BLOCKADE OF CD7 EXPRESSION IN T CELLS FOR EFFECTIVE CHIMERIC ANTIGEN-RECEPTOR TARGETING OF T-CELL MALIGNANCIES

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Supplemental Figures

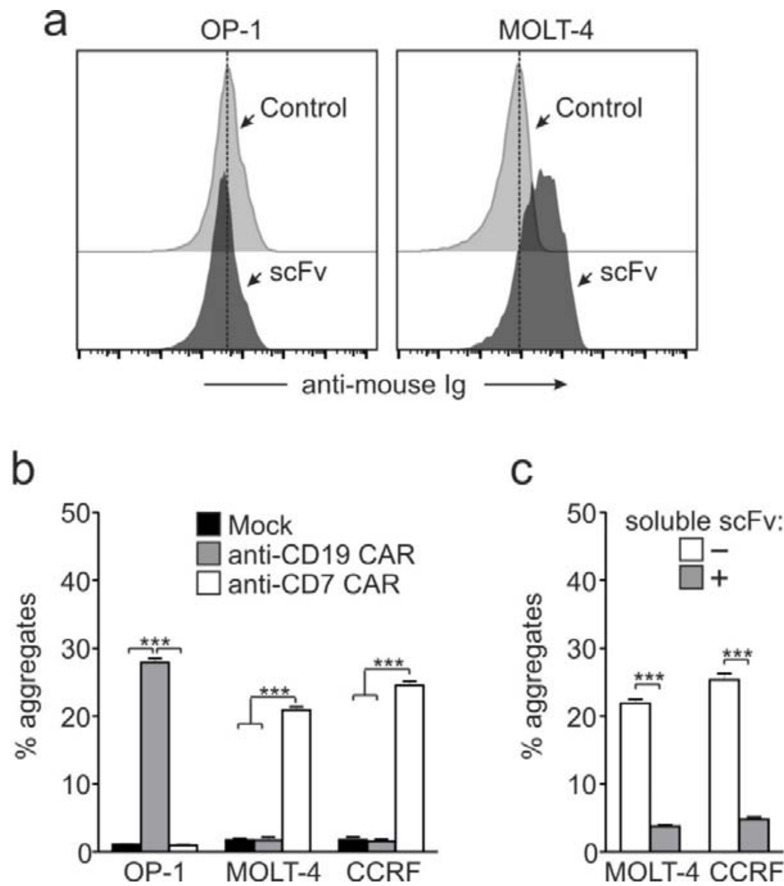

Figure S1. Specificity and function of the anti-CD7-41BB-CD3ζ CAR. **a.** OP-1 (CD7-) and MOLT-4 (CD7+) were incubated with supernatant collected from Jurkat cells transduced with anti-CD7 scFv, or transduced with a vector containing GFP only ("Control"). After washing, cells were incubated with biotin-conjugated goat anti-mouse F(ab')2 antibody followed by streptavidin-APC (Jackson ImmunoResearch). Flow cytometric histograms illustrate binding of the anti-CD7 scFv to MOLT-4 but not OP-1. **b.** Jurkat cells were transduced with anti-CD7-41BB-CD3ζ CAR, anti-CD19-41BB-CD3ζ CAR, or a vector containing GFP alone. These cells were co-cultured at 1:1 E:T with the CD7+ MOLT-4 or CCRF-CEM cells, or with the CD7- cells OP-1. Target cells were labelled with calcein red-orange AM (Invitrogen). After 30 minutes incubation, the percentage of cell doublets was measured by flow cytometry. Bars illustrate mean (± SD) of triplicate measurements. **c.** CAR-mediated cell aggregation is inhibited by pre-incubating target cells with a soluble form of the anti-CD7 scFv. *** P <0.001

