Using CD69 PET Imaging to Monitor Immunotherapy-Induced Immune Activation

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SUPPLEMENTARY FIGURES S1 - S4

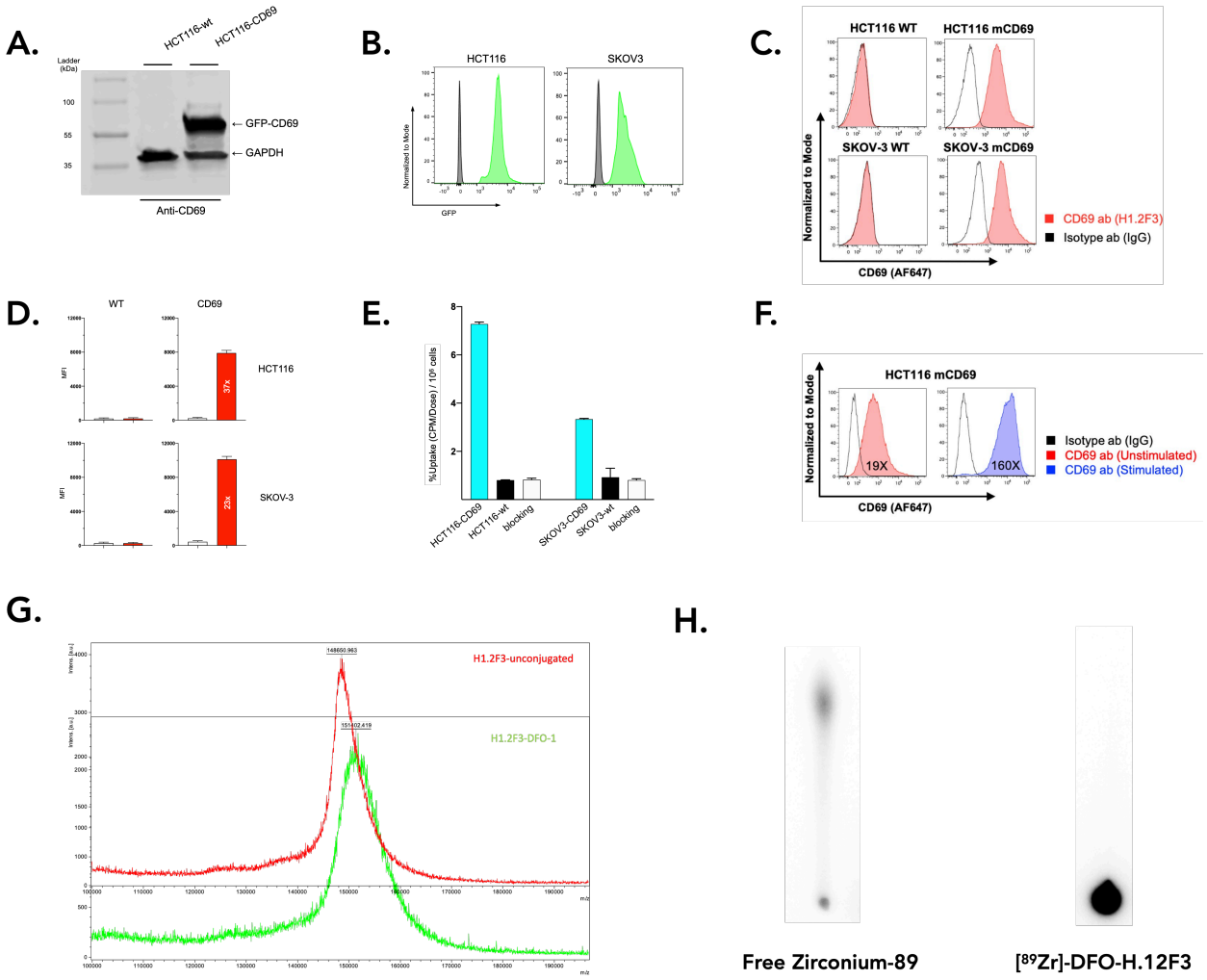


Figure S1. Validating CD69 expression and the specificity of H1.2F3, an Armenian anti-CD69 monoclonal antibody, using mCD69-transduced human cancer cell lines, *in vitro*. **(A)** Western blot *in vitro* validation of CD69-GFP fusion protein expression in HCT116-CD69-GFP. **(B)** Flow cytometry validation of CD69-GFP fusion protein expression in HCT116-CD69-GFP transduced cell line. HCT116-WT was used as a negative control. **(C)** Flow cytometry of validation of CD69-GFP surface expression using surface labelling with an AF647-conjugated H1.2F3 monoclonal antibody. An isotype control antibody was used as a negative control. **(D)** Mean fluorescence intensity (MFI), n = 3, of CD69-GFP surface expression for HCT116-CD69 and SKOV-3-CD69 cells using surface labelling with an AF647-conjugated H1.2F3 monoclonal antibody. Error bars represent standard deviation (SD). **(E)** [^{89}Zr]-DFO-H1.2F3 *in vitro* uptake