Online supplemental material for

Selection of therapeutically effective T cell receptors from the diverse tumor-bearing repertoire

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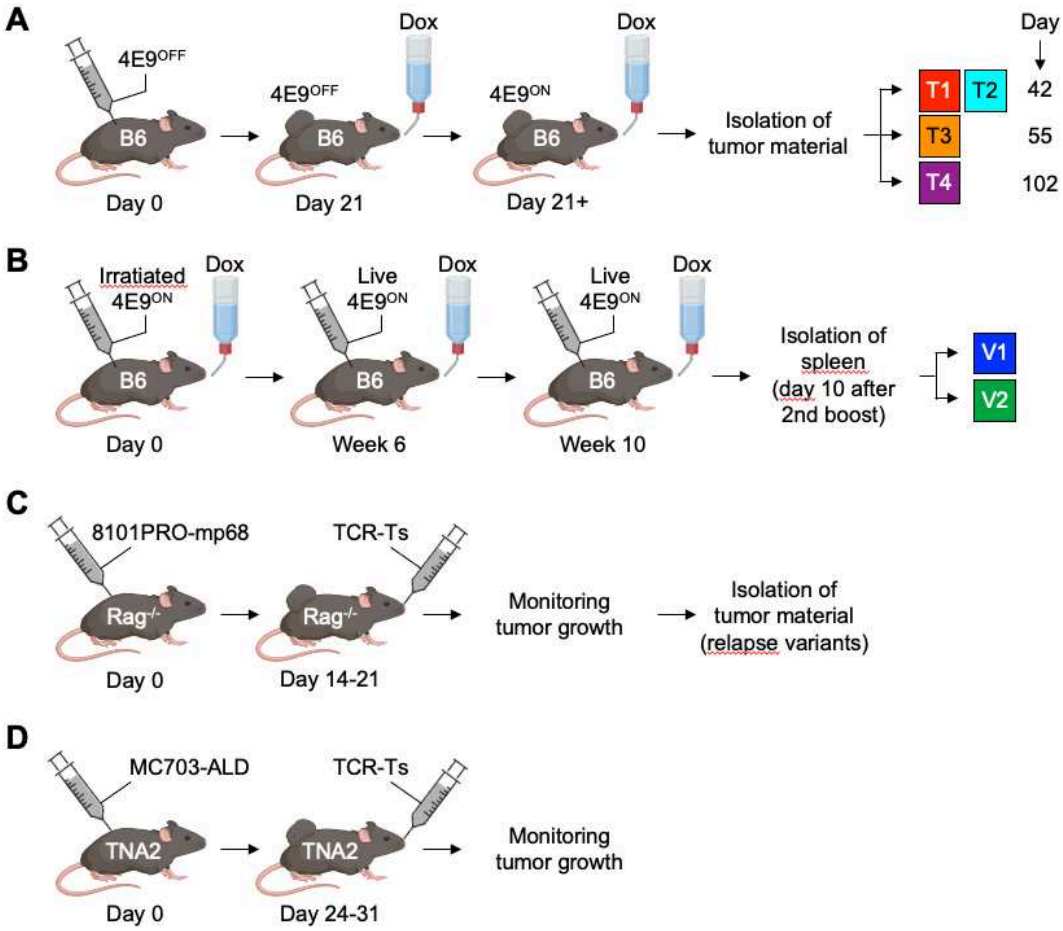