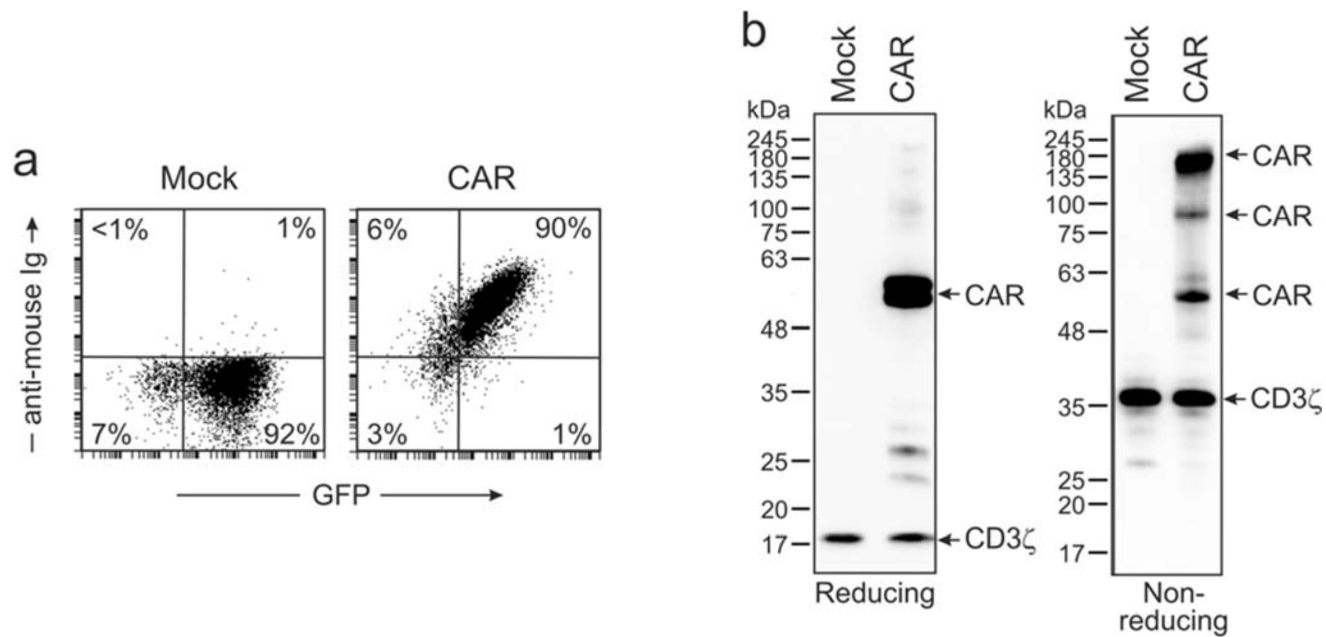

Figure S2. Expression of anti-CD7-41BB-CD3ζ CAR in human peripheral blood T lymphocytes. **a.** Representative flow cytometric dot plots of T lymphocytes activated for 7 days with Dynabeads Human T-Activator CD3/CD28 (ThermoFisher Scientific) and IL-2, and transduced with the anti-CD7 CAR. Flow cytometric dot plots illustrate GFP fluorescence and CAR expression, the latter revealed by staining with biotin-conjugated goat anti-mouse F(ab')2 antibody followed by streptavidin-APC (Jackson ImmunoResearch). **b.** Western blot analysis of CAR expression. Cell lysates of mock- and CAR-transduced T cells were separated on a 10% polyacrylamide gel under reducing or non-reducing conditions. The blotted membrane was probed with a mouse anti-human CD3ζ antibody (8D3; BD Biosciences) followed by goat anti-mouse IgG conjugated to horseradish peroxidase (R&D Systems). Antibody binding was revealed with Clarity Western ECL Substrate (Bio-Rad).

