Supplemental Methods:

Generation of WT1- and MSLN-specific T cell clones:

The WT1-specific TCR 3D was recovered from a high avidity CD8 T cell clone that was generated by priming mice with splenocytes that were adherent *in vitro* and activated with LPS (10μg/ml) and anti-CD40 antibody (5μg/ml), then pulsed with the immunodominant H-2K^{d} epitope of WT1 RMFPNAPYL ($WT1_{126\text{-}134}$). These mice were then boosted with a recombinant *Listeria monocytogenes* (LM_{WT1}) encoding the RMFPNAPYL epitope within a piece of the ovalbumin gene in an attenuated *actA*- and *inlB*-deficient strain of LM (created by Aduro BioTech). $WT1_{126\text{-}134}$ -specific T cells were expanded by stimulating with irradiated peptide-pulsed splenocytes and IL-2 (25 u/mL) every 7 days and cloned by limiting dilution. Individual clones were analyzed for peptide-MHC multimer staining, and functional avidity was determined by assessing cytokine (IFNγ and TNFα) production in response to APCs pulsed with decreasing concentrations of the relevant peptide, and TCRα and TCRβ chain genes were isolated from the clones with the highest functional avidity for $WT1_{126\text{-}134}$.

Mesothelin-deficient mice ($Msln^{-/-}$) were previously generated[1], and generously provided by Dr. Ira Pastan (National Cancer Institute, Bethesda, MD). Msln-specific T cells were isolated from wildtype B6 and Msln-/- mice targeting a previously described H2-D^{b} epitope $Msln_{406\text{-}414}$ [2]. The highest affinity clones were determined by assessing cytokine (IFNγ and TNFα) production in response to APCs pulsed with decreasing concentrations of the relevant peptide. The retroviral expression constructs Mig-MSLN-*wt* and Mig-MSLN-

ko consist of the retroviral vector MigR1 expressing codon-optimized, P2A-linked TCRα and TCRβ genes cloned from the highest affinity Mesothelin specific T cell clones that could be generated from wildtype B6 and Mesothelin deficient mice, respectively.

Library Construction and FACS for Isolation of Improved Affinity 3D TCR variants: The 3D TCR was used as a template for generating three separate degenerate amino acid libraries by overlap extension PCR as described previously[3]. Each library was three codons in length, spanning the complementarity determining region (CDR)-3α (TAP/MDS/NYQ). 58-/- cells were retrovirally transduced, expanded, and then sorted with anti-mouse Cβ (Clone H57-597), followed by the WT-1/D^b dimer (100 to 200 nM). After three sorts with WT-1/D^b dimer a positive population emerged, and the retroviral insert was recovered by PCR. The PCR product was sequenced, yielding two unique species; a predominant sequence, called Q103H (CDR3α: TAPMDSNY**H**), and a less predominant sequence, called N101P, Q103Y (CDR3α: TAPMDS**P**Y**Y**).

Histopathology: Tissues were fixed in 10% neutral formalin, embedded in paraffin and routine hematoxylin and eosin (H&E) staining was performed by the Experimental Histopathology Shared Resource of the Fred Hutchinson Cancer Research Center. Sections were then analyzed for evidence of lymphocyte infiltration and histological damage by a pathologist.

References:

1. Bera TK, Pastan I. Mesothelin is not required for normal mouse development or reproduction. *Mol Cell Biol*. 2000;20(8):2902–2906.
2. Hung C-F, Tsai Y-C, He L, Wu T-C. Control of mesothelin-expressing ovarian cancer using adoptive transfer of mesothelin peptide-specific CD8+ T cells. *Nature*. 2007;14(12):921–929.
3. Chervin AS, Aggen DH, Raseman JM, Kranz DM. Engineering higher affinity T cell receptors using a T cell display system. *J Immunol Methods*. 2008;339(2):175–184.