Online supplemental figure 1 - Timelines of conducted animal experiments. (A) A total of 3-5x10^6 4E9^{OFF} cancer cells were injected subcutaneously into C57BL/6 mice (B6). Tumor size was measured three times per week. After three weeks, mice with established 4E9^{OFF} tumors were given drinking water containing 200 µg/ml doxycycline (Dox). By this time, the tumors were at least palpable. The growth of 4E9^{ON} tumors was monitored further, and tumor material was collected on specified days. (B) C57BL/6 mice (B6) received drinking water containing 200 µg/ml doxycycline (Dox). The mice were injected with 3-5x10^6 lethally irradiated 4E9^{ON} cancer cells. Doxycycline treatment of 4E9 cells began 48 h before tumor cell inoculation. Mice received two subcutaneous booster injections of 3-5x10^6 live 4E9^{ON} cancer cells at weeks 6 and 10. Ten days after the second boost, mice were sacrificed for spleen isolation. (C-D) 3-5x10^6 8101PRO-mp68 or MC703-ALD cancer cells were injected subcutaneously into the flanks of either Rag1$^{-/-}$ or TNA2 mice, respectively. Tumor size was measured three times per week. Tumor-bearing mice were treated by intravenous injection of 1x10^6 TCR-Ts. The growth of 8101PRO-mp68 or MC703-ALD tumors was monitored further. 8101PRO-mp68 tumor

material was collected when relapse occurred. The images were created using graphic elements from BioRender.com.

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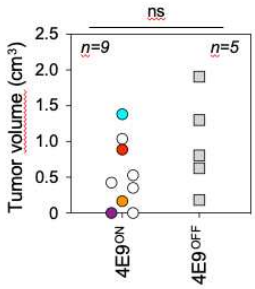
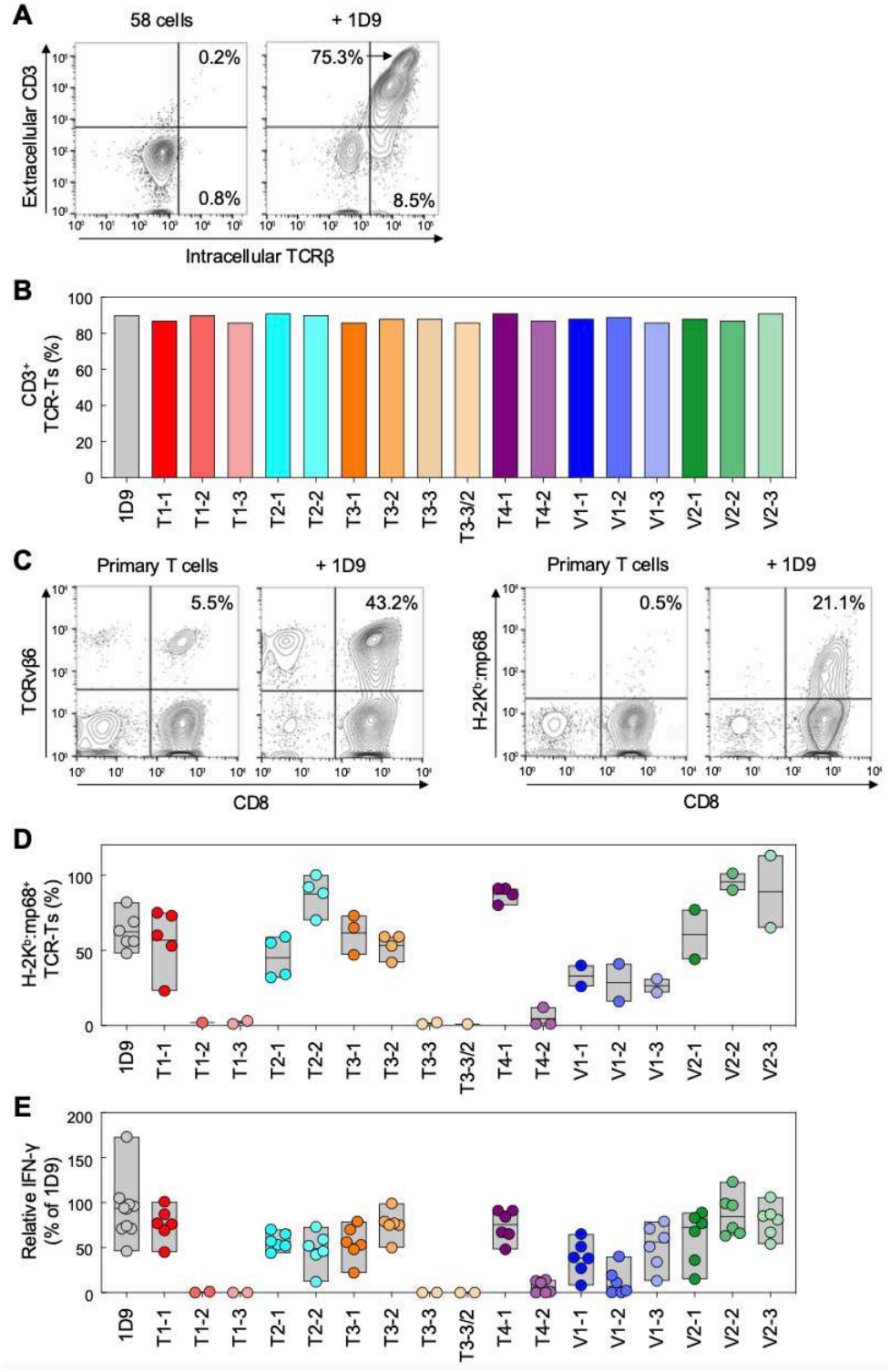
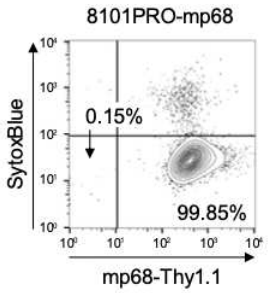