study for CD69-transduced cells, WT cells, and blocked controls. Uptake was measured on gamma counter and normalized to percent injected dose per million cells (%ID/10^6 cells). **(F)** Flow cytometry analysis of CD69 expression for PMA/Ionomycin treated HCT116-CD69 cells. **(G)** Representative Matrix-assisted laser desorption/ionization-time of flight **(**MALDI-TOF) analysis of DFO-conjugated H1.2F3 antibody to determine the degree of labeling (DOL). **(H)** Representative radio thin layer chromatograph (Radio-TLC) showing final radiotracer product after purification.

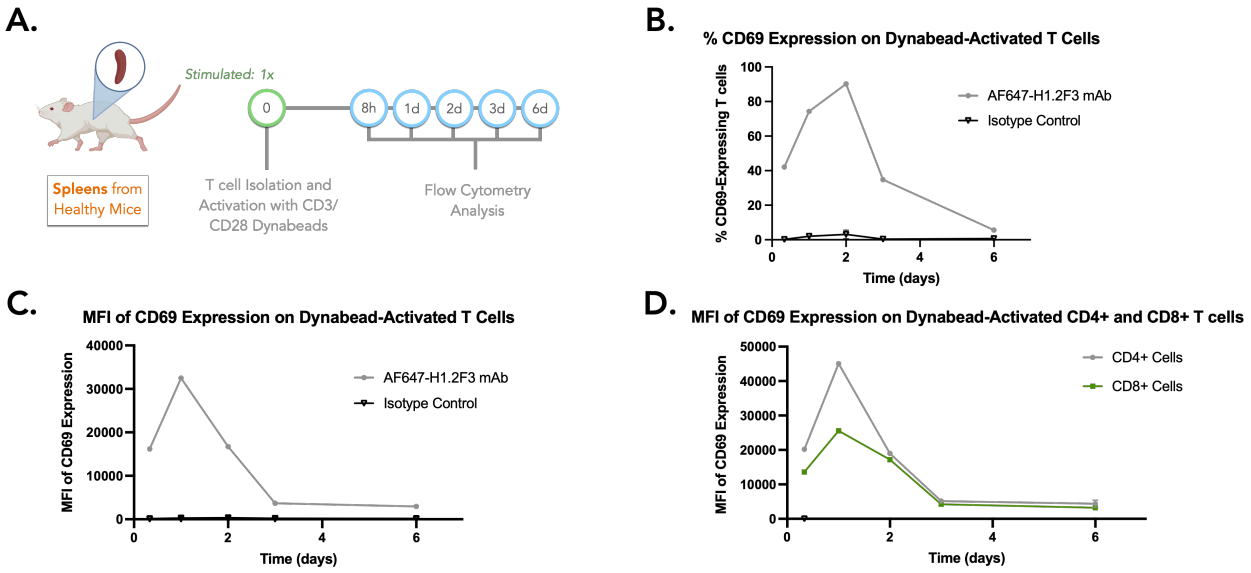


Figure S2. Assessing CD69 expression in primary mouse T cells following activation with CD3/CD28 Dynabeads.
(A) Study design for quantifying activation of primary mouse T cells that have been stimulated with CD3/CD28 Dynabeads for 2 hours starting on Day 0. **(B)** Percent (%) CD69 expression on primary mouse T cells. **(C)** Mean Fluorescence Intensity (MFI) of CD69-positive T cells **(D)** MFI of CD69 expression from CD4- and CD8-positive cells. Error bars represent standard deviation (SD).

