GENERATION OF FUNCTIONAL CD8+ T CELLS FROM iPSCs IN A SCALABLE STIRRED TANK BIOREACTOR VIA NOTCH SIGNALING PROVIDED BY DLL4/VCAM-COUPLED MICROBEADS TO SUPPORT THE DEVELOPMENT OF OFF-THE-SHELF CAR-T CELL THERAPEUTICS

Notch THERAPEUTICS

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OVERVIEW

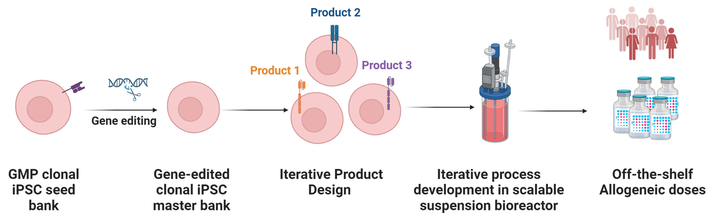
Induced pluripotent stem cells (iPSCs) have emerged as a consistent cell source for 'off-the-shelf' therapeutic T cells, with particular interest in CD8+ T cells for CAR-T cell applications. Traditionally, Notch signaling, crucial for T-lineage differentiation, has been delivered through DLL4-expressing feeder cells or DLL4 protein-coated vessels in static culture, but these methods have proven inefficient for generating CD8+ T cells from iPSCs at therapeutic scale. We have previously shown a method involving our proprietary DLL4/VCAM-1-conjugated magnetic beads ('Engineered Thymic Niche'; ETN) for precisely modulating Notch signaling in a scalable suspension bioreactor-based culture.

In this study, we have used ETN technology for the generation of functional CD8+ CAR-T cells in a stirred tank bioreactor (STR), starting with a clonal iPSC cell line with a CD19-CAR inserted in at the TRAC locus. We combine multiplexed genome engineering with iterative process development in the STR to improve yield and generate *in vivo* scale CD8+ CAR-T cells in 250 mL bioreactor. CD8+ CAR-T cells generated from suspension STR are functionally capable of multiple rounds of *in vitro* tumor cell lysis and *in vivo* tumor growth inhibition potential comparable to primary T cells.

This is a significant advancement in generation of 'off-the-shelf', highly functional CD8+ CAR-T cells using a clonal iPSC line in a small footprint automated bioreactor which has greater capacity for linear scale-up and process control than traditional T cell manufacturing systems. This PoC study is a step toward the development of robust, cost-effective, safe, and efficacious allogeneic immunotherapies.

Notch's proprietary platform allows for the development of off-the-shelf iPSC therapeutics, with a focus on multiplexed gene deletion and transgene insertion for iterative product design and small footprint suspension bioreactor system for iterative process improvements and readily scalable manufacturing solution.

Notch Platform for Allogeneic T-cell Production



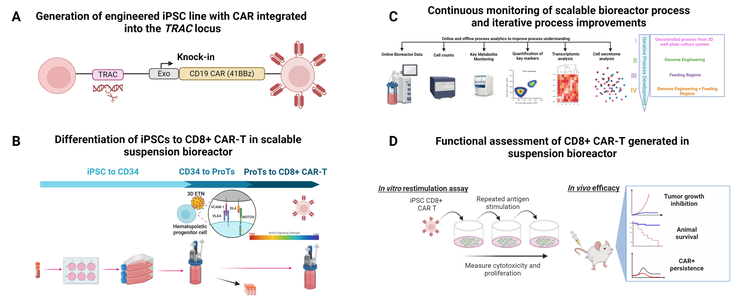
SUMMARY OF METHODS

A. iPSCs were engineered to be TRAC-/- with site-specific integration of a CD19 CAR (41BBz) under exogenous promotor control into the TRAC Locus.

B. Engineered iPSCs were differentiated to CD34+ hematopoietic progenitor cells before undergoing differentiation to CD8+ CAR-T cells in a suspension culture via precise control of Notch signaling in the presence of 3D Engineered Thymic Niche (ETN) beads.

