iPSC-DERIVED CD4+, CD8+ αβT CELLS THAT EXPAND UPON ANTIGEN STIMULATION AND EXHIBIT IN VIVO TUMOR CONTROL

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INTRODUCTION

The clinical success of autologous CAR-T cells relies upon acute cytotoxicity, expansion upon target engagement, cytokine secretion, and persistence but so far iPSC-derived cells cannot recapitulate adult αβT cell functionality. Notably, autologous CAR-T cells are a mix of mature CD4+ and CD8+ cells, where each plays a supporting or direct cytotoxic role, respectively.

We report the scalable generation of feeder-free CD4+ and CD8+ αβ iPSC-CAR-T cells that specifically expand upon target engagement, secrete the critical cytokine IL-2, and successfully control tumor cells in CD19 systemic tumor models. In addition to rapid tumor control, iPSC-derived CAR-T cells showed temporary control of tumor re-challenge 36 days after initial tumor clearance and persistence in the bloodstream three weeks after infusion. In a subsequent study, we were able to demonstrate tumor control and in-vivo expansion of iPSC-derived CAR-T cells after tumor re-challenge at day 15 after initial infusion. Finally, iPSC-derived CAR-T cells show function at doses as low as 10e6/mouse. In sum, we have developed a process that can generate engineered iPSC CAR T cells that perform comparably to primary T cells in a full suite of preclinical assays, unlocking the potential of the platform to solve key challenges faced by allogeneic cell therapies.

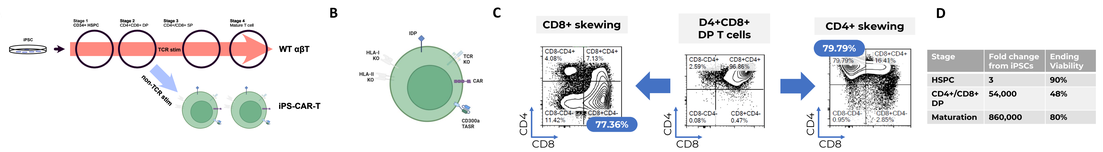


Figure 1. iPSC-CAR-T differentiation platform offers defined control over the mix of CD4+ and CD8+ single positive αβT cells. A Schematic of the challenge posed by elimination of the TCR and introduction of the CAR into engineered cells. **B.** Engineered iPSC-CAR-T lines include an anti-BCMA or anti-CD19 CAR in addition to a) the AlloEvasion 5.0 pair of IDP and CD300a TASR, b) knockout of HLA class I and II, and c) knockout of the TCRab complex. **C.** CAR engineered, Stage 2 CD4+CD8+ DP T cells are differentiated to single positive CD4+ or CD8+ T cells in a process that can be purposely shifted by alteration of conditions. **D.** Fold change and viability across the differentiation process.

iPSC-CAR-T CELLS TRAVERSE THYMOCYTE DEVELOPMENT

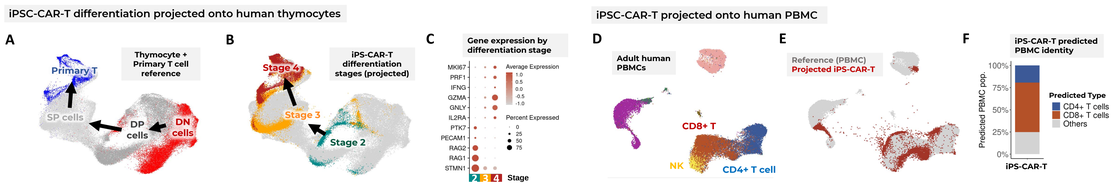
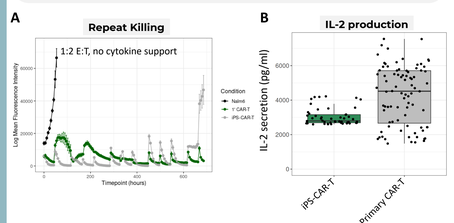


Figure 2. Century iPSC-CAR-T differentiation traverses a biologically recognizable path. A. UMAP projection of a subset of a public thymocyte development atlas (Park et al.) integrated with internal adult primary T cell data. Colored by development stage. **B.** Projection of iPS-CAR-T data into reference UMAP space. **C.** Gene expression by differentiation stage shows expected down regulation of RAG1/2 and increase in cytotoxic-related genes. **D.** UMAP projection of human adult PMBCs colored by cell type annotation. **E.** Projection of iPS-CAR-T cells into reference **F.** Projected iPS-CAR-T are predicted to have CD8+ T cell and CD4+ T cell PBMC identity.