Online supplemental figure 2 - 21 days after mp68 expression was induced, 4E9^{ON} tumor sizes showed no significant differences when compared to 4E9^{OFF} tumors. Tumor sizes measured on day 42 after tumor cell injection (see figure 1) were plotted, and the 4E9^{ON} and 4E9^{OFF} groups were compared using an unpaired Student's t-test.

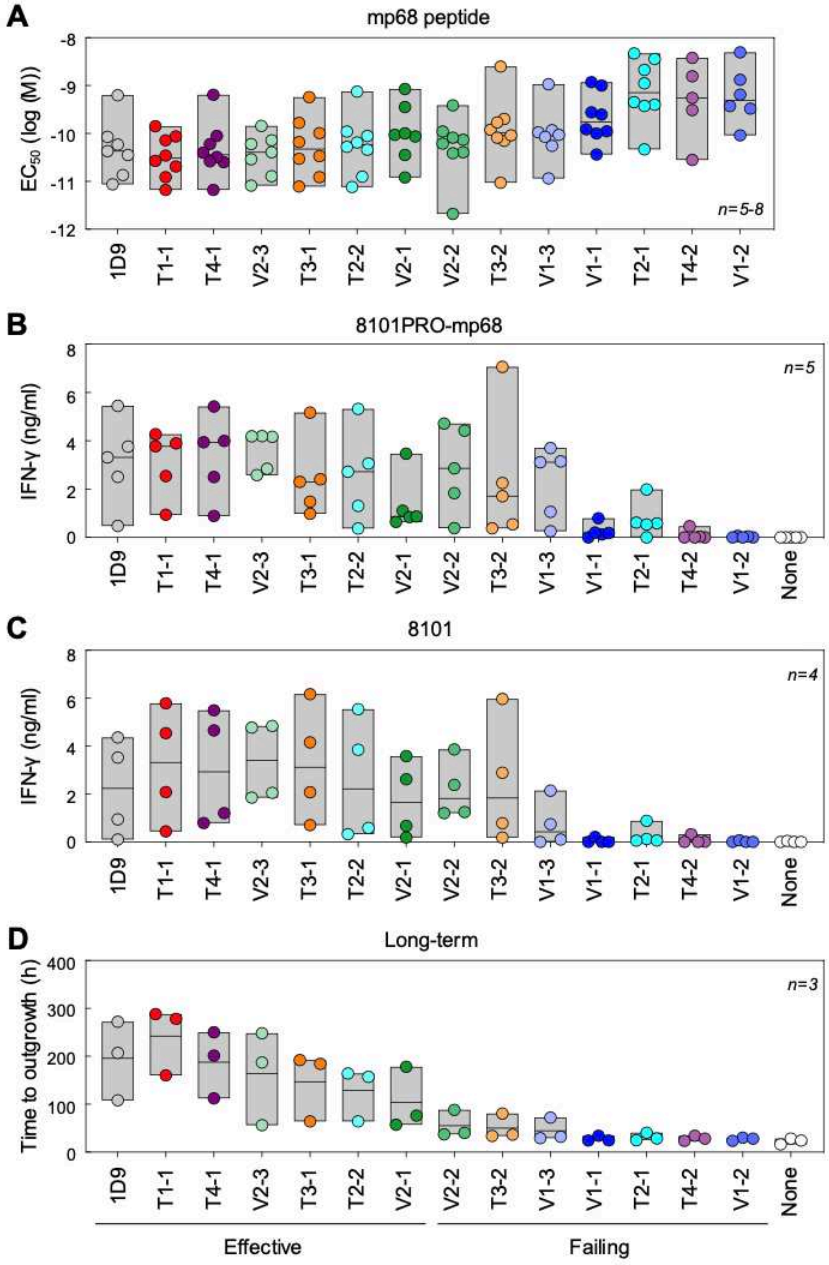


Online supplemental figure 3 - The majority of mp68-specific TCRs from either TILs of 4E9^{ON} tumors or immunized mice are functionally expressed in 58 and primary mouse T cells. (A-B) TCR-Ts generated from 58 cells show a correlation between CD3 and TCR

expression. (A) 58 cells were engineered with TCR 1D9 and surface expression of CD3 as well as intra-/extracellular presence of mouse constant TCRβ was determined by flow cytometry. (B) The percentage of $CD3^+$ 58 cells after engineering with indicated TCRs is shown as bar graph. (C-E) 13 of 16 mp68-specific TCRs generate functional TCR-Ts when generated from primary mouse T cells. TCR-Ts were analyzed to determine TCR surface expression (C), the percentage of TCR-Ts binding mp68-tetramers (D), and secretion of IFN-γ upon co-culture with splenocytes loaded with mp68 peptide ($1x10^{-6}$ M) (E). Values in (E) are shown in relation to TCR-Ts expressing 1D9. Each dot represents the results for an independent experiment.

