SUPPLEMENTAL METHODS

Antibody discovery: Hybridomas were labeled with CloneDetect in semi-solid methyl cellulose and isolated using a ClonePix 2 (Molecular Devices). For kinetics against Fc-conjugated mHER2 (P70424 aa1-653) or hHER2 (P04626 aa1-652) (Sino Biological), hybridoma supernatants were injected on Carterra LSA (Carterra Bio) over ligands captured by immobilized anti-human IgG Fc. For kinetics against the monomeric His-tagged mHER2 (mHER2.His; P70424 aa1-653; Sino Biological) or His-tagged domain-IV HER2 (P70424 aa490-631; Sino Biological), anti-mHER2 antibodies were immobilized by amine-coupling and soluble mHER2.His was injected over the array. Sensograms were fitted using the Carterra Data Analysis Software. Antibody 1G3 was subcloned and purified using protein G HP column (Cytiva). To derive the IgG sequences of 1C9 and 1G3, total RNA was isolated from hybridomas, cDNA was generated and sequenced (GenScript ProBio). To sequence other domain IV-specific IgGs, variable regions from hybridoma RNA were amplified using rat-specific primers (30), based on a published template-switching strategy (31), and Sanger sequencing was performed (FHCC Genomics). Genes encoding the respective CARs were codon optimized using GeneArt GeneOptimizer (ThermoFisher) and synthesized by Twist Bioscience

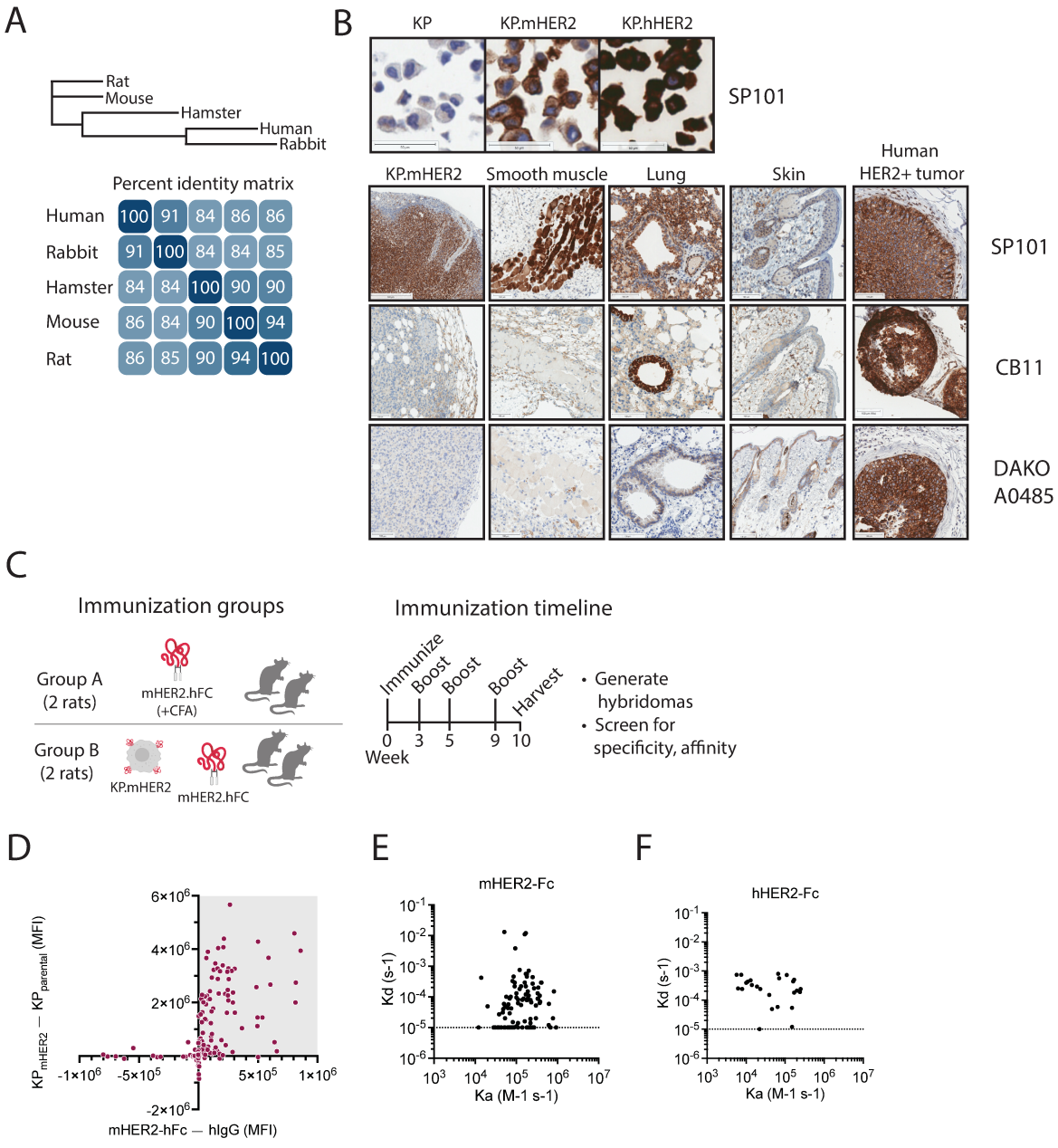
Immunohistochemistry: Antigen retrieval was performed in Decloaker (Biocare) using Tris-EDTA or Diva (for 1G3) at 95°C for 20min, and slides were treated with H_2O_2 for 5min and Background Punisher (Biocare) for 5min, then stained on IntelliPATH autostainer (Biocare). 1G3 was diluted to 10 µg/mL in Da Vinci Diluent (Biocare) and applied for 30min followed by ImPRESS

