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Supplemental Information

Targeting of Aberrant αvβ6 Integrin Expression in Solid Tumors Using Chimeric Antigen Receptor-Engineered T Cells

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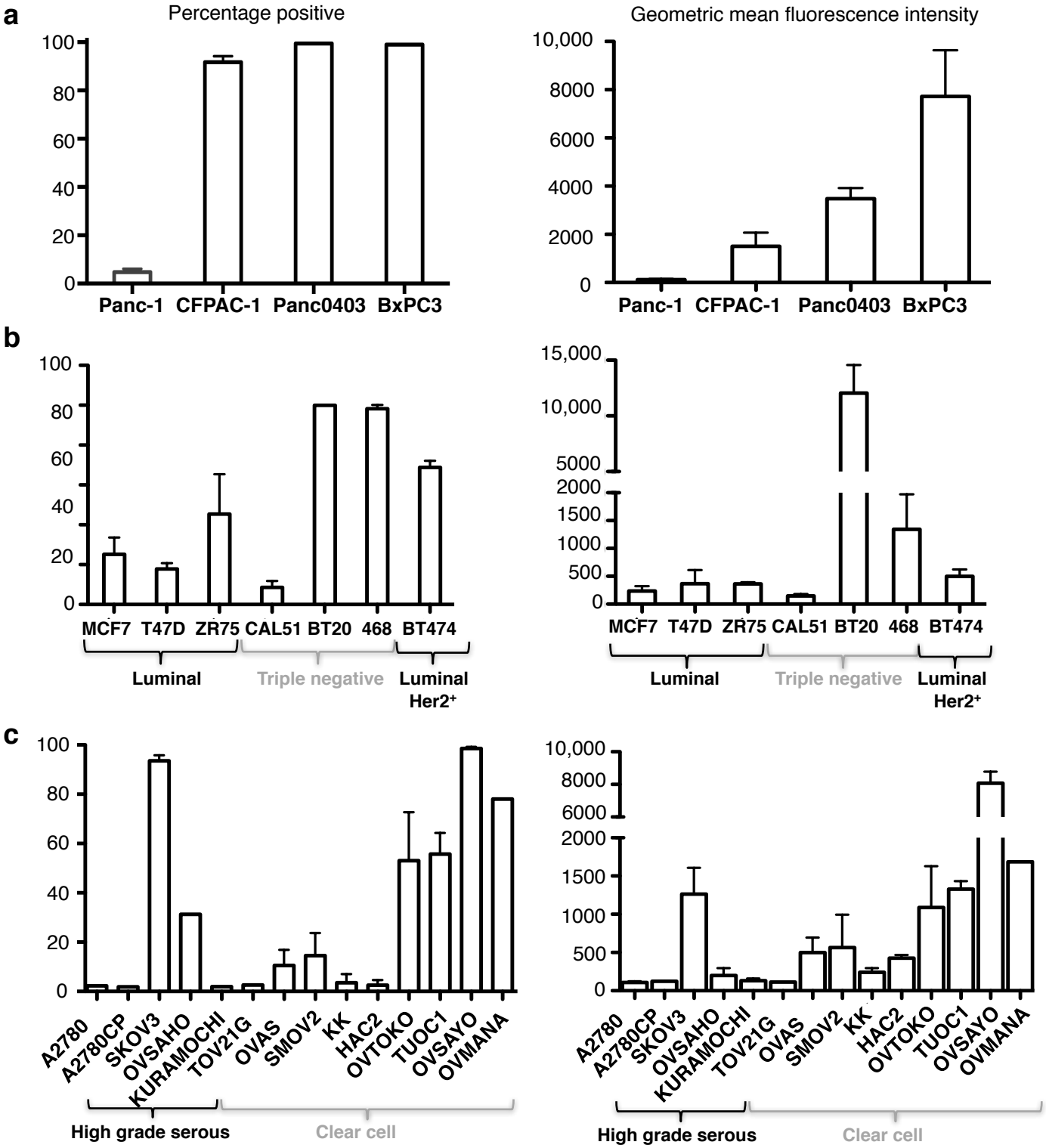
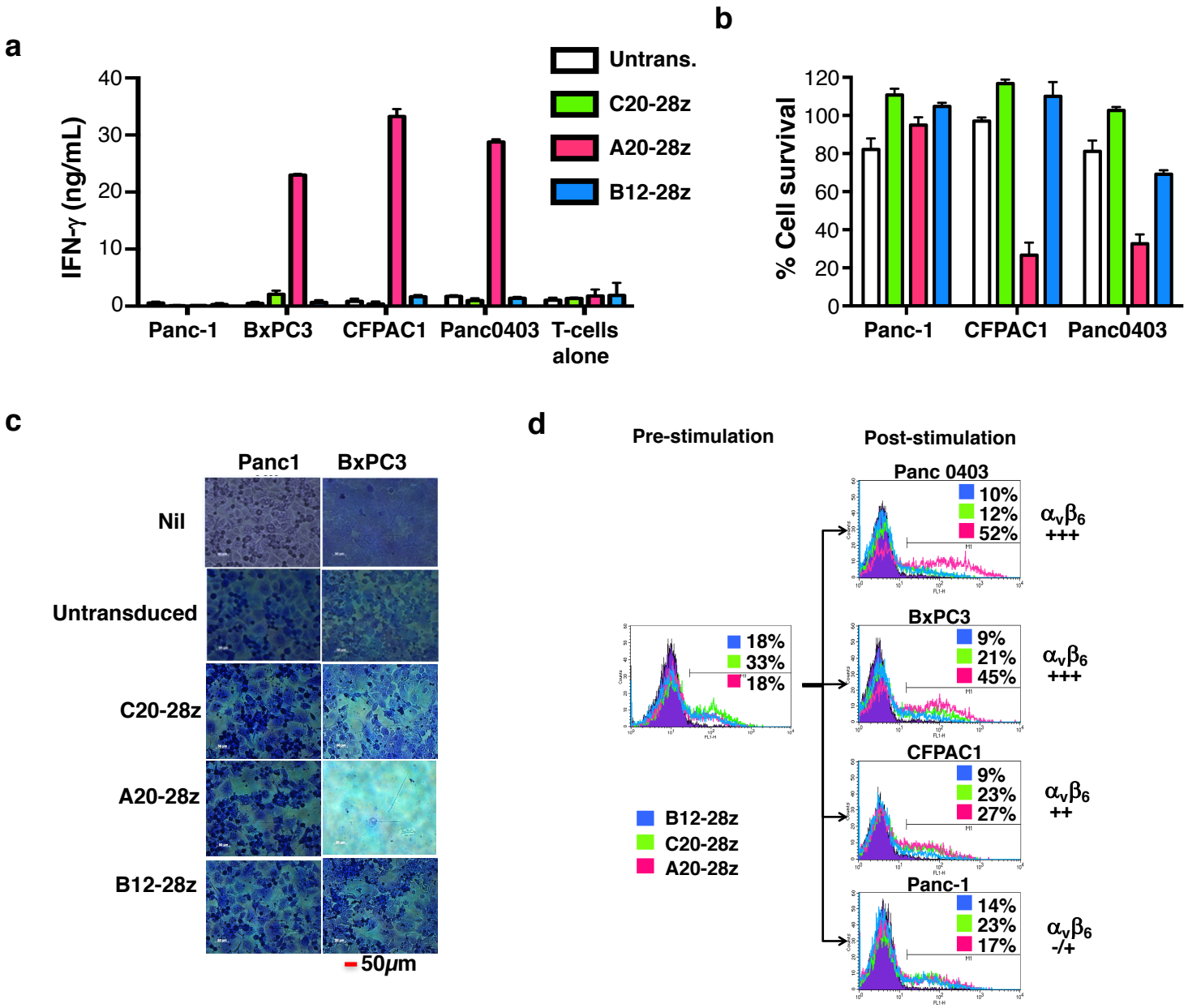