iPSC-CAR-T DEMONSTRATE αβT CELL FUNCTION IN VITRO



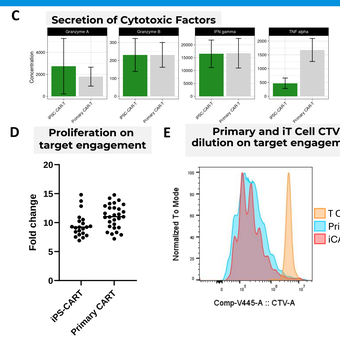


Figure 3. Century iPSC-CAR-T cells demonstrate robust T cell function in vitro. A. Sustained cytotoxicity observed in a repeat killing assay with NALM6 targets at an E:T ratio of 1:2. **B.** IL-2 production detected by ELISA after 48-hour exposure to targets is similar to primary T cell levels. **C.** Quantification of secreted proteins important for cytotoxicity show similar levels between iPS-CAR-T and primary CAR-T. Data shown for effectors with CD19 CAR challenged with Nalm6 targets for 48 hours. **D.** Antigen-mediated effector expansion evident after 5-day target exposure by cell counts after verification that targets are absent. **E.** iPSC-CAR-T cell proliferation measured by CTV dilution is similar to primary CAR-T cells after a NALM6 target cell challenge at an E:T of 1:1 in an assay without cytokine support (Day 5 readout).

SINGLE iPSC-CAR-T INFUSION CONTROLS TUMOR, PERSISTS IN VIVO

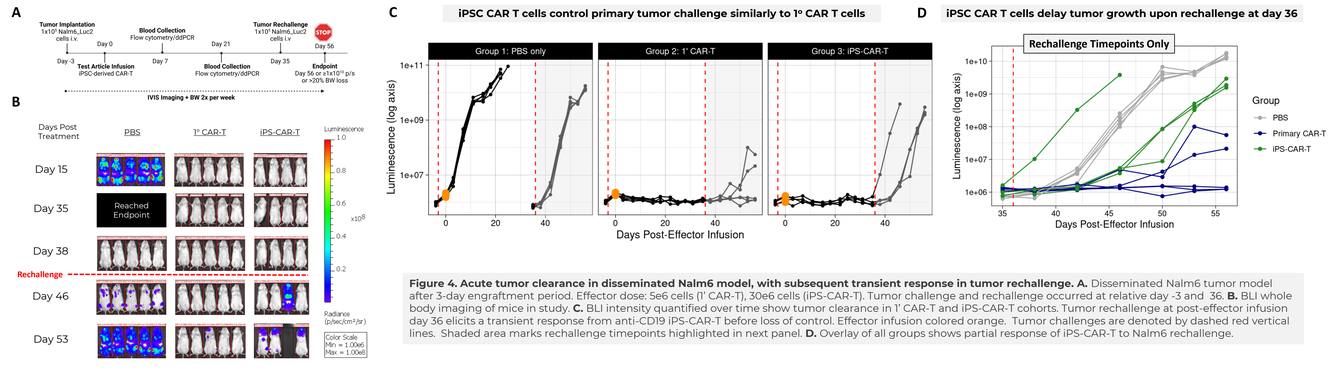


Figure 4. Acute tumor clearance in disseminated Nalm6 model, with subsequent transient response in tumor rechallenge. A. Disseminated Nalm6 tumor model after 3-day engraftment period. Effector dose: 5e6 cells (1' CAR-T), 30e6 cells (iPS-CAR-T). Tumor challenge and rechallenge occurred at relative day -3 and 36. **B.** BLI whole body imaging of mice in study. **C.** BLI intensity quantified over time show tumor clearance in 1' CAR-T and iPS-CAR-T cohorts. Tumor rechallenge at post-effector infusion day 36 elicits a transient response from anti-CD19 iPS-CAR-T before loss of control. Effector infusion colored orange. Tumor challenges are denoted by dashed red vertical lines. Shaded area marks rechallenge timepoints highlighted in next panel. **D.** Overlay of all groups shows partial response of iPS-CAR-T to Nalm6 rechallenge.