Goat anti-Rat IgG (Vector Laboratories) then 1,3'diaminobenzidine (DAB, IntelliPATH) for 10min and hematoxylin (IntelliPATH) for 1min. For quantification of HER2 on KP^{mHER2} tumors, clone D8F12 (Cell Signaling) was used. Slides were dehydrated on the Prisma Plus with xylene mountant and scanned in brightfield at 40X on Aperio AT Turbo (Leica). Relative abundance of CD3+ or mHER2+ cells were analyzed using HALO (Indica Labs). For DAB staining of CD3, clone CD3-12 (Biorad) was used.

Retroviral packaging: To produce retrovirus supernatant, Plat-E (Cell Biolabs) was transiently transfected using calcium phosphate or Xfect (Takara) and supernatants collected 2 days later, filtered through 0.45 µm filter, and titered to achieve T cell transduction efficiency of ~50%.

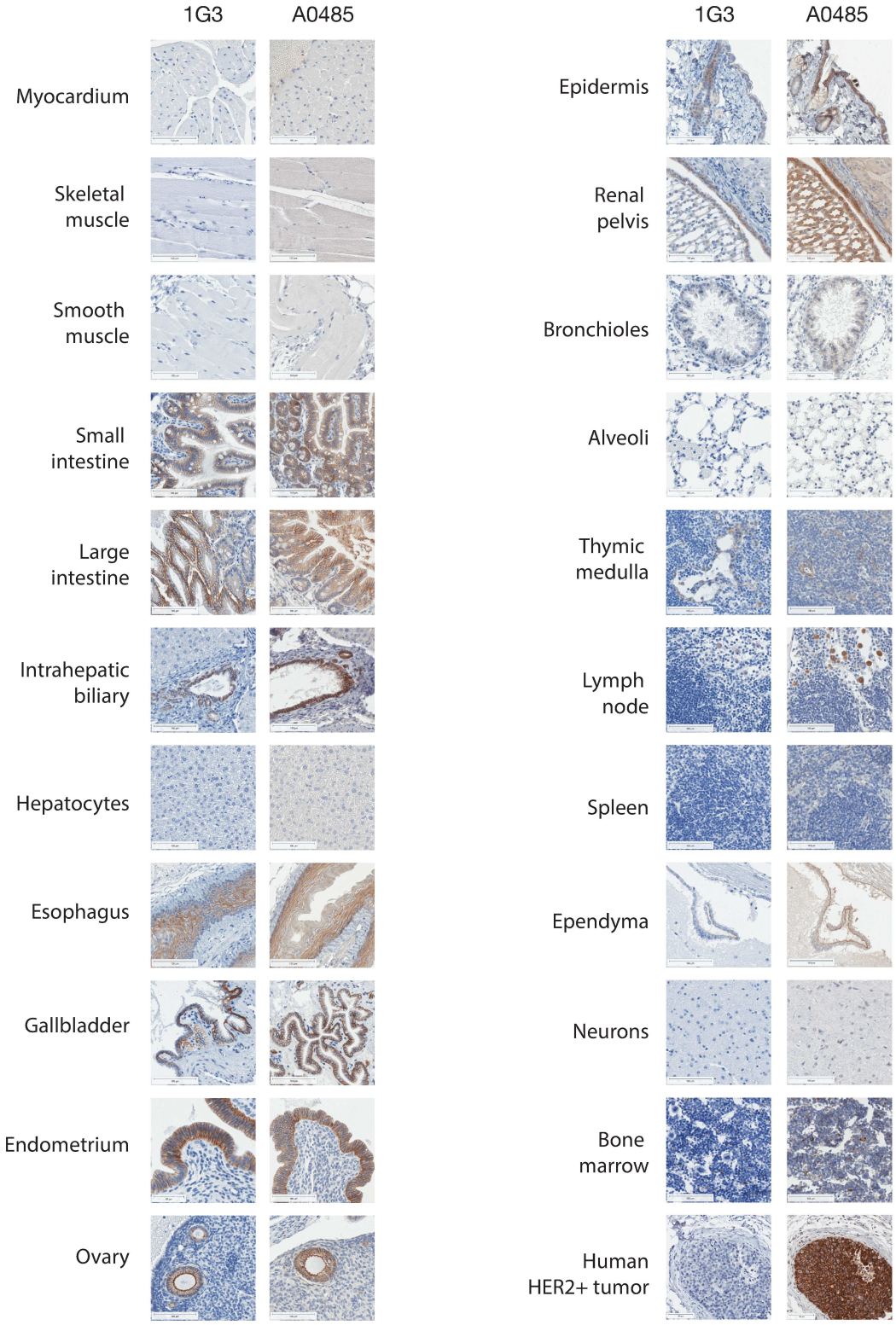
Flow cytometry antibodies: CD8α-FITC (53-6.7; Biolegend unless otherwise noted), CD19-PerCP/Cy5.5 (1D3), CD4-PE-Cy7 (GK1.5), CD45.2-APC/Fire750 (104), CD45.1-PB (A20), Thy1.1-APC (OX7), and myc-PE (9B11, Cell Signaling) and acquired on Celesta (BD). CAR TILs were stained with antibodies against CD45.1-AF700 (A20), CD8α-BUV395 (53-6.7, BD), CD4-BUV496 (GK1.5, BD), Thy1.1-BUV661 (OX7, BD) or CD19-BUV661 (1D3, BD), CD45-BUV805 (30-F11, BD), Tim3-BV421 (RMT3-23), PD-1-BV605 (29F.1A12), CD39-PE-Cy7 (Duha59), Lag3-BV785 (C9B7W), CD62L-APC/Fire750 (MEL-14), CD44-FITC (IM7), CD69-PerCP/Cy5.5 (HI.2F3), SLAMF6-PE (13G3, BD) or CD69-PE (H1.2F3), Ki67-BV711 (B56, BD) or CX3CR1-BV711 (SA011F11), and KLRG1-APC (2F1) and acquired on Symphony (BD).