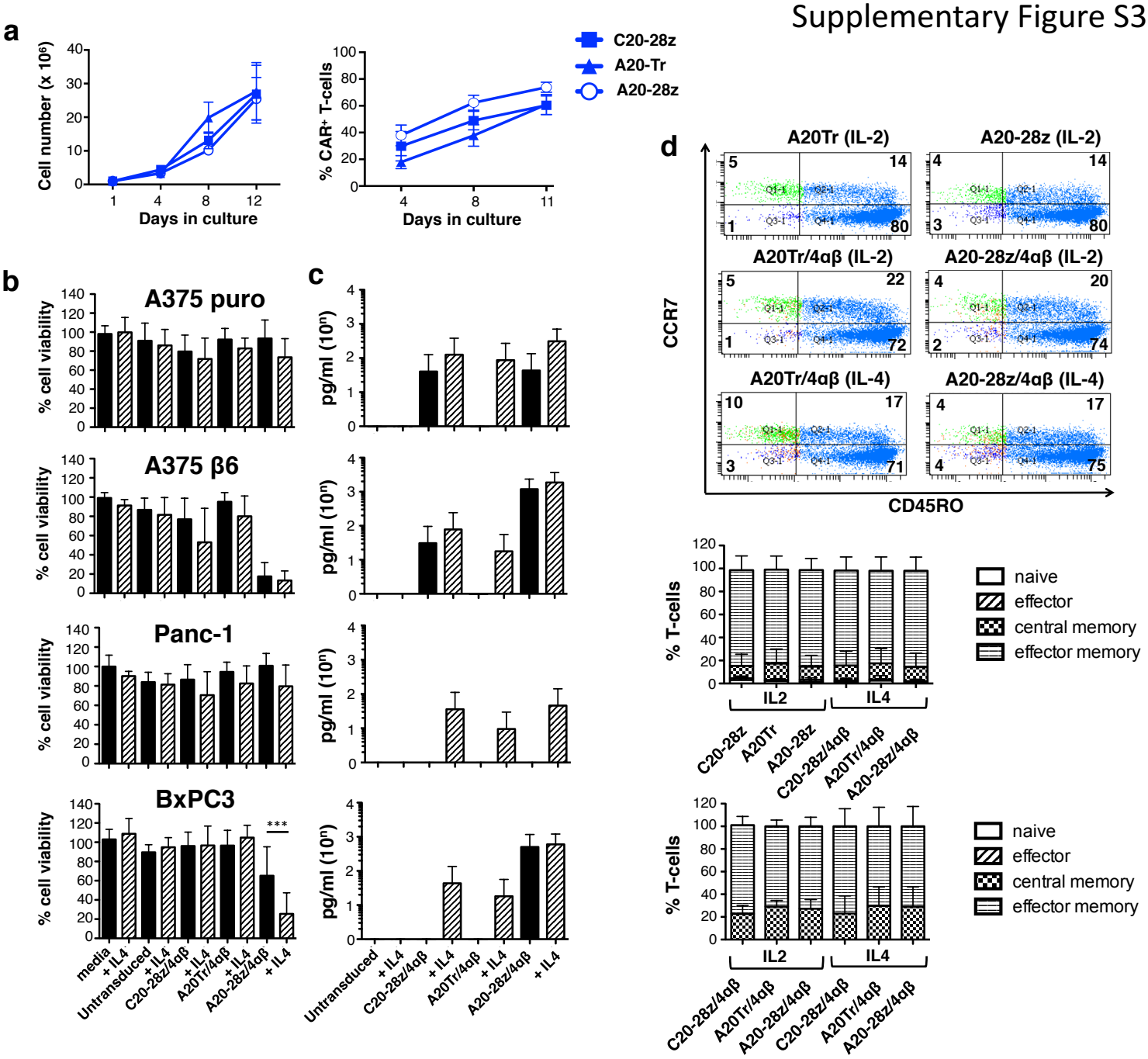
Supplementary Figure S1: Expression of αvβ6 integrin by tumor cell lines. Immortalized pancreatic (**a**), breast (**b**) and ovarian cancer cell lines (**c**) cells were analyzed for expression of the β6 integrin subunit by flow cytometry after incubation with the 6.3G9 antibody followed by goat anti-mouse IgG-PE. Cells stained with secondary antibody alone served as negative control. Data show the mean ± SD from 2-8 independent experiments.

