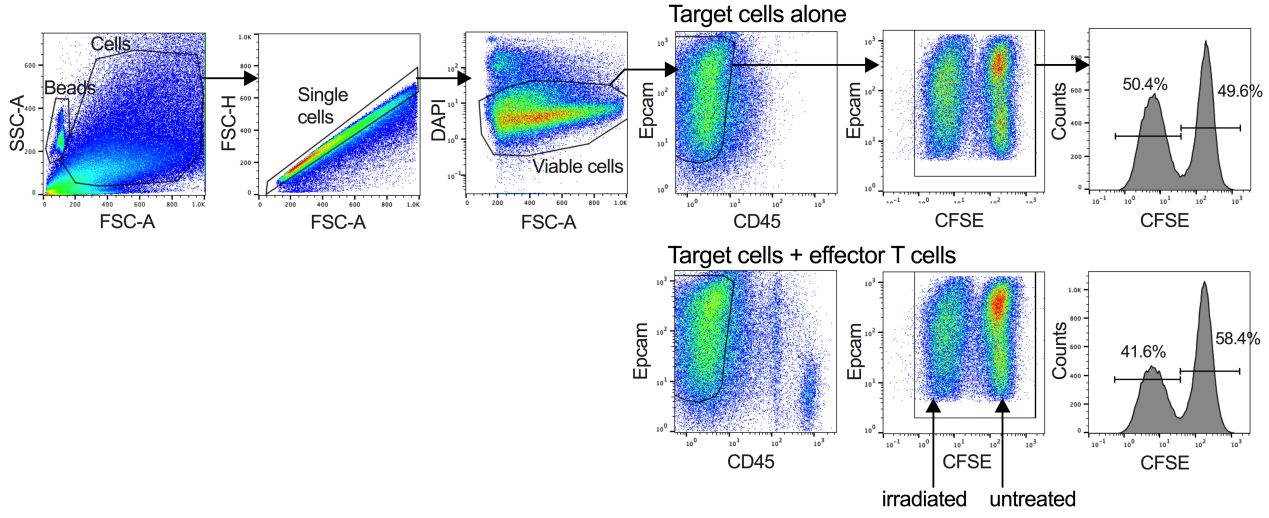
Supplemental Figure 3. Identification of the CAND1 neoepitope at the surface of irradiated 4T1 cells. MS/MS spectra of the CAND1 neopitope detected in untreated (**A**) or irradiated (**B**) 4T1 cells. Predicted fragment ions are shown in Supplemental Table 2.



Supplemental Figure 4. MHC-II expression and identification of the ADGRF5-II neoepitope at the surface of irradiated 4T1 cells. (**A**) MHC-II expression on untreated, IFNγ-treated and irradiated (3x8 Gy) 4T1 cells, analyzed by flow cytometry. Grey histograms represent unstained samples. Percentage of MHC-II^{+} cells are calculated by subtracting the background from unstained cells. (**B-C**) MS/MS spectra of the ADGRF5-II neopitope detected in untreated (**B**) or irradiated (**C**) 4T1 cells. Predicted fragment ions are shown in Supplemental Table 2.



Supplemental Figure 5. Tumor growth control induced by Neo-vax in combination with radiotherapy. Mice were vaccinated and treated as described in Figure 5A. Data are pooled from 4 independent experiments (n=32-33 per group). Statistically significant effect of Neo-vax in combination with RT was assessed by mixed-effects model; ***p<0.001.



Supplemental Figure 6. Flow cytometry gating strategy for in vitro killing assay of 4T1 tumor cells. After gating on single cells, DAPI-negative cells were selected, and the gating was performed on Epcam^{+} CD45^{-} cells. After further gating on the CFSE^{+} population, proportions of CFSE^{low} and CFSE^{high} cells can be calculated. Gating was also performed on counting beads in order to get the absolute counts.