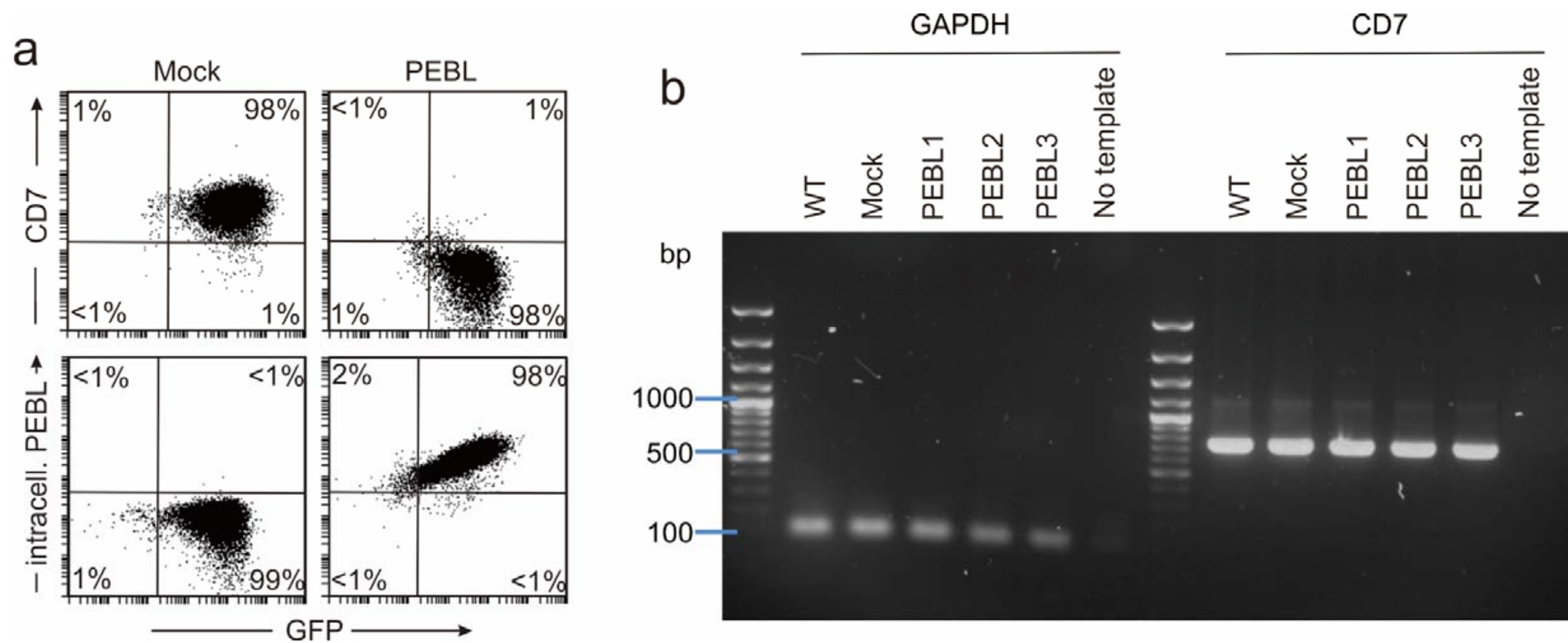

Figure S3. Downregulation of CD7 protein expression with anti-CD7 PEBLs. **a.** Flow cytometric dot plot illustrate GFP expression (x axes), CD7 expression (y axes, top row), and intracellular anti-CD7 PEBL -1 expression (y axes, bottom row). T lymphocytes were retrovirally transduced with anti-CD7 PEBL-1 or a vector containing GFP alone ("Mock").. T-cells were stained with an anti-CD7 antibody (M-T701; BD Biosciences) conjugated to phycoerythrin. Intracellular expression of PEBL-1 was tested with a PE-conjugated anti-Myc antibody (9B11; Cell Signaling Technology) which binds to the sequence EQKLISEEDL incorporated in the ER-binding motif. Prior to antibody labelling, cells were permeabilized with 8E reagent (a permeabilization reagent developed in our laboratory). **b.** RT-PCR analysis of CD7 mRNA expression. cDNA derived from total mRNA extracted from Jurkat cells transduced with PEBL1-3, GFP alone ("mock"), or untransduced ("WT") was used as template. CD7 cDNA (723 bp) was amplified with the following primers: Forward, ATGGCCGGGCCTCCG, Reverse, TCACTGGTACTGGTTGGG. Electrophoresis was performed on a 1% agarose gel with SYBR Safe Gel Stain (ThermoFisher). No template control is also shown. A 87bp (676-762th nucleotide) region of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified in parallel as a control.