Supplementary Figure S2

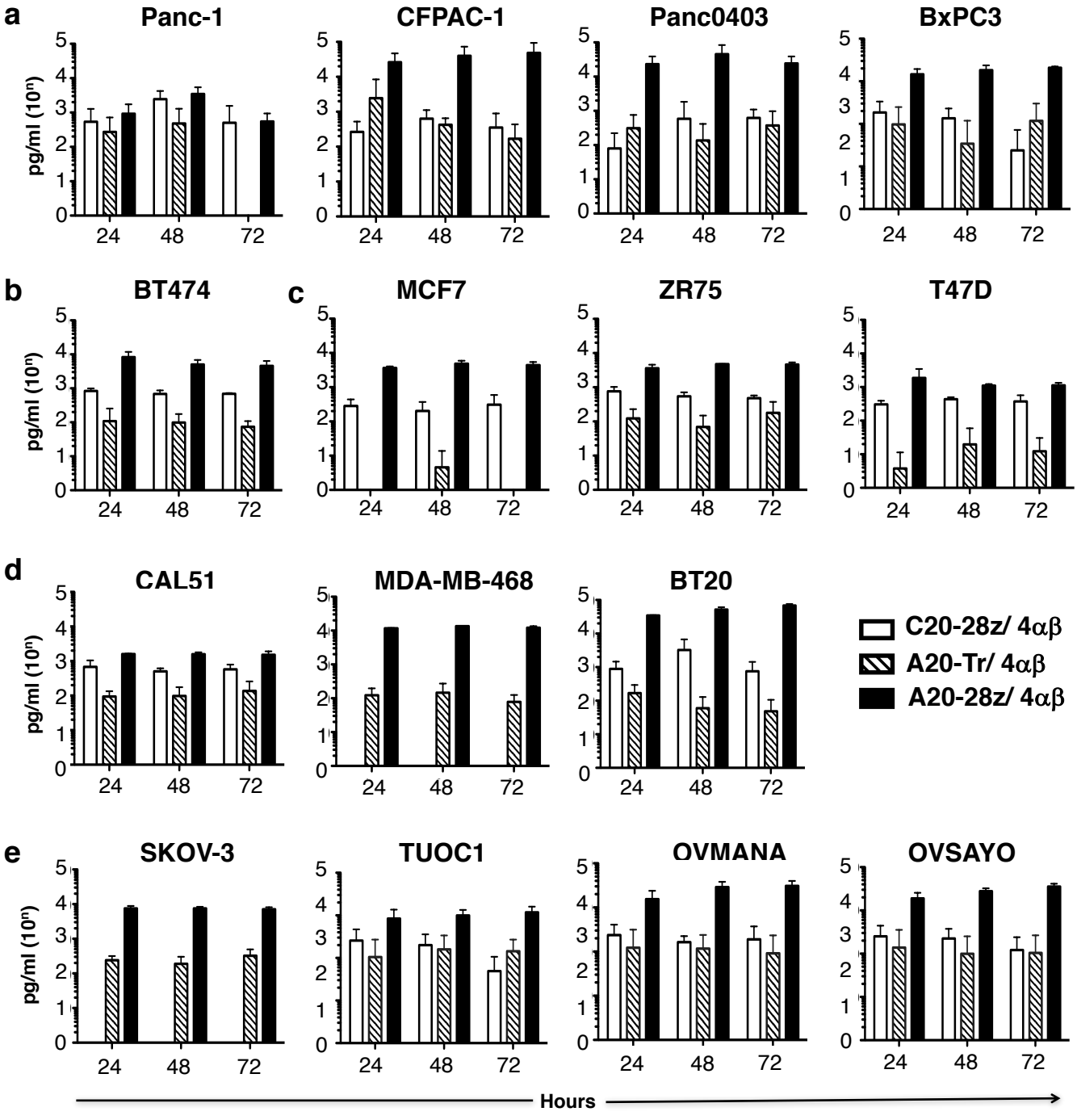


Supplementary Figure S2: *In vitro* comparison of anti-tumor activity of candidate αvβ6-specific CARs. (**a**) T-cells that expressed indicated CARs were co-cultivated at a 10:1 ratio with the specified pancreatic cell lines. After 24h, IFN-γ was measured in harvested supernatants while residual tumor cell viability was assessed by MTT assay (**b**). Both datasets show mean ± SD of 6 replicates. (**c**) 1 x 10^6 T-cells that expressed indicated CARs were co-cultivated with a confluent monolayer (24 well plate) of the indicated pancreatic tumor cell line, making comparison with untransduced T-cells or no addition (nil). After 24h, cultures were supplemented with IL-2 (100U/mL), which was added thereafter three times per week. Residual tumor monolayers were stained by crystal violet on day 8. (**d**) T-cells described in **c** were analyzed by flow cytometry for CAR expression before and after culture with the indicated monolayer for 8 days. Crystal violet and CAR T-cell enrichment data are representative of three independent cultures with αvβ6-expressing pancreatic tumor cell lines.