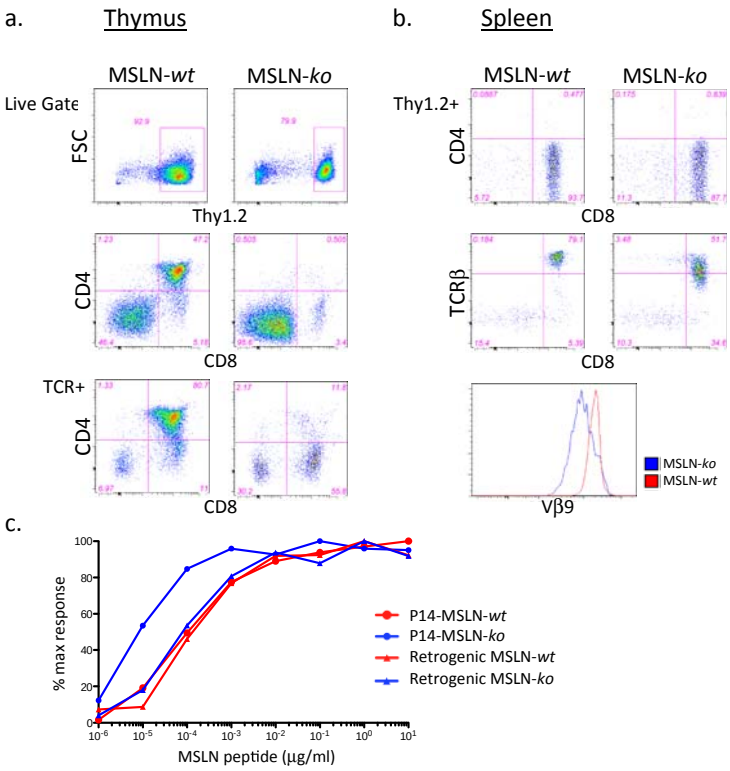

Fig. S1. *Analysis of MSLN-specific TCRs and TCR retrogenic mice:* (a) *MSLN*-specific TCR genes were cloned from high affinity T cell clones isolated from wildtype (*MSLN-wt*) or *MSLN*$^{-/-}$ (*MSLN-ko*) mice. *MSLN*-specific TCR expression constructs were transduced into Rag2$^{-/-}$ BM cells (Thy1.2), which were transferred into irradiated Thy1.1 B6 recipients and analyzed >5 weeks post-transfer. (a) Thymocytes were gated on cells expressing Thy1.2 by flow cytometry as a marker of donor-derived cells and analyzed for CD4 and CD8 expression. Increased negative selection is evident in the *MSLN-ko* retrogenic mice, but a population of SP T cells survives and remains detectable. (b) Splenocytes from retrogenic mice were gated on cells expressing Thy1.2, and these donor cells were then analyzed for the expression of CD4, CD8, TCRβ, and the relative expression of Vβ9, the β-chain utilized by both the *MSLN-wt* and *MSLN-ko* TCRs. *MSLN-ko*-expressing CD8 T cells exhibited significantly reduced levels of TCR expression, similar to the highest affinity 3D TCR variant, 3D-PYY. (c) *MSLN*-specific retrogenic T cells and TCR-transduced P14 T cells were stimulated with *MSLN* peptide and expanded one week *in vitro*, and then assayed for functional avidity by measuring IFNγ production in response to APCs pulsed with decreasing concentrations of MSLN peptide. The functional avidity of peripheral P14 T cells transduced with the *MSLN-ko* TCR demonstrated an ~10-fold increase in antigen sensitivity compared to CD8^{+} T cells expressing the *MSLN-wt* TCR, whereas the *MSLN-ko*-expressing retrogenic T cells exhibited a functional avidity similar to that seen with T cells expressing the *MSLN-wt* TCR.