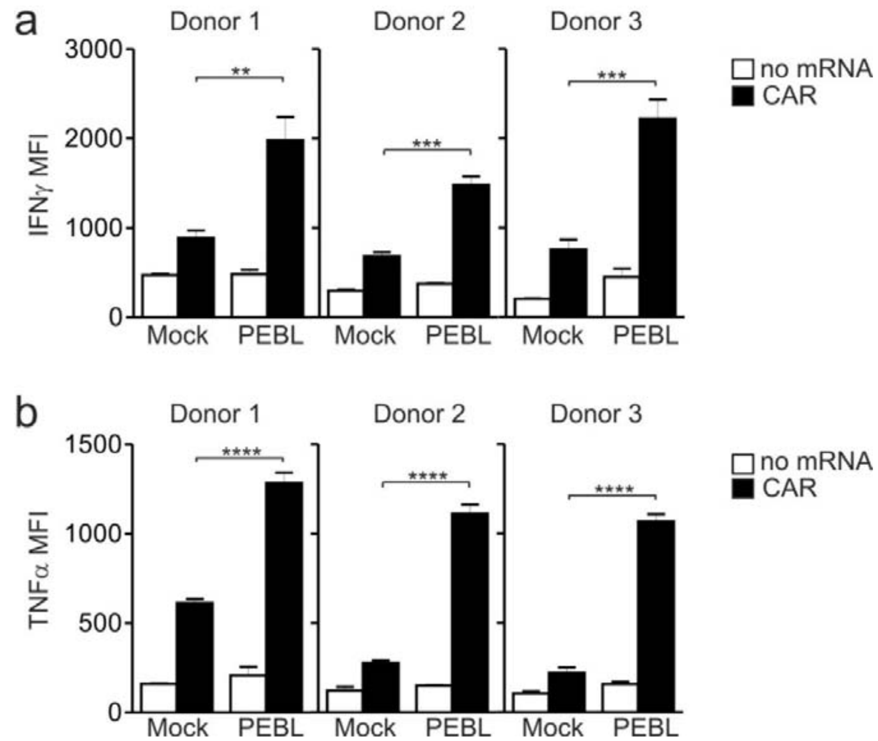

Figure S4. Anti-CD7 CAR signal elicits higher cytokine secretion in T cells with CD7 knock-down expression by anti-CD7 PEBL. T lymphocytes from 3 donors were transduced with anti-CD7 PEBL or GFP alone ("Mock") were electroporated with either anti-CD7-41BB-CD3ζ mRNA or no mRNA. Intracellular IFNγ (**a**) and TNFα (**b**) expression in T cells after 6 hours of co-culture with MOLT4 was measured. Bars represent mean (± SD) of triplicate MFI measurements. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

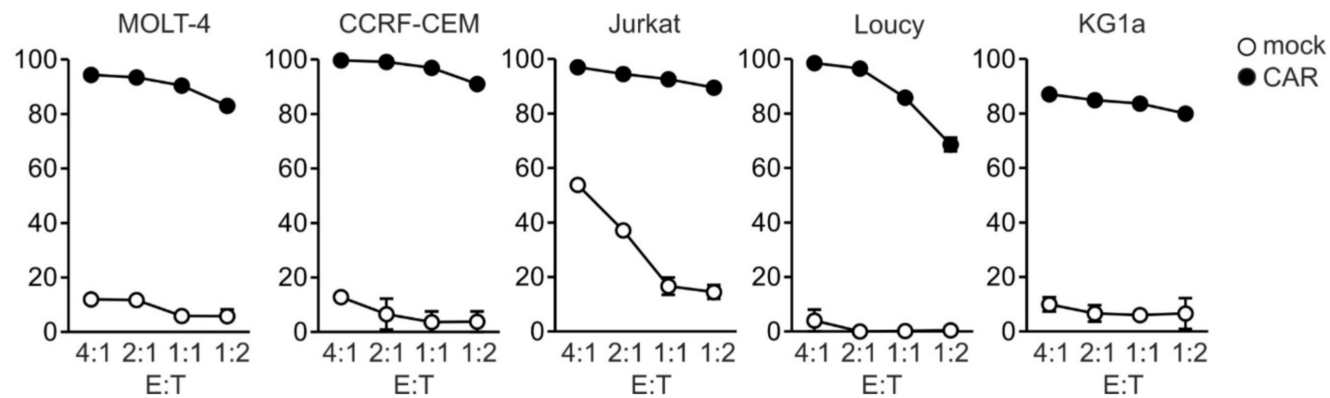

Figure S5. CD7-negative T-cells expressing anti-CD7-41BB-CD3ζ CAR exert anti-tumour cytotoxicity against CD7+ cell lines. Shown are results of 4-hour cytotoxicity assays performed with T cells transduced with anti-CD7 PEBL and then transduced with either CD7-41BB-CD3ζ or GFP only ("Mock"). Symbols represent mean (± SD) of triplicate experiments at the indicated E:T ratios. P <0.001 for all comparisons.