Supplementary Figure S3

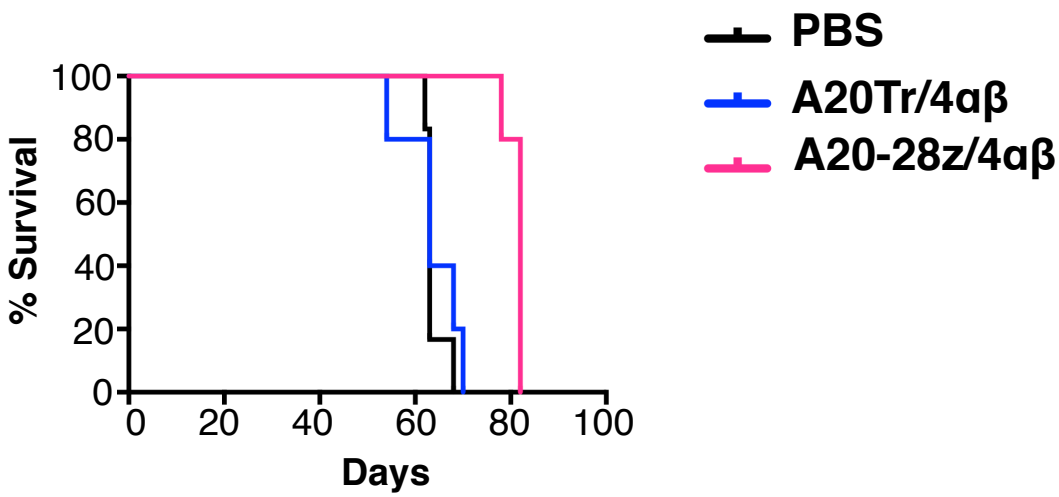


Supplementary Figure S3: Expansion and enrichment of CAR T-cells in IL-4. (a) Culture of 4αβ-expressing CAR T-cells in IL-4, demonstrating expansion and enrichment of the indicated CAR T-cell populations (mean ± SD, n=5-6 independent replicates). Tumour cells were co-cultivated at a 1:1 ratio with the indicated CAR/4αβ-engineered T-cells in the absence or presence of exogenous cytokine (30ng/ml), following *ex vivo* expansion and enrichment of CAR T-cells using IL-4. **(b)** Cytotoxicity and **(c)** IFN-γ release was analysed at 48hrs. Data show the mean mean ± SD of 5 independent experiments, each performed in triplicate. **(d)** The phenotype of T-cells cultured in either IL-2 or IL-4 for 12 days was assessed by flow cytometry. Data show representative plots and pooled data from T-cells derived from 5 independent donors.

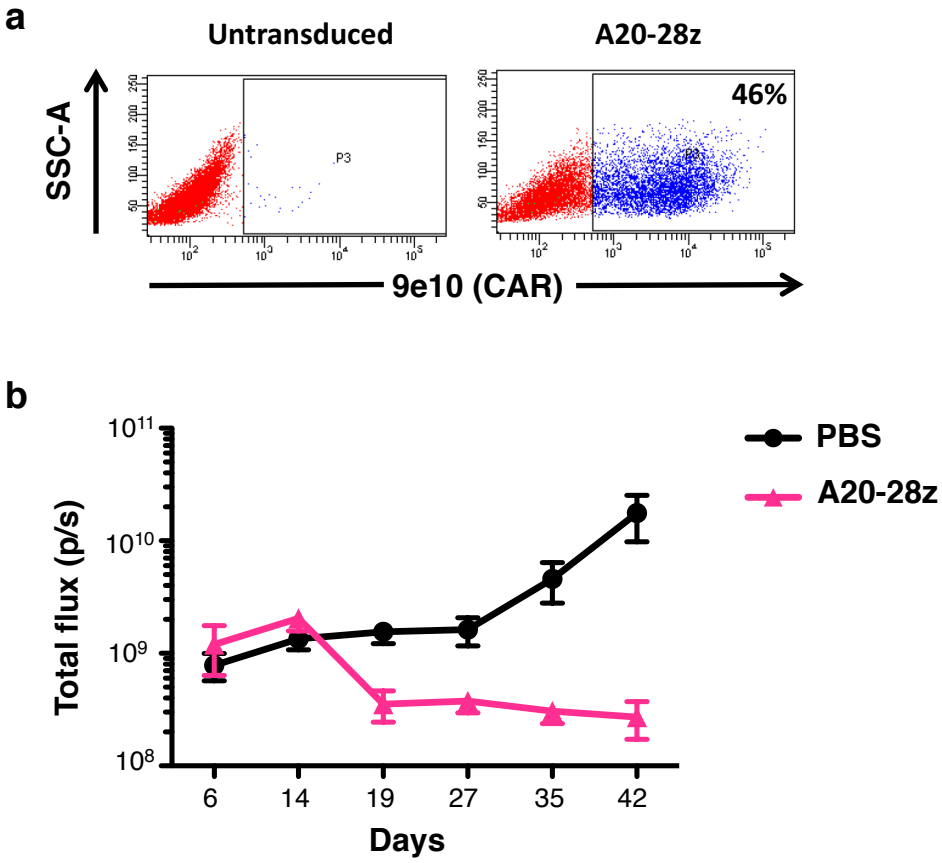


Supplementary Figure S4: Production of interferon (IFN)-γ by αvβ6 re-targeted CAR T-cells. Firefly luciferase-expressing pancreatic (**a**), HER2^{+} breast (**b**), luminal breast (**c**), triple negative breast (**d**), or ovarian tumor cells (**e**) were co-cultivated at a 1:1 ratio with the indicated CAR/4αβ-engineered T-cells in the absence of exogenous cytokine, following *ex vivo* expansion and enrichment of CAR T-cells using IL-4. Supernatant was harvested at each time-point and analyzed for IFN-γ. Data shows the mean ± SD of 3-6 independent replicates.