Supplementary Figure 1: Generation and characterization of anti-murine HER2 IgGs



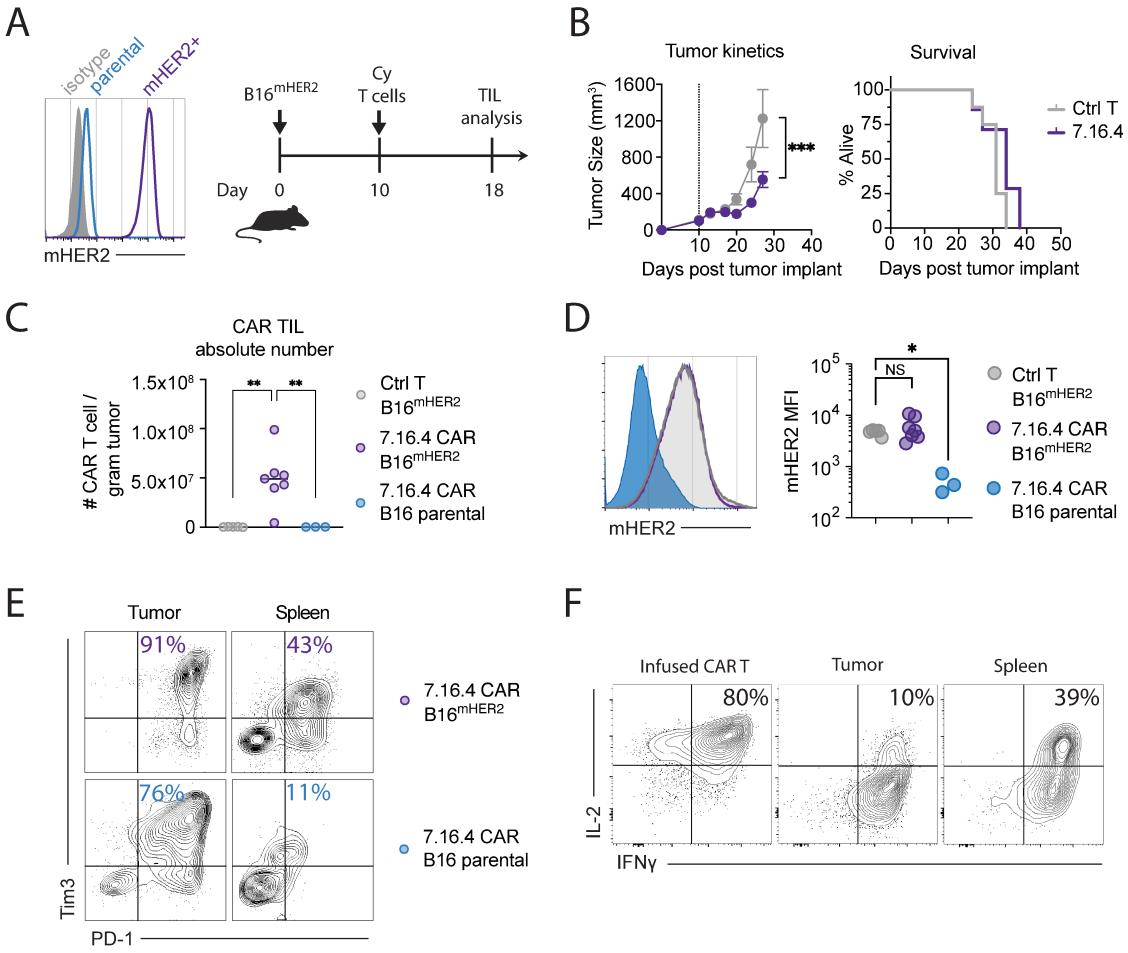
Supplementary Figure 1: Raising monoclonal rat antibodies against murine HER2. (**A**) Clustal peptide sequence alignment of the extracellular domains of HER2 in human (Uniprot P04626), mouse (Uniprot P70424), rat (Uniprot P06494), rabbit (Uniprot G1SZL0), and golden hamster (Uniprot Q60553). (**B**) Top: representative IHC staining with rabbit anti-human HER2 (clone SP101) using pellets of parental KP cells or KP cells previously transduced with murine HER2 (KP^{mHER2}) or human HER2 (KP^{hHER2}). Bottom: IHC staining with rabbit anti-human HER2 clones SP101, CB11 (mouse isotype), or DAKO A0485 (polyclonal rabbit isotype) was performed on a subcutaneously implanted KP^{mHER2} tumor, murine subcutaneous smooth muscle, normal murine lung, normal murine skin, and human HER2+ tumor. CB11 and DAKO were generated using immunogens derived from the intracellular domain of hHER2 and therefore do not stain KP^{mHER2} tumors. (**C**) Schematic depicting the immunization and boosting strategy of rats with recombinant human-Fc-conjugated mHER2 combined with complete Freund's adjuvant (group A) or with recombinant protein combined with KP^{mHER2} cells (group B). (**D**-**F**) Cytometric binding analysis with parental KP, KP^{mHER2}, bead-tethered human IgG, and bead-tethered mHER2.Fc (**D**), binding kinetics for biosensor-chip-captured mHER2-Fc (**E**), or binding kinetics for captured hHER2.Fc (**F**) performed with crude hybridoma supernatants. Hybridoma samples with sufficient IgG titer and specificity were selected for further analysis in E, F, and subsequent studies. Hybridoma supernatant titers were considered adequate if the analyte-binding capacity of ligand (Rmax) exceeded 90 RU, whereas the binding was considered mHER2-specific (shaded in grey in panel **D**) if the candidate exhibited a positive MFI differential of KP^{mHER2} staining compared to parental KP and positive MFI differential of mHER2-hFc+ beads compared to hIgG+ beads to exclude off-target specificity to human Fc. Individual values of MFI, K_a, K_d, and K_D used in **D**-**F** are listed in Supplementary Table 1.