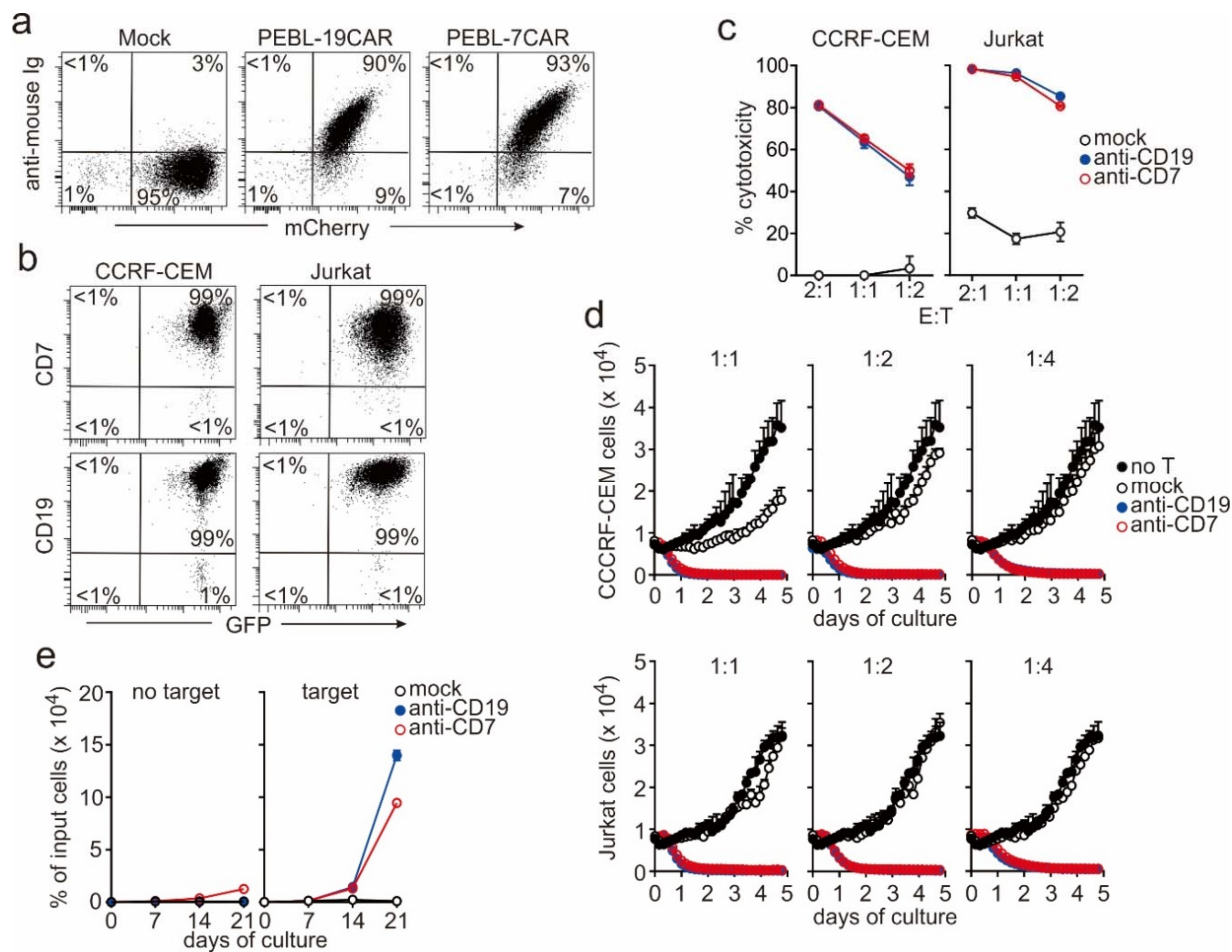

Figure S6. Functional comparison of anti-CD7-41BB-CD3ζ and anti-CD19-41BB-CD3ζ CARs. **a.** Expression of anti-CD19 and anti-CD7 CARs (in an mCherry-containing vector) in peripheral blood T cells previously transduced with anti-CD7 PEBL. Flow cytometry dot plots illustrate mCherry expression and staining of T cells with biotin-conjugated goat anti-mouse F(ab')2 antibody followed by streptavidin

conjugated to allophycocyanin (Jackson ImmunoResearch). Results with T cell transduced with a vector containing mCherry alone ("Mock") are also shown. **b.** Expression of CD19 in CCRF-CEM and Jurkat cells transduced with a vector containing CD19 and GFP. CD19 was detected with anti-CD19 APC (Miltenyi Biotech). **c.** Four-hour cytotoxicity assays targeting CD19+ CCRF-CEM or CD19+ Jurkat cells with anti-CD19 or anti-CD7 PEBL-CAR-T cells at different E:T ratios. Symbols indicate mean (± SD) of triplicate measurements. $P<0.001$ for data with either CAR versus mock-transduced T cells at all E:T ratios. **d.** Long-term cytotoxicity of anti-CD19 or anti-CD7 PEBL-CAR-T cells at different E:T ratios as measured by live cell image analysis with IncuCyte Zoom System (Essen BioScience). Symbols indicate mean (± SD) of 3 measurements of CD19+ CCRF-CEM (top) or CD19+ Jurkat cells (bottom) in wells containing CAR-T cells, mock-transduced T cells, or no T cells. Measurements were performed at 4-hour intervals. **e.** Proliferative capacity of anti-CD19 and anti-CD7 PEBL-CAR-T cells with and without co-culture with CD19+ Jurkat cells. Anti-CD7 PEBL-transduced T-cells, sequentially transduced with anti-CD19 or anti-CD7 CARs or mCherry alone, were cultured alone or in presence of irradiated CD19+ Jurkat cells, added weekly and 120 IU/mL IL-2. Symbols indicate mean (± SD) percentage of cell recovery relative to number of input cells in triplicate cultures.