Supplementary Figure S5

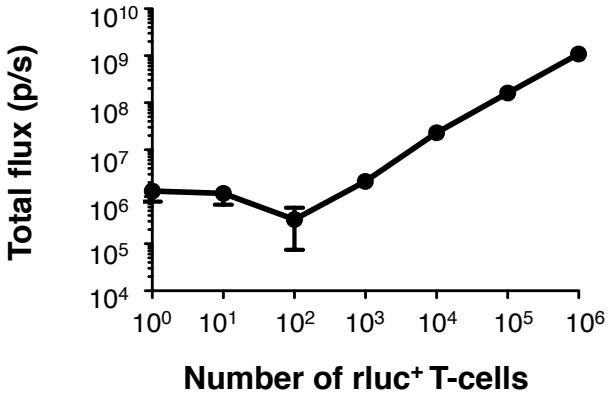


Supplementary Figure S5: Survival curve. Mice were injected i.p. with 1 x 10^6 SKOV-3-ffluc cells and tumors were allowed to establish for 21 days before i.p. treatment with 10 x 10^6 of the indicated gene-modified T-cells. Control mice received PBS. Animals were culled when humane endpoints were reached. p = 0.0014 comparing A20-28z/4$\alpha\beta$ treated mice with both other groups using the Log-rank (Mantel-Cox) test.

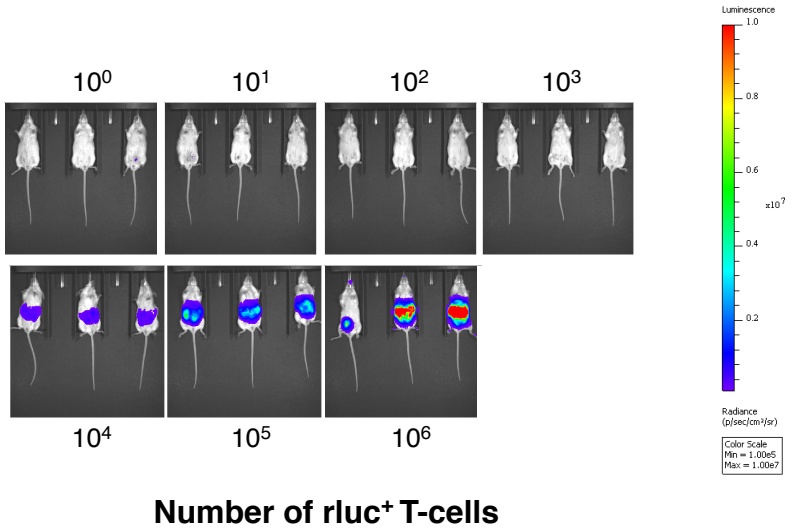


Supplementary Figure S6: *In vivo* anti-tumor activity of αvβ6 re-targeted CAR T-cells against Panc0403 PDAC xenografts. (**a**) T-cells were transduced with a retroviral vector encoding for A20-28z (e.g. without 4αβ). After culture for 6 days in IL-2, cells were analyzed by flow cytometry. 9e10 detects a myc epitope tag in the CAR ectodomain. SSC – side scatter. Gates were set using untransduced T-cells cultured in IL-2. (**b**) Mice were injected i.p. with 2 x 10^6 Panc0403-ffluc cells and tumors were allowed to establish for 14 days before i.p. treatment with 20 x 10^6 of A20-28z T-cells or PBS as control. Bioluminescence imaging using d-luciferin (substrate for ffluc) was used to monitor tumor status. Data show the mean ± SD of tumor-derived total flux (n=5 mice per group). The arrow indicates the day of treatment with CAR T-cells.