IN VIVO TARGET-MEDIATED EXPANSION OF iPSC-CAR-T CELLS

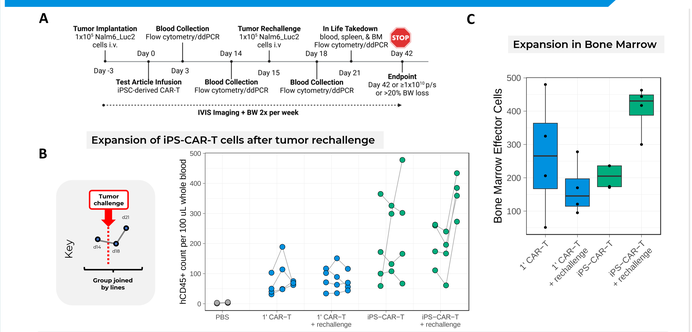


Figure 5. Target-mediated expansion of iPS-CAR-T cells evident in circulating cells and bone marrow upon rechallenge. A. Disseminated Nalm6 tumor model after 3-day engraftment period with rechallenge at day 15 post effector-infusion. Effector dose: 5e6 cells (1' CAR-T), 30e6 cells (iPS-CAR-T). Effector counts for iPS-CAR-T before and after rechallenge with Nalm6 targets 15 days after the initial effector infusion in circulation **(B)** and bone marrow **(C)**. All iPS-CAR-T-infused mice demonstrated complete tumor control through day 21.

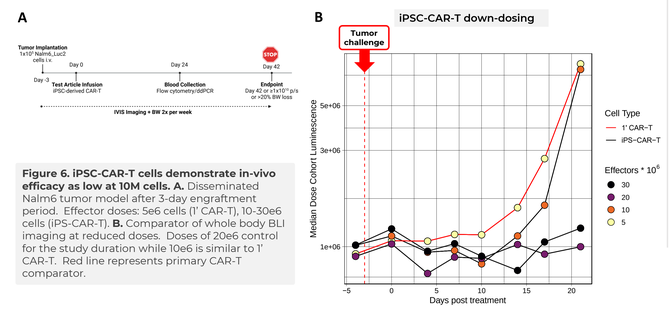


Figure 6. iPSC-CAR-T cells demonstrate in-vivo efficacy as low at 10M cells. A. Disseminated Nalm6 tumor model after 3-day engraftment period. Effector doses: 5e6 cells (1' CAR-T), 10-30e6 cells (iPS-CAR-T). **B.** Comparator of whole body BLI imaging at reduced doses. Doses of 20e6 control for the study duration while 10e6 is similar to 1' CAR-T. Red line represents primary CAR-T comparator.

GENE SIGNATURE OF IMPROVED IN VIVO PERFORMANCE IDENTIFIED

In vivo performance = measured as progression-free survival (PFS) scores

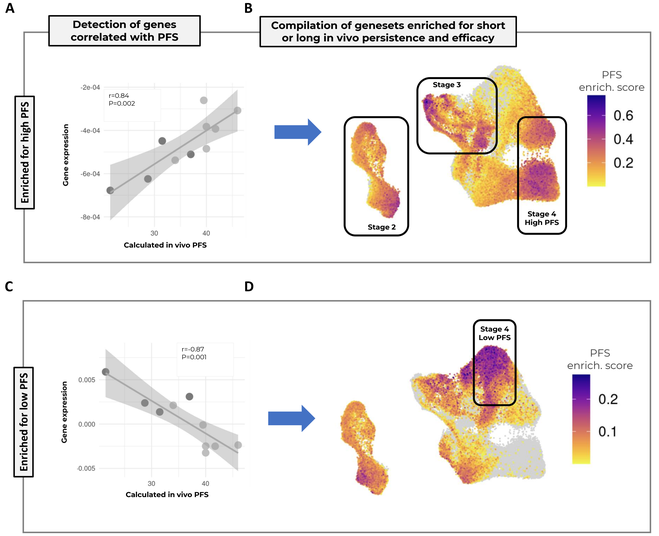


Figure 7. Single-cell transcriptomics identifies gene signatures associated with in vivo persistence and efficacy of iPSC-derived CAR-T cells. A, B. Genes whose expression levels positively correlate with in vivo performance (measured as progression-free survival, PFS) scores were identified. These genes were compiled into gene sets enriched in long-lived, high-PFS cells and mapped onto the UMAP of Stage 2, Stage 3, and Stage 4 iPS-CAR-Ts. **C, D.** Genes negatively correlated with in vivo PFS were similarly identified and compiled into gene sets enriched in short-lived, low-PFS cells. Both high and low PFS genesets are fully curated in-house, enabling their direct application toward improving the iPSC-CAR-T platform.