Supplementary Figure 2: IHC staining of murine tissues with 1G3 and DAKO A0485



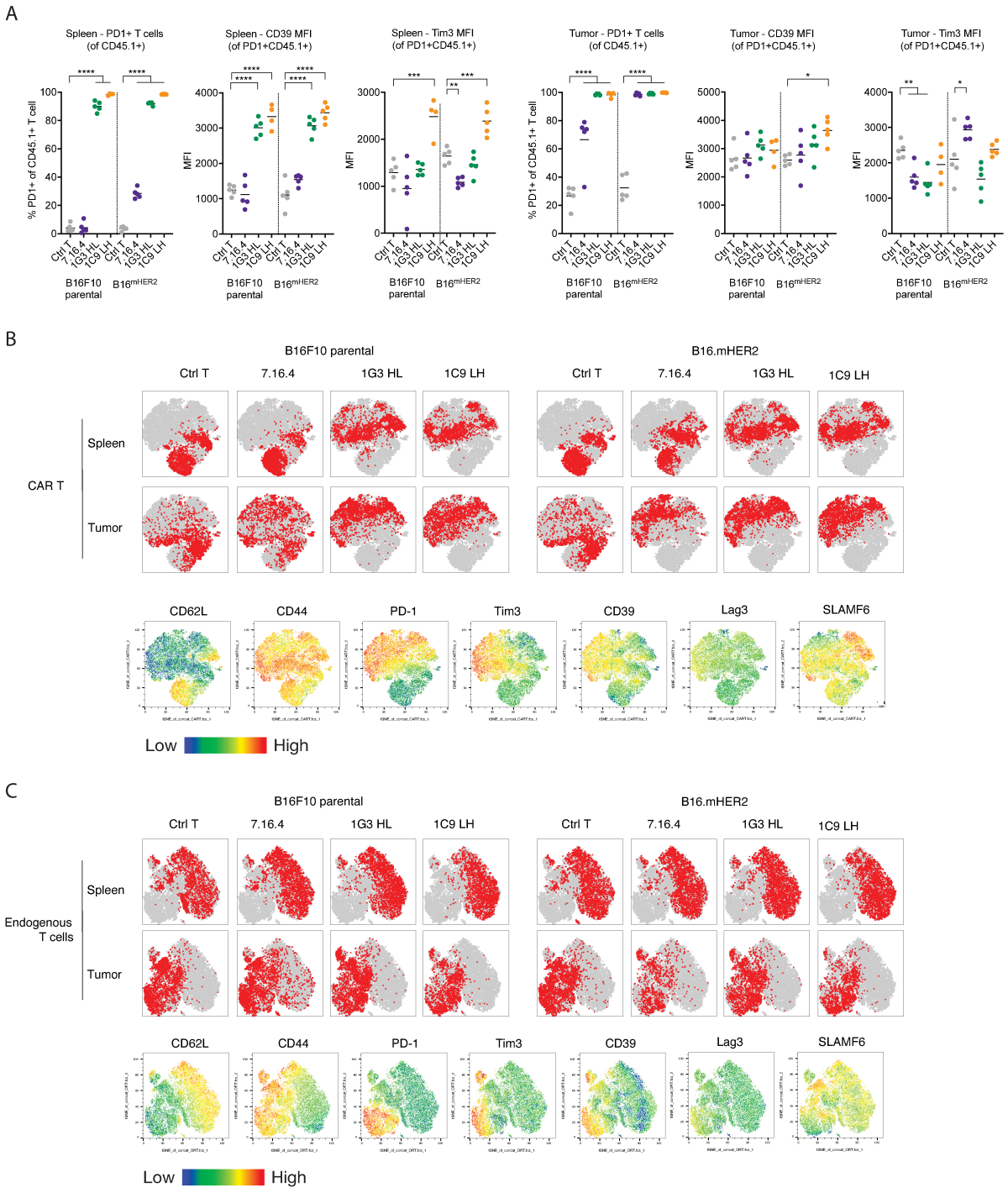
Supplementary Figure 2: mHER2 staining with 1G3 exhibits robust signal-to-noise ratio compared to DAKO A0485. Representative IHC staining with 1G3 and DAKO A0485 on normal tissues of a 6-week-old C57BL/6J female and on human HER2+ tumor tissue.