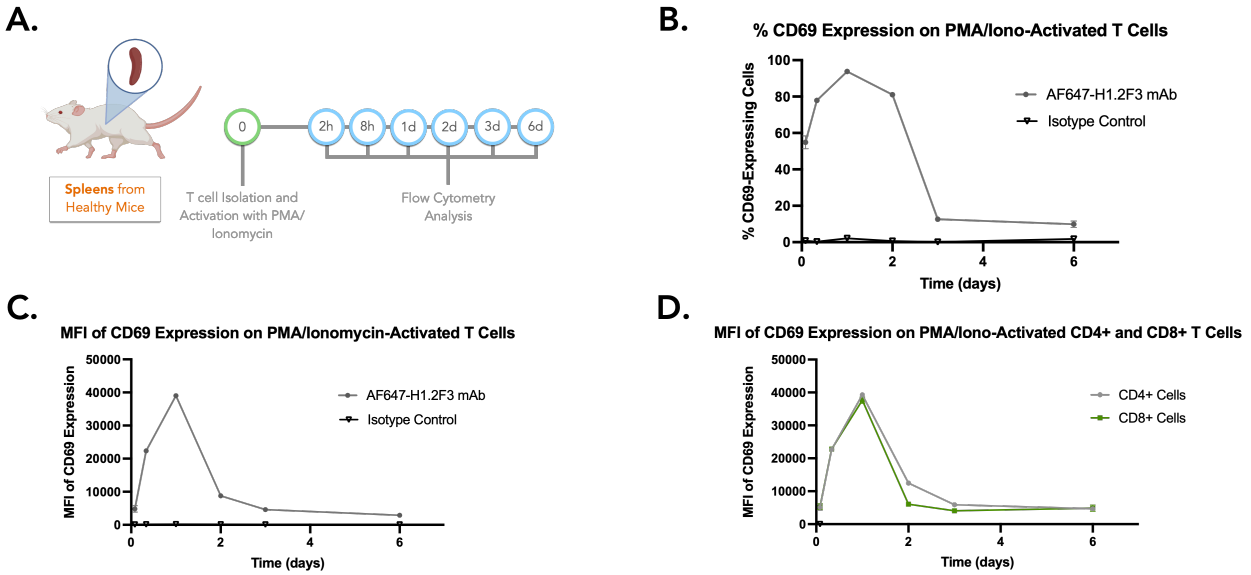


Figure S3. Assessing CD69 expression in primary mouse T cells following activation with PMA/Ionomycin.

(A) Study design for quantifying activation of primary mouse T cells that have been stimulated with PMA/Ionomycin (50 ng/mL PMA and 1 µg/mL Ionomycin) for 2 hours on Day 0. **(B)** Percent (%) CD69 expression on primary mouse T cells. **(C)** Mean Fluorescence Intensity (MFI) of CD69-positive T cells **(D)** MFI of CD69 expression from CD4- and CD8-positive cells. Error bars represent standard deviation (SD).