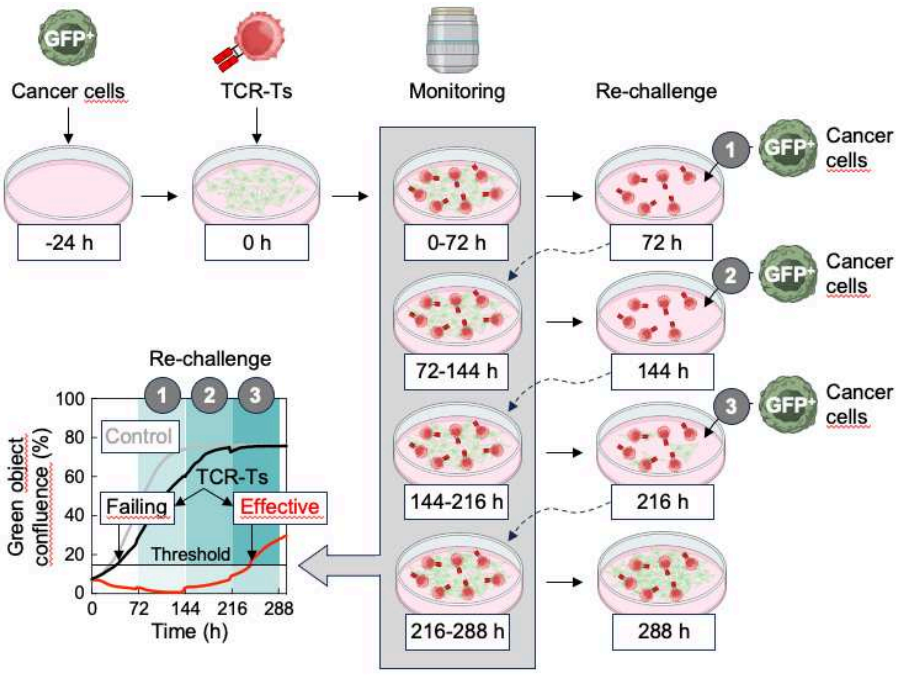


Online supplemental figure 4 - 8101PRO-mp68 cancer cells show surface expression of Thy1.1 and contain a small fraction of parental 8101PRO cancer cells that are Thy1.1-negative. Surface expression of Thy1.1 on 8101PRO-mp68 cancer cells was determined by flow cytometry. SytoxBlue was used to discriminate live and dead/apoptotic cells in culture. The given percentages show the fraction of 8101PRO-mp68 (99.85%) and parental 8101PRO (0.15%) cancer cell population that was used for tumor inoculation.