Supplementary Figure 3: Anti-murine HER2 CAR T cells transiently control the growth of HER2+ B16F10 tumors and rapidly develop T cell dysfunction



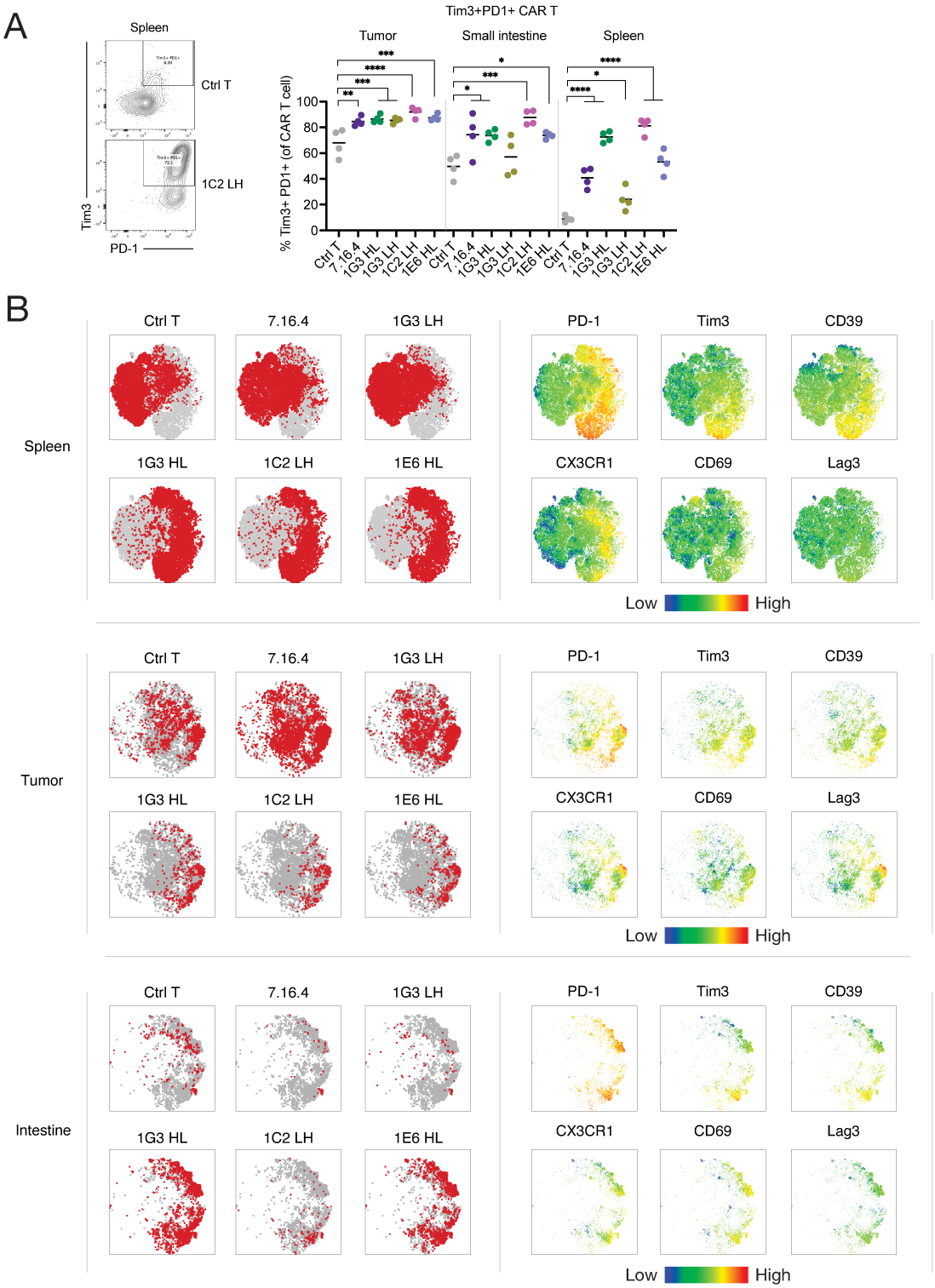
Supplementary Figure 3: Anti-murine HER2 CAR T cells only transiently control the growth of HER2+ B16F10 tumors and rapidly exhibit T cell dysfunction. (**A**) Left: surface expression of murine HER2 on parental B16F10 (light blue) or B16F10 cells transduced with mHER2 (purple) compared to rat isotype control (grey), determined by flow cytometry after staining with 1G3 followed by PE anti-rat antibody. Right: Schematic of the adoptive transfer protocol. Mice were implanted subcutaneously with 4×10^5 B16 tumor cells, administered 200 mg/kg cyclophosphamide (Cy) 10 days post tumor implantation, and 6 h later given 1×10^6 CD45.1+ CAR T cells or control T cells. (**B**) $B16^{mHER2}$ tumor volumes and mouse survival after treatment with rHER2.CAR or control T cells. (**C**) Tumors analyzed on day 8 post infusion for the absolute numbers of CAR and Ctrl T cells, gated on CD8+ CD45.1+ cells. (**D**) Surface levels of mHER2 on digested $B16^{mHER2}$ tumors excised on day 8 after T cell therapy. mHER2 expression was determined by flow cytometry after staining with 1G3 followed by APC-conjugated anti-rat antibody. Tumor cells were gated on live CD45- TA99+ CD105+ cells. (**E**) Flow cytometry analysis of the expression of PD-1 and Tim-3 on CAR T cells in the tumors (left) and spleens (right) of tumor-bearing mice treated with the indicated CAR T cells. (**F**) Tumor-infiltrating or spleen-derived 7.16.4 CAR T cells were isolated on day 8 post tumor injection, stimulated with PMA and ionomycin for 4 h, stained for intracellular cytokine, and analyzed by flow cytometry for dual expression of IL-2 and IFNγ. Day 0 infusion product shown for comparison. Symbols represent means ± SEM (B) or individual mice (C, D), with n=3-7 mice/group. *<0.05, **<0.01, ***<0.001, ****<0.0001 by ANOVA (Dunnett post hoc).