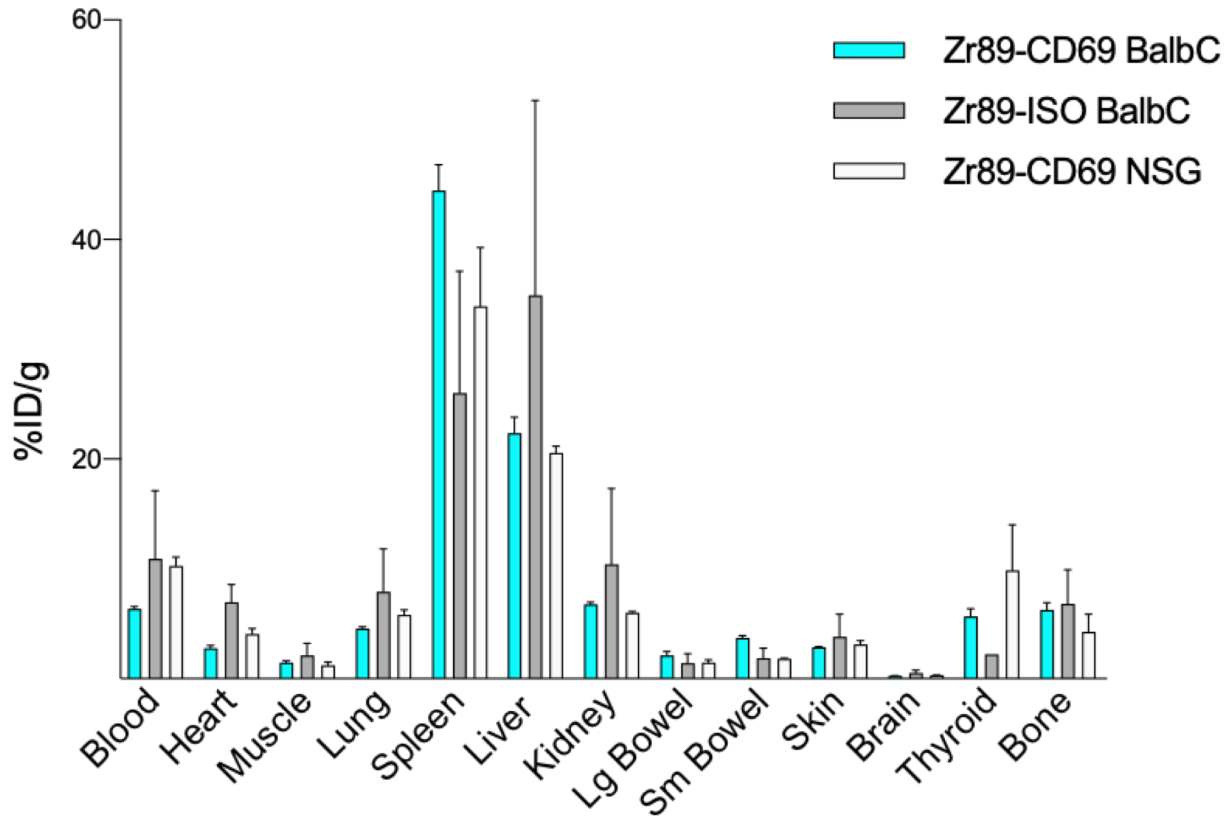
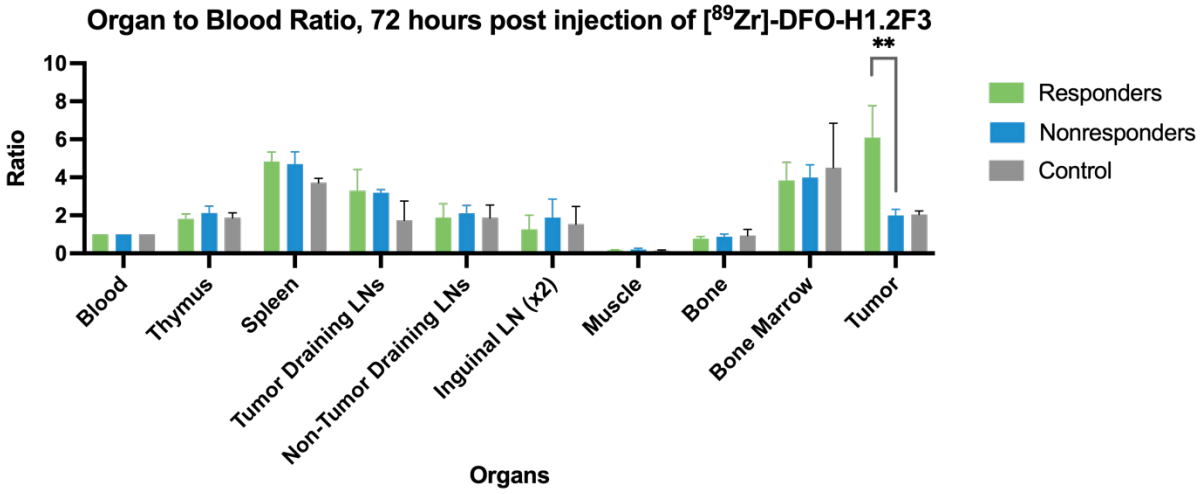


Figure S4. Quantifying [^{89}Zr]-DFO-H1.2F3 ex vivo biodistribution in healthy mice. Healthy mouse biodistribution analysis 72 hours post intravenous injection of [^{89}Zr]-DFO-H1.2F3 into BALB/c (n = 3) and NSG (n = 3) mice, as well as [^{89}Zr]-DFO-ISO isotype control into BALB/c (n = 2) mice.