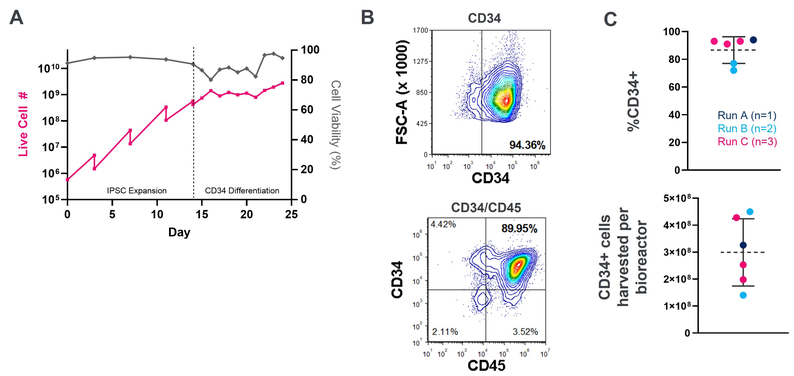
C. Process in suspension bioreactor was monitored and intensified by using a broad multiparametric analysis of cell attributes to continually improve process knowledge with a focus on yield, phenotype and scalability.

D. Antigen-mediated cytotoxicity and T cell proliferation were assessed *in vitro* using live cell imaging of iPSC-T cells co-cultured via serial restimulation with CD19-expressing tumor cells. An *in vivo* efficacy study was conducted in NSG mice engrafted with a disseminated A549 CD19-expressing tumor cell line, with bi-weekly readouts of tumor growth inhibition, animal survival, and body weight, and weekly readouts of CAR-T persistence.



RESULTS

Figure 1: Optimized Process conditions in STR generated iPSC-derived CD34 cells with high yield, purity, and reproducibility

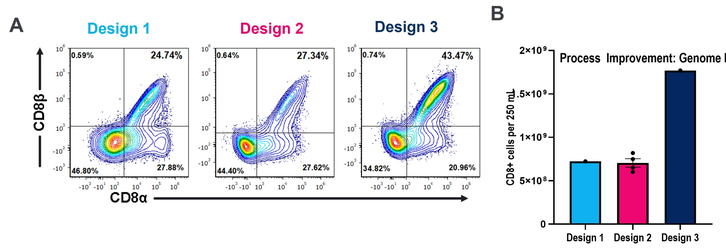


A. Representative growth curve demonstrates the high cell yield and viability achieved throughout initial iPSC passaging, expansion and subsequent CD34 differentiation processes.

B. Flow cytometry plots showing the phenotype of a representative hematopoietic progenitor cells (HPCs) generated in a suspension bioreactor (STR) vessel from edited clonal iPSCs. CD34+ cells had >90% purity at harvest without CD34 enrichment process and they co-express CD45+ and CD43+ phenotype (not shown).

C. iPSC-derived CD34s were generated from three independent process runs (6 total bioreactors) with >85% purity at harvest and yield of 3E8 CD34+ cells per 140 mL working volume in 250 mL STR vessel

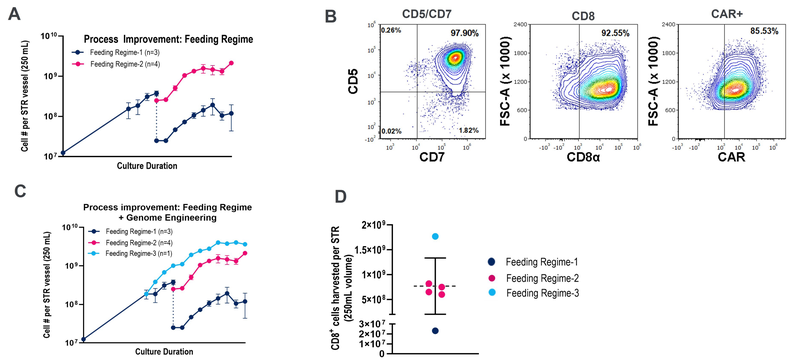
Figure 2: Genome engineering of iPSCs improved yields and purities of desired CD8+ CAR-T cells in STR



A. At the end of CD34+ to CD8 differentiation, cells had committed to a CD8ab cytotoxic T cell phenotype. Rapid genome engineering by multiplexed gene deletion and transgene insertion combined with optimized suspension bioreactor culture iteratively improved CD8 phenotype of cells at harvest, pre-processing for CD8 enrichment.

B. Gradual refinement in the yield of CD8+ CAR-T cells generated at the end of the process. While genome engineering increased number of T-lineage committed cells, improvements in the STR process and post-harvest unit operations (CD8 enrichment and ETN removal) improved overall yield to ~1.5 billion CD8+ CAR-T cells per STR vessel.