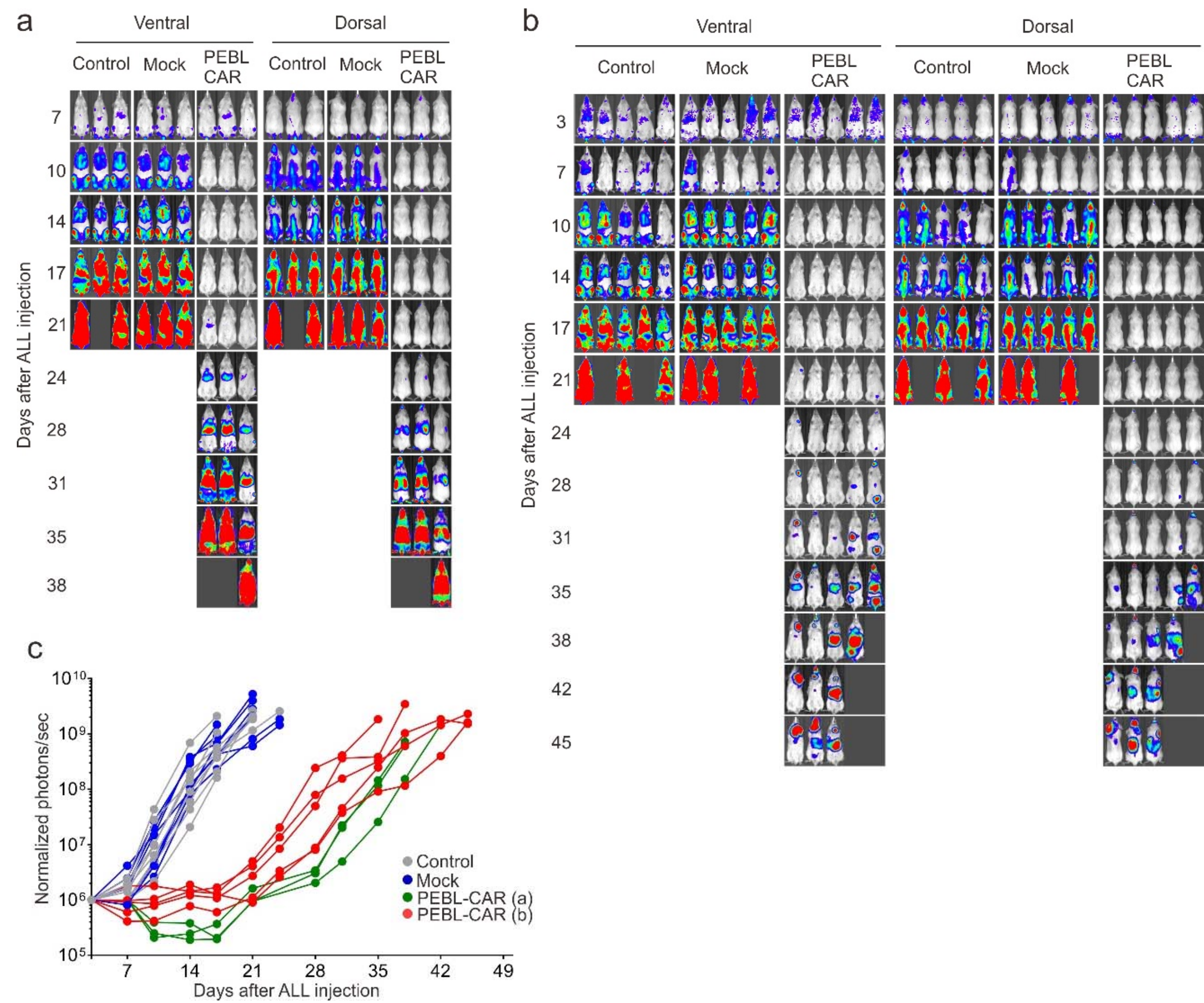

Figure S7. PEBL-transduced T-cells expressing anti-CD7-41BB-CD3ζ CAR exert antitumor activity in mouse models. NOD-SCID-IL2RGnull