A.

Organ	Responders			Nonresponders			Significance	
	Mean	SD	N	Mean	SD	N	P values	Symbol
Blood	7.10	0.698	6	5.23	0.68	4	0.003	**
Spleen	34.0	2.66	6	24.2	0.517	4	<0.001	***
Tumor Draining LNs	23.1	6.75	6	16.6	1.65	4	0.064	n.s.
Non-Tumor Draining LNs	13.3	5.14	6	11.0	2.17	4	0.358	n.s.
CT26 Tumor	43.1	12.8	6	10.3	1.21	4	<0.01	**

B.



C.

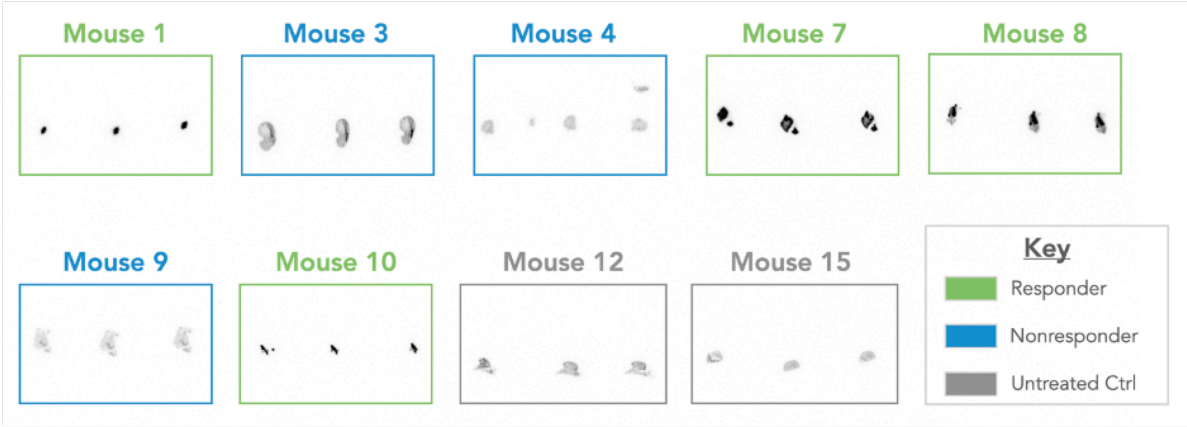
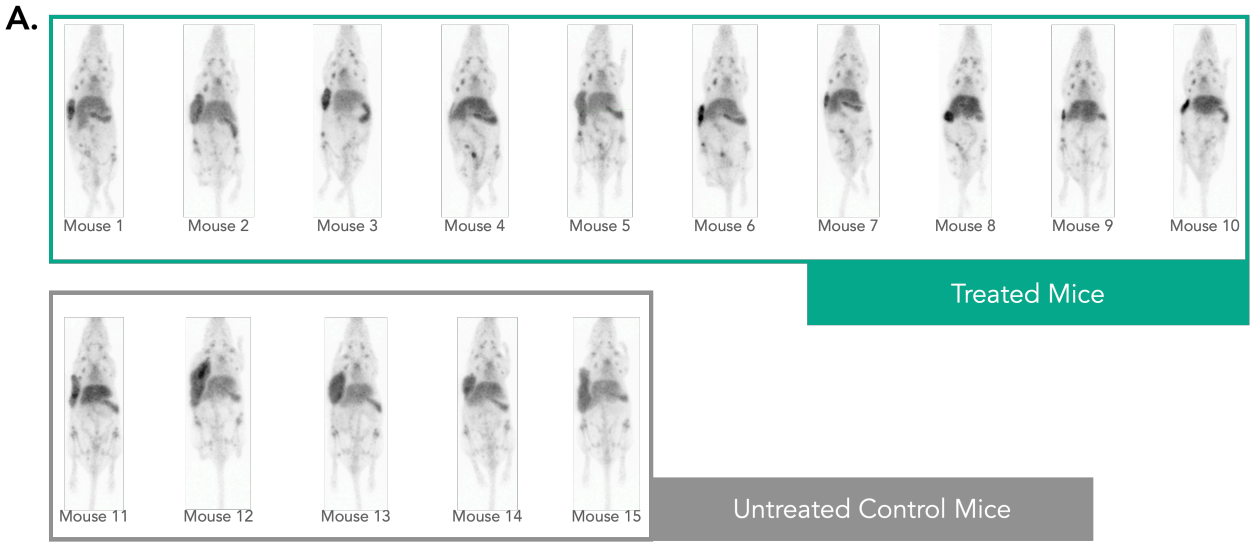
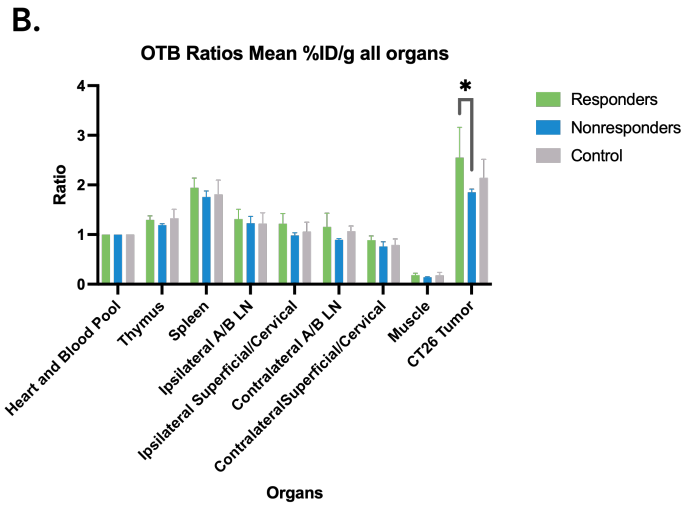
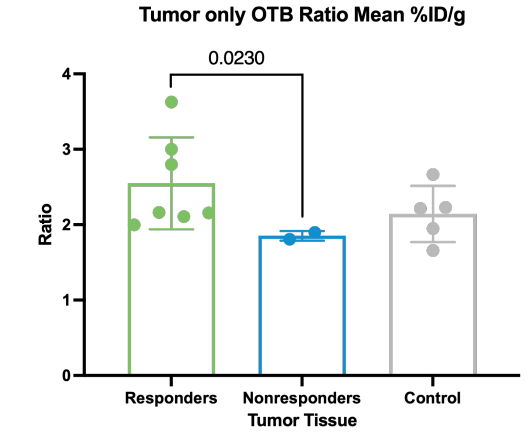