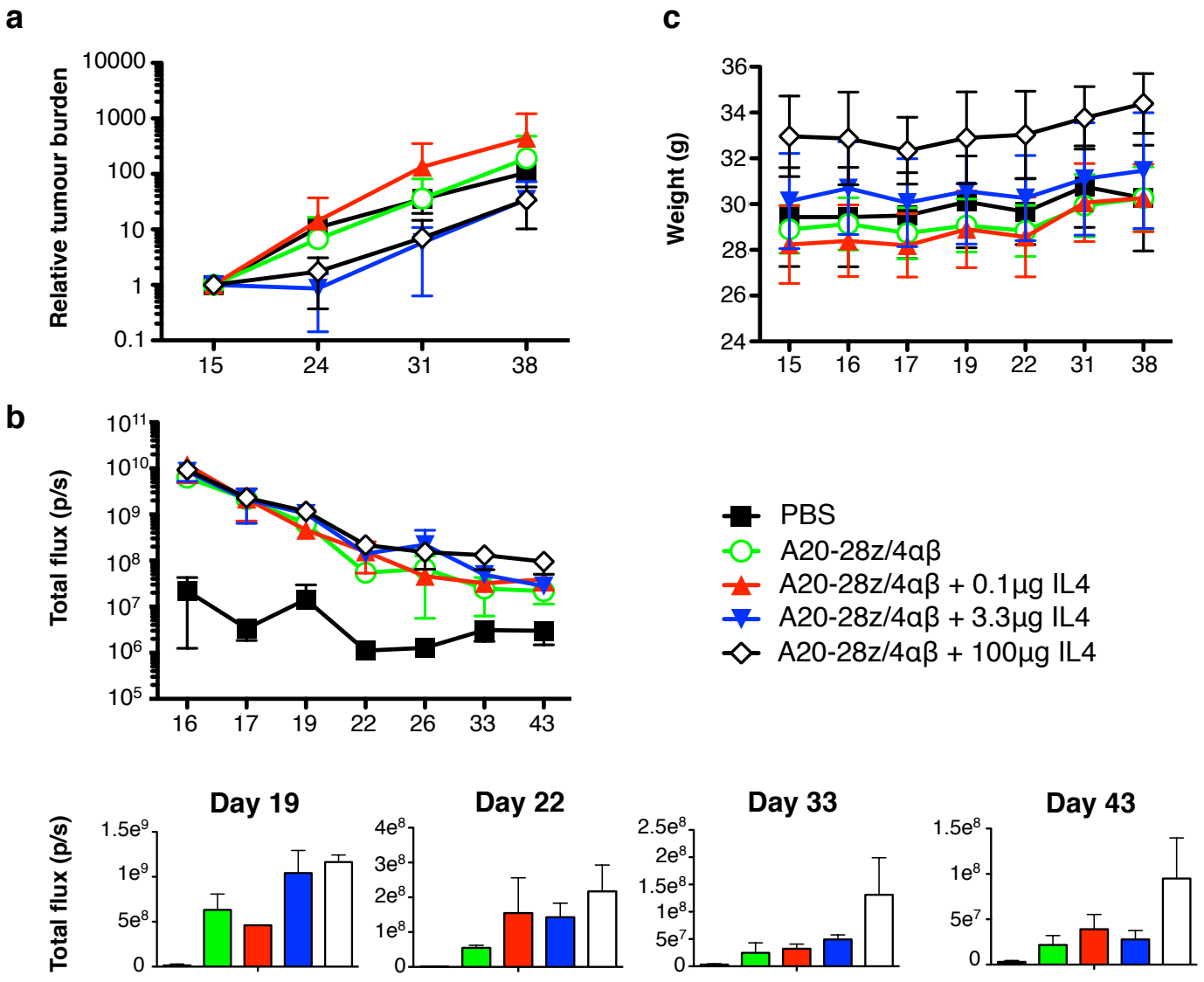
a



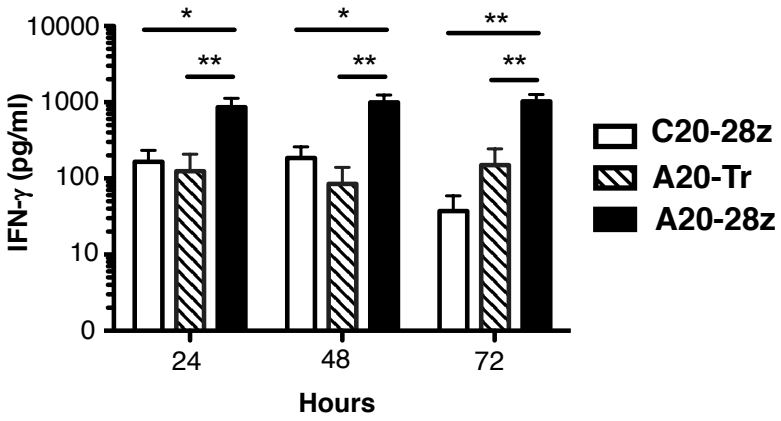
b



Supplementary Figure S7: *In vivo* imaging of adoptively transferred T-cells that express *Renilla* luciferase. (**a**) T-cells were transduced with a retroviral vector encoding rluc/GFP. The indicated number of rluc+ T-cells were injected i.p. into SCID-Beige mice, followed by i.p. injection of substrate (coelenterazine) and BLI imaging after 30 minutes. The graph shows the mean ± SD of 3 mice/group. Images of individual mice are shown in **(b).**