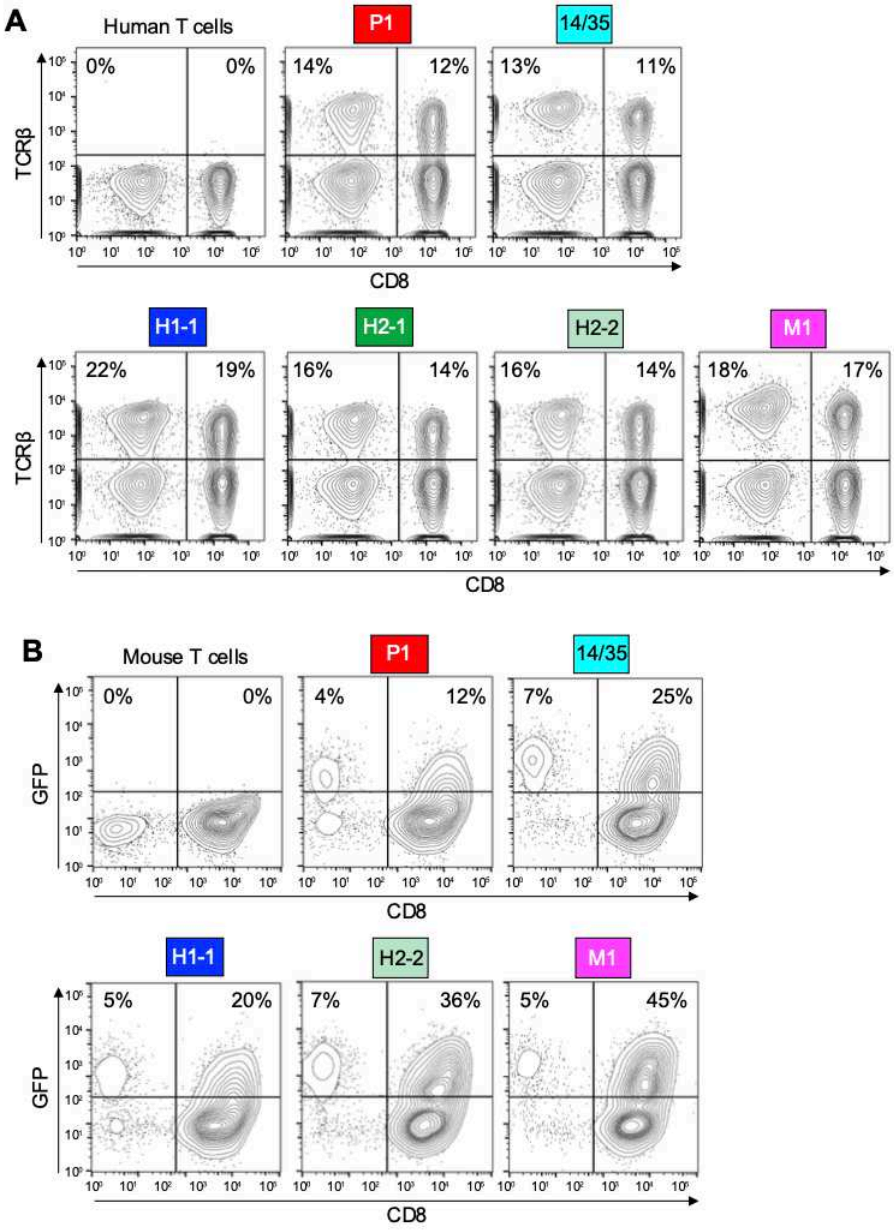


Online supplemental figure 5 - The therapeutic efficacy of TCR-Ts *in vivo* is predicted by long-term *in vitro* analysis. TCRs from either TILs of 4E9^{ON} tumors (T1-T4) or immunized mice (V1-V2) were classified as effective or failing based on their *in vivo* efficacy (figure 3). The groups of effective and failing TCRs are indicated on the bottom of the figure. (A) TCR-Ts were incubated with splenocytes loaded with graded amounts of mp68 peptide for 24 h. IFN-γ release was determined by ELISA and EC_{50} values were determined. EC_{50} is the concentration of mp68 peptide required to elicit half-maximal IFN-γ release by TCR-Ts. (B-C) TCR-Ts were incubated with 8101 cancer cells showing ectopic (8101PRO-mp68, B) or native mp68

expression (8101, C) for 24 h. IFN-γ release was determined by ELISA. (D) TCR-Ts were incubated with 8101-12-GFP cancer cells. Growth of GFP-labelled cancer cells was monitored using Incucyte SX5. TCR-Ts were re-challenged with cancer cells every 72 h. Outgrowth was defined as time point when cancer cell confluence was ≥10% (see also online supplemental figure 6). Each dot represents a single experiment. The number of experiments performed is shown in the upper right of each diagram (n=3-8). Bars indicate mean and range of data set.



Online supplemental figure 6 - Timeline of long-term *in vitro* analysis of TCR-Ts. Cancer cells ($2x10^3$ 8101-12-GFP or WM-902B+A2) were seeded in flat-bottom 96-well plates, followed by the addition of $1x10^4$ $CD8^+$ TCR-Ts 24 h later. Non-engineered T cells were added to maintain a constant total T cell count per well. Co-cultures were