Figure S5. Tabular evaluation of significance, organ to blood ratios and autoradiography images for 15-day Biodistribution study using the CT26 syngeneic tumor immunotherapy. **(A)** Tabular evaluation of significance with respect to comparisons between Responder and Nonresponder groups for select organs of interest. **(B)** Organ to blood ratios for major organs. P value for comparison between Responder and Nonresponder tumor uptake, P = 0.0015. **(C)** All

autoradiography images taken during the study, for *ex vivo* validation of CD69 expression and localization in whole tumor sections from Responders, Nonresponders and Untreated Control groups. Two-tailed unpaired t test with or without Welch's correction was used to compare groups. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.


A.
Mouse 1
Mouse 2
Mouse 3
Mouse 4
Mouse 5
Mouse 6
Mouse 7
Mouse 8
Mouse 9
Mouse 10
Treated Mice
Mouse 11
Mouse 12
Mouse 13
Mouse 14
Mouse 15
Untreated Control Mice


B.
OTB Ratios Mean %ID/g all organs
Ratio
Responders
Nonresponders
Control
*
Heart and Blood Pool
Thymus
Spleen
Ipsilateral A/B LN
Ipsilateral Superficial/Cervical
Contralateral A/B LN
ContralateralSuperficial/Cervical
Muscle
CT26 Tumor
Organs


C.
Tumor only OTB Ratio Mean %ID/g
0.0230
Ratio
Responders
Nonresponders
Control
Tumor Tissue

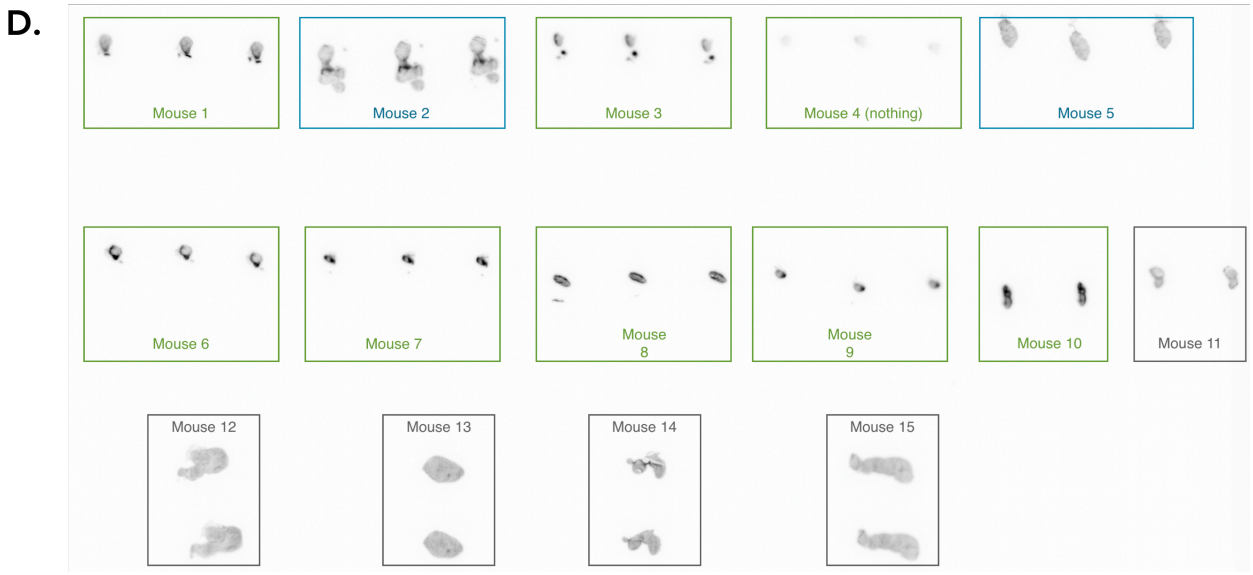
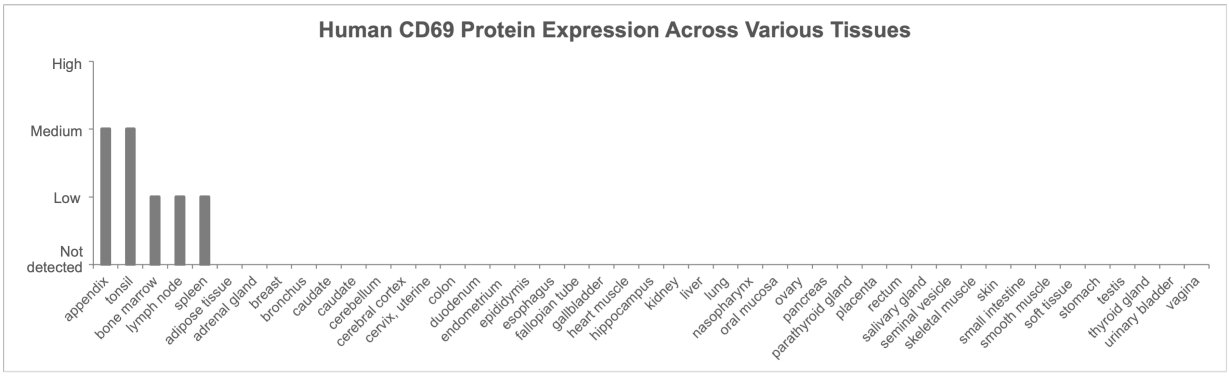