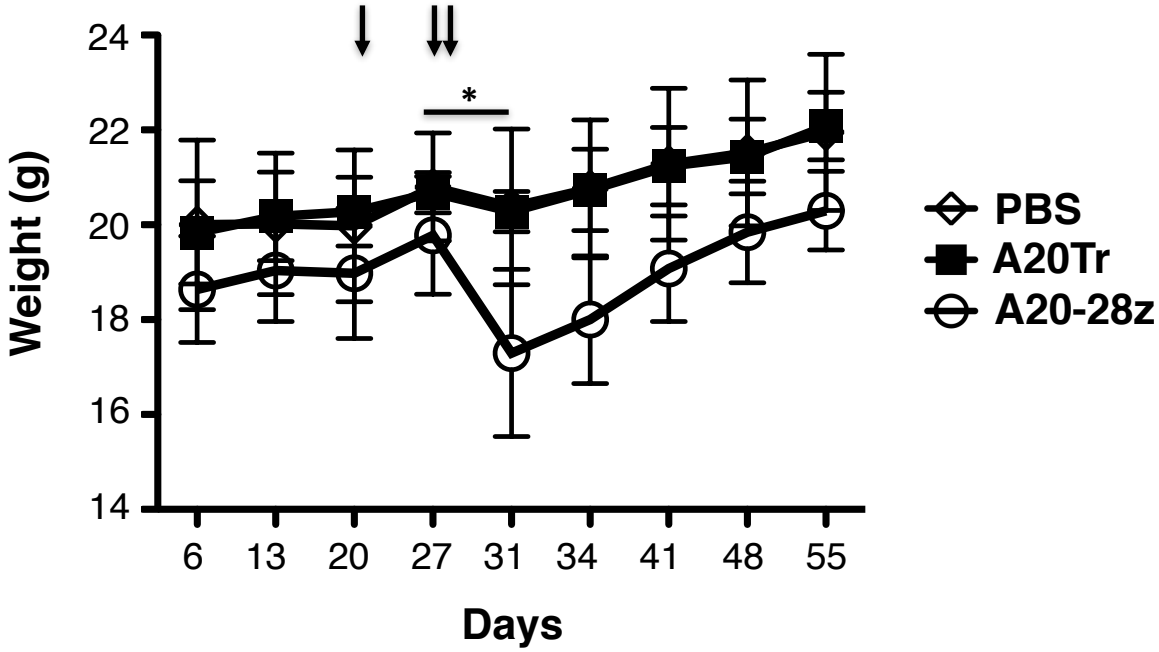


Supplementary Figure S8: Tumor stress test to evaluate effect of exogenous IL-4 treatment on T-cell activity *in vivo.* 1x10^5 BxPC3-ffluc cells were injected i.p. in NSG mice. Tumors were allowed to establish for 16 days prior to injection of 2.5 x 10^6 CAR T-cells i.p. Either PBS or the indicated dose of human IL-4 was administered 3 times per week thereafter using the i.p. route. T-cells were transduced with A20-28z/4αβ CAR and a retroviral vector encoding rluc/GFP to enable imaging of CAR T-cells. **(a)** Tumour burden was assessed weekly by BLI. Tumour burden is expressed relative to pre-treatment levels. **(b)** T-cells were imaged using coelenterazine administered i.p.. All data show the mean± SD of 3 mice/group. **(c)** Weight of mice during the study.



Supplementary Figure S9: Production of IFN-γ by αvβ6 re-targeted human CAR T-cells when stimulated by mouse tumor cells that naturally express αvβ6 integrin. 4T1 cells were co-cultivated at a 1:1 ratio with the indicated CAR/4αβ-engineered T-cells in the absence of exogenous cytokine, following *ex vivo* expansion and enrichment of CAR T-cells using IL-4. Supernatant was harvested at each time-point and analyzed for IFN-γ. Data shows the mean ± SD of 3 independent experiments, each performed in duplicate. * $p<0.05$; ** $p<0.01$.

Supplementary Figure S10



Supplementary Figure S10: *In vivo* safety testing of human αvβ6-retargeted CAR T-cells. SCID Beige mice received the indicated CAR T-cell populations that had been enriched to homogeneity following *ex vivo* culture in IL-4. Bolus doses of 20 million CAR T-cells were administered by iv (tail vein) injection at timepoints indicated by the overhead arrows. Animals were weighed at the indicated intervals (mean ± SD, n=5 mice per group). *p <0.05 for comparison of weight of A20-28z-treated mice on day 31 versus day 27.