monitored for a total of 288 h. Fresh tumor cells ($2x10^3$) were added to the cultures every 72 h (Re-challenge). Monitoring was done via continuous imaging (every 2 h, 10x objective) using an Incucyte SX5. Tumor outgrowth was defined as the point when confluence reached 10% (8101-12-GFP) or 15% (WM-902B+A2). The graphical inset (lower left) illustrates the growth of GFP-labeled cancer cells in co-cultures with either effective or failing TCR-Ts. Cancer cells cultured without TCR-Ts served as control. The intersection of the growth curve with the selected threshold determines the data points presented in figures 4 and 5, as well as supplemental figure 5. The images were created using graphic elements from BioRender.com.



Online supplemental figure 7 - Expression of CDK4^{R24L}-specific TCRs in human- and mouse-derived T cells. TCR-Ts were generated from primary human (A) and primary mouse T cells (B). Contour plots show representative results for transduction efficacies in experiments summarized in figure 5. (A) Expression of transgenic TCRs (P1, 14/35, H1-1, H2-1, H2-2, and M1) in TCR-Ts generated from primary human T cells was assessed by measuring surface expression of TCRβ and CD8 using flow cytometry. The percentages in the upper quadrants show the fraction of CD8$^+$ and CD8$^-$ T cells with transgenic TCR expression. (B) To monitor TCR expression in primary mouse T cells, the indicated TCRs were expressed together with

GFP. The percentage of transduced $CD8^+$ and $CD8^-$ T cells (indicated in the upper quadrants) was measured by assessing expression of CD8 and GFP using flow cytometry.

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