Supplementary Figure 4: High-affinity HER2 CAR T cells exhibit a highly differentiated phenotype in both spleen and tumor



Supplementary Figure 4: High-affinity CAR T cells exhibit a terminally differentiated phenotype in the spleen and tumor, irrespective of antigen expression levels. (**A**) Flow cytometric analysis of Thy1.1+ CAR T cells, gated on CD8+ CD45.1+ T cells, from the spleen and tumor on day 7 post infusion. Symbols represent individual mice with n=4-5. *<0.05, **<0.01, ***<0.001, ****<0.0001, ANOVA (Dunnett post hoc). (**B**) *Top*: tSNE visualization of the indicated CAR T cells (Red) compared to the total analyzed CAR T cells in the tissue (grey). *Bottom*: Surface marker expression on individual CAR T cells derived from the indicated tissues. Data from flow cytometry was processed using the Downsample plugin (FlowJo Exchange) and FlowJo v10 tSNE algorithm (iterations = 1000; perplexity = 30; learning rate = 401; KINN algorithm = exact, vantage point tree; gradient algorithm = Barnes-Hut). Each dot represents a single cell colored in red to represent the indicated T cell subset or by scaled color gradient to represent the indicated marker intensity. (**C**) Endogenous Thy1.1- CD45.1- CD8+ T cells analyzed as in **B** and shown as internal controls. See Figure 4 for data source.

Supplementary Figure 5: High-affinity CAR T cell exhibit a terminally differentiated phenotype in the spleen, tumor, and small intestine.



Supplementary Figure 5: Non-toxic high-affinity CAR T cell exhibit a terminally differentiated phenotype in the spleen, tumor, and small intestine. (**A**) Flow cytometric analysis of Tim3 and PD-1 co-expression on CAR T cells in tumor, intestine, and spleen tissues 8 days post infusion. Symbols represent individual mice with n=4. *<0.05, **<0.01, ***<0.001, ****<0.0001 by ANOVA (Dunnett post hoc). (**B**) *Left*: tSNE visualization of T cells indicating respective CAR T cells (Red) compared to the total analyzed CAR T cells in the tissue (grey). *Right*: Surface marker expression on individual CAR T cells derived from the indicated tissues. Data from flow cytometry was processed using the Downsample plugin (FlowJo Exchange) and FlowJo v10 tSNE algorithm (iterations = 1000; perplexity = 30; learning rate = 401; KINN algorithm = exact, vantage point tree; gradient algorithm = Barnes-Hut). Each dot represents a single cell colored in red to represent the indicated T cell subset or by scaled color gradient to represent the indicated marker intensity. See Figure 6 for data source.