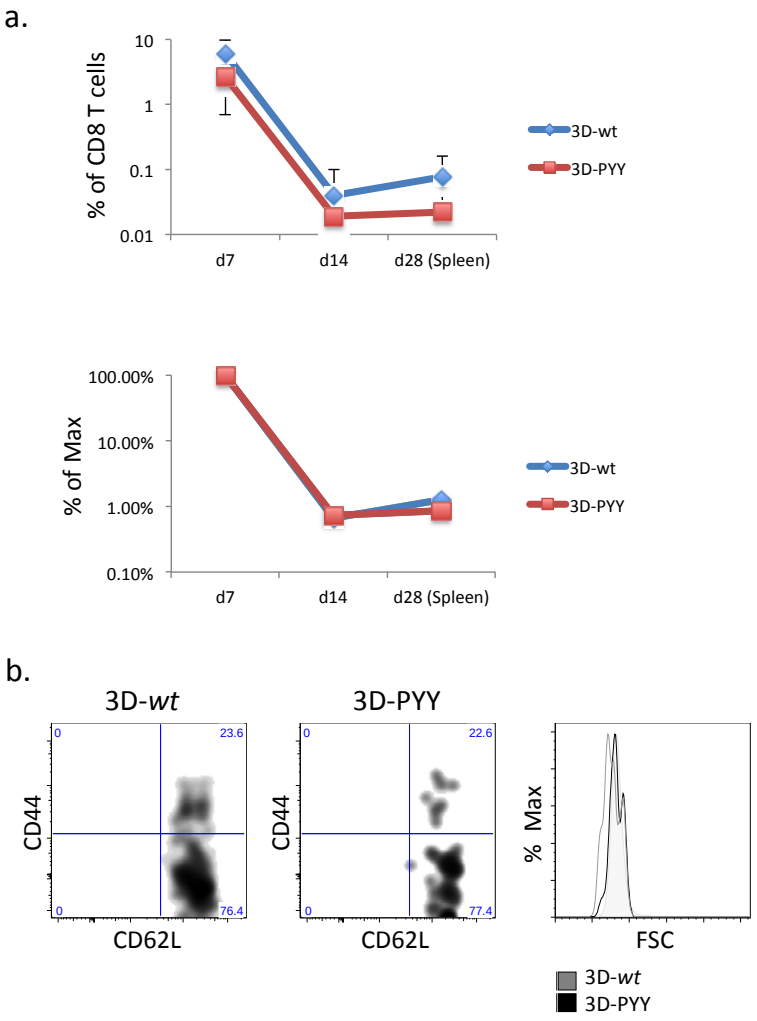

Fig. S2. *Kinetics of T cell persistence in vivo:* Thy1.1 congenic P14 T cells were retrovirally transduced to express either the 3D-*wt* or 3D-PYY TCR. Transduced T cells were restimulated with WT1 peptide on day 7 and then injected 1:1 with WT1 peptide-pulsed Thy1.2$^+$ splenocytes into three Thy1.2 B6 recipients (9x10^6/mouse) per condition 6 days later. Recipient mice received IL-2 (10^4 units/mouse) for 10 days post-injection. (a) The persistence of transferred CD8$^+$ T cells as a percentage of total CD8 T cells in the blood is shown for day 7 and day 14 post T cell transfer, as well as the percentage of total CD8 T cells in the spleen on day 28. These data are also plotted as a percentage of the maximum for each TCR to highlight the similar kinetics of T cell contraction. (b) Expression of CD44 and CD62L, as well as the FSC profile was analyzed for persistent CD8$^+$ T cells on day 28 post-T cell transfer. The shaded histogram represents total host-derived CD8 T cells.

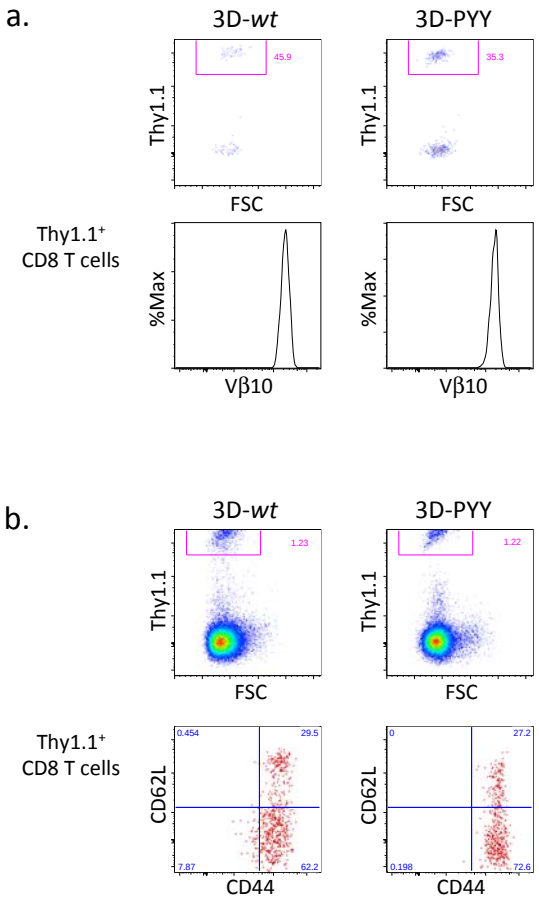

Fig. S3. *Transfer and persistence of TCR-transduced T cells into irradiated hosts:* (a) Thy1.1 congenic P14 T cells were retrovirally transduced to express either the 3D-*wt* or 3D-PYY TCR. Transduced T cells were restimulated with WT1 peptide on day 7 and then injected 1:1 with WT1 peptide-pulsed Thy1.2$^+$ splenocytes into irradiated (550 Rad) Thy1.2 B6 recipients 6 days later (9x10^6/mouse, three mice per condition). Recipient mice received IL-2 (10^4 units/mouse) for 10 days post-injection. A representative plot for each condition shows the high percentage of transferred Thy1.1$^+$ CD8$^+$ T cells within the CD8+ fraction of the blood of all recipient mice on day 7 post T cell transfer, as well as the uniform expression of the transgene encoded Vβ10 TCR on transferred T cells. (b) Recipient mice were boosted with LM expressing the WT1 RMFPNAPYL epitope within a piece of the ovalbumin gene *(LM_{WT1})* 8 days after T cell transfer, and analyzed for persistence and/or autoimmune mediated expansion of the transferred CD8$^+$ T cells 21 days post-treatment with LM_{WT1}. Expression of the memory markers CD44 and CD62L by persistent CD8$^+$ T cells was also analyzed on day 21 post-treatment with LM_{WT1}.