mice were infused intravenously with 1 x 10^6 CCRF-CEM cells labeled with luciferase. 2 x 10^7 PEBL-CAR T cells were administered intravenously on day 7 **(a)** or on day 3 and day 7 **(b)** after leukemic cell infusion to 3 and 5 mice, respectively. The remaining mice received either mock-transduced T cells, or RPMI-1640 instead of cells ("Control"). All mice received 20,000 IU IL-2 once every two days intraperitoneally (i.p.). In vivo imaging of leukemia cell growth was performed after D-luciferin i.p. injection. Ventral images of mice on day 3 in **b** are shown with enhanced sensitivity to demonstrate leukemia cell engraftment in all mice. **c.** Leukemia cell growth expressed as photons per second over time normalised to average of ventral plus dorsal signals in all mice before CAR-T cell infusion. Each symbol corresponds to bioluminescence measurements in each mouse.

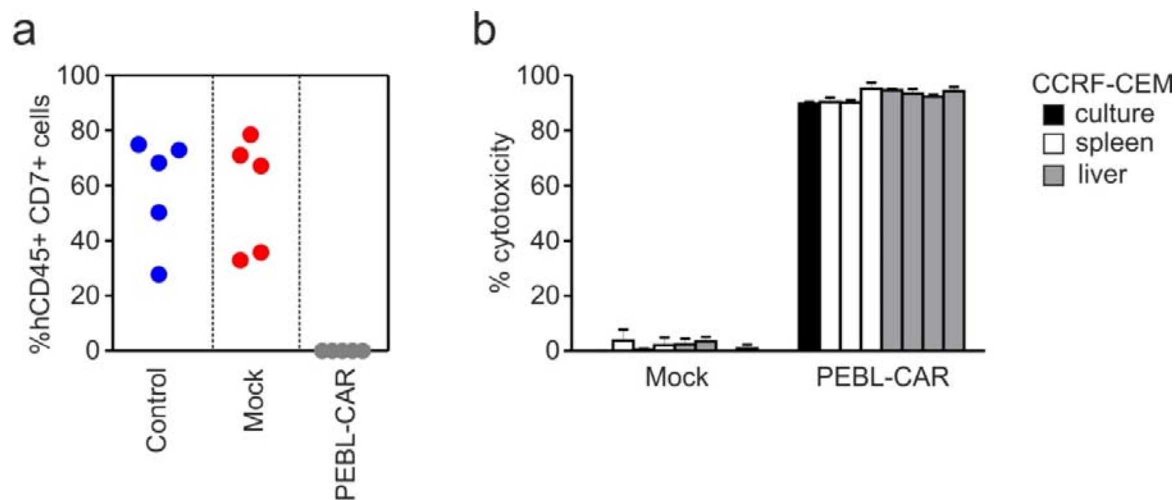

Figure S8. PEBL-transduced T cells expressing anti-CD7-41BB-CD3ζ CAR exert antitumor activity in mouse models and remain active against cells collected at relapse. **a.** Percentage of CCRF-CEM cells among white blood cells in blood from NOD-SCID-IL2RGnull mice infused i.v. with CCRF-CEM cells labelled with luciferase and then treated intravenously with either PEBL-CAR-transduced T-cells, mock-transduced T-cells, or RPMI-1640 instead of cells ("Control"), as described for Fig. 6b. For "Control" and "Mock", blood was obtained from euthanized mice that had reached bioluminescence threshold of 10^{10} photons/second 17-23 days after leukemia cells infusion. For PEBL-CAR mice, blood was obtained via cheek prick on day 24 after CCRF-CEM infusion. **b.** CCRF-CEM cells collected at relapse from the spleen and liver of mice treated with PEBL-CAR were cultured for 2 days. They were then used as targets in 4-hour cytotoxicity assay at E:T 1:1 using PEBL-CAR- or mock-transduced T-cells originally used for infusion. Comparison was also made with the same batch of CCRF-CEM-expressing luciferase cells used to generate the xenograft. Percentage cytotoxicity was determined from plate measurements of bioluminescence signal after addition of BrightGlo luciferase assay system (Promega). Bars show mean (± SD) of triplicate measurements; each white and grey bar corresponds to cells from one mouse.