D.
Mouse 1
Mouse 2
Mouse 3
Mouse 4 (nothing)
Mouse 5
Mouse 6
Mouse 7
Mouse 8
Mouse 9
Mouse 10
Mouse 11
Mouse 12
Mouse 13
Mouse 14
Mouse 15

Figure S6. Maximum intensity projection (MIP) PET images, PET image ROI Organ to blood (OTB) ratios, and autoradiography images for 15-day Biodistribution study using the CT26 syngeneic tumor immunotherapy.
(A) MIP of all PET images taken for the study. All images were normalized to the same standardized uptake values (SUVbw) in MIM. **(B)** Organ to blood ratios for major organs for Responders, Nonresponders and Untreated Control groups. **(C)** Tumor to blood ratios Responders, Nonresponders and Untreated Control groups. **(D)** All autoradiography images taken during the study, for ex vivo validation of CD69 expression and localization in whole tumor sections from Responders, Nonresponders and Untreated Control groups. Two-tailed unpaired t test with Welch's correction was used to compare groups. *, $P < 0.05$

A.



B.

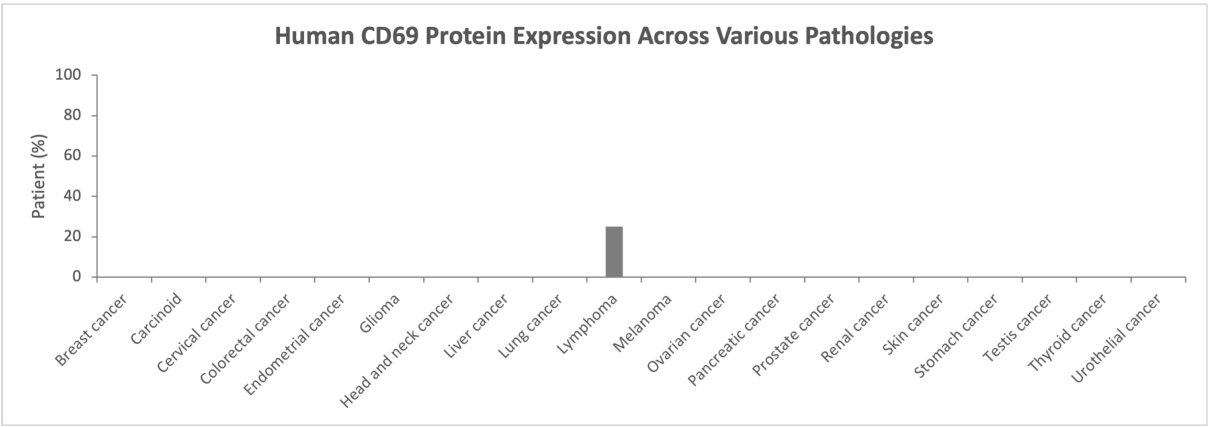


Figure S7. Human CD69 protein expression summary across various pathologies.
(A) Data show scores of CD69 expression (Not detected, Low, Medium, High) across various tissue types, which were stained, imaged, and analyzed. For the appendix, there was medium CD69 expression in sections taken from lymphoid tissue, but no expression was detected in the glandular cells. For the tonsil, medium CD69 expression was detected for germinal and non-germinal cells, but no expression was detected for squamous epithelial cells. Consistently low CD69 expression was detected for sections taken from the bone marrow, lymph nodes, and spleen. The remaining tissue types were negative for CD69 expression. Data was acquired from the Human Protein Atlas (image credit). Image/CD69/Protein Expression Summary (HPA050525 and CAB002503 antibodies), v20.1.proteinatlas.org, Ensembl version: 92.38. **(B)** Data shows CD69 positivity (Patients, %) across various types of cancer. For the Lymphoma pathology, bone marrow and lymphoid tissues were stained, imaged, and analyzed. 3 of 12 patients showed medium-high CD69 expression and 2 of 12 patients showed low expression. CD69 expression was not detected for the remaining 7 of 12. The remaining cancer tissue types were also negative for CD69 expression. Data was acquired from the Human Protein Atlas (image credit). Image/CD69/Protein Expression Summary (CAB002503), v20.1.proteinatlas.org, Ensembl version: 92.38.