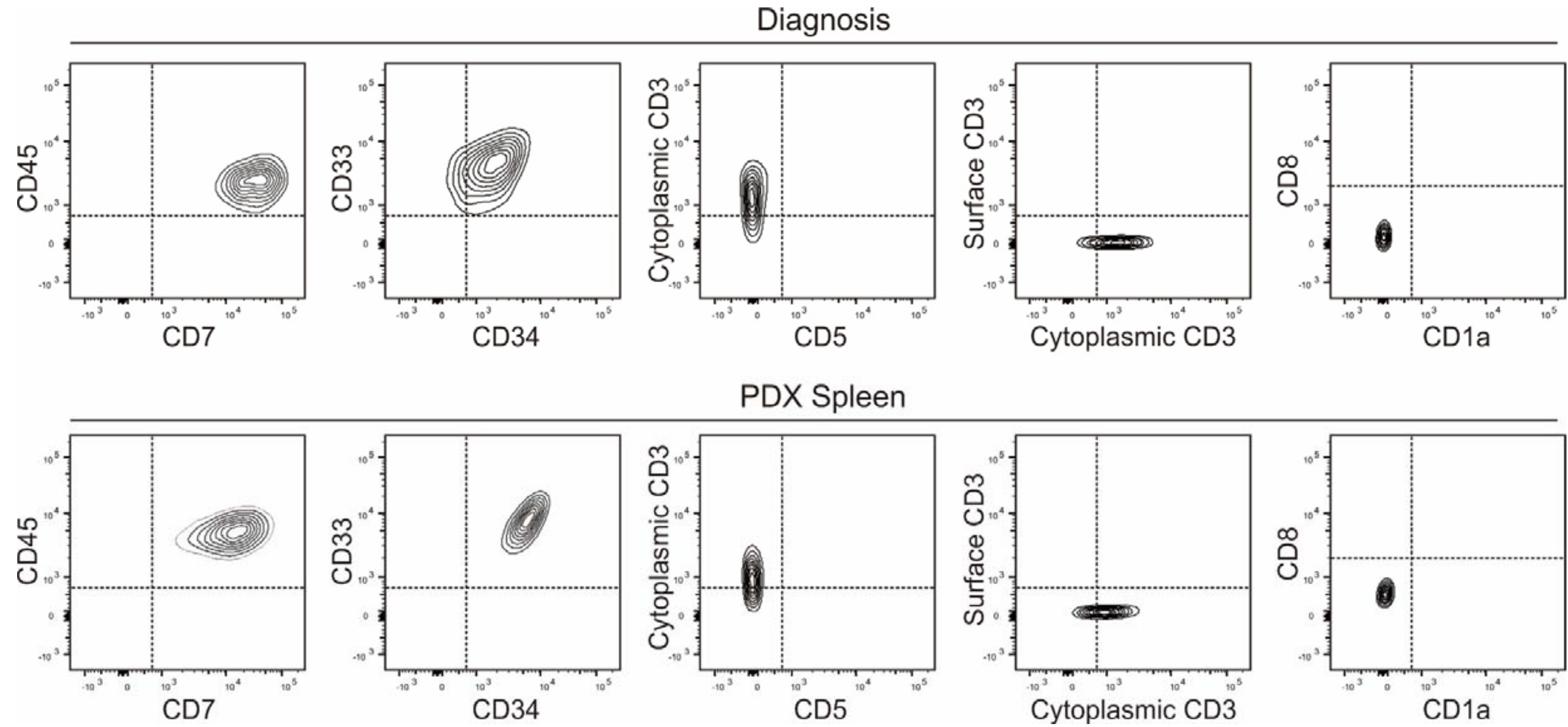

Figure S9. Immunophenotypic features of ETP-ALL at diagnosis and after propagation in NOD-SCID-IL2RGnull mice. Flow cytometric contour plots show the immunophenotype diagnostic bone marrow samples of the ETP-ALL used to develop the PDX model in this study **(a)**, and that of the ETP-ALL cells recovered from the spleen of one of the control mice shown in Fig. 7 **(b)**. The following antibodies were used: CD7-PE, CD45-APC-H7, CD34-PerCP, CD8-BV510, CD5-PE-Cy7, CD3-PerCP (for cytoplasmic staining), CD3-V450 (for surface staining), all from BD Biosciences; CD33-BV421 (Biolegend); CD1a-PE (Beckman Coulter). Quadrants were drawn based on staining with isotype-matched non-reactive antibodies conjugated to the same fluorochromes.