Figure 3: Rapid iterative process implementation enables generation of *in vivo* scale batch in 250 mL bioreactor



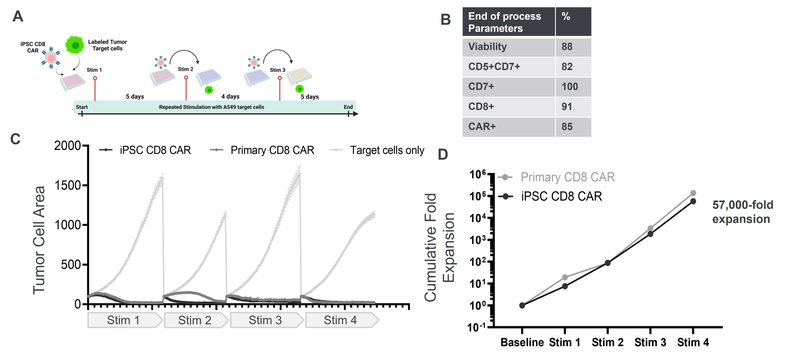
A. To improve feeding process in STR and support high density culture, an optimized feeding regime was implemented. In comparison to feeding regime 1, regime 2 improved overall yield of cells by 10-fold to over a billion cells per vessel.

B. By end of process, post CD8 enrichment, cells had committed to a CD8 cytotoxic T cell phenotype with ~98% CD5/7+, 90% CD8 and high CD19 CAR expression.

C. Further process intensification in STR with improvement in the feeding regime led to increased cell densities to almost 15M cells/mL. This translated to a total cell number of 3.6B cells in the culture volume of 250 mL.

D. Step-by-step parameter optimization and feeding regime improvement led to generation of an *in vivo* scale batch in a single 250mL bioreactor with reproducible high yield and phenotype.

Figure 4: iPSC-derived T cells produced in suspension bioreactors demonstrate cytotoxicity and proliferation comparable to primary CD8 T cells



End of process Parameters	%
Viability	88
CD5+CD7+	82
CD7+	100
CD8+	91
CAR+	85

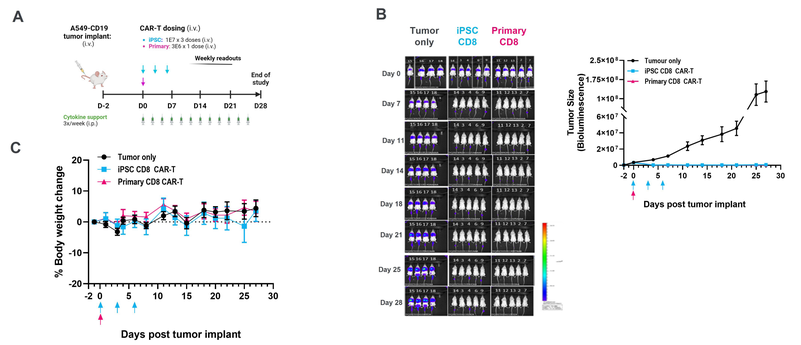
A. Serial restimulation assay schematic. iPSC CD8 CAR-T or Primary CD8 CAR-T (effector cells) are seeded with Nuclight™ green-labeled A549 CD19+/+ tumor targets at multiple E:T ratios and co-cultured for 5 days in an Incucyte to monitor cytotoxicity (Stim 1). Following harvest at day 5, effector cells from the 2:1 E:T ratio are counted and reseeded at a 2:1 E:T for a subsequent stimulation with new tumor targets. This process was repeated for a total of 4 rounds of activity.

B. Summary of key phenotypes of cells used in the functional assay.

C. Incucyte-based monitoring of target cell killing. At an optimal E:T ratio for iPSC CD8 (2:1), iPSC CD8 CAR-Ts demonstrate repeated killing of tumor targets comparable to Primary CD8 CAR-Ts for up to 4 rounds of activity.

D. Proliferation was assessed by quantification of number of T cells per well before and after each round of activation allowing for calculation of fold change. iPSC CD8 CAR-Ts continue to proliferate during each round of stimulation.

Figure 5: iPSC-derived CAR-Ts maintain complete remission *in vivo* that is comparable to primary CAR-Ts in a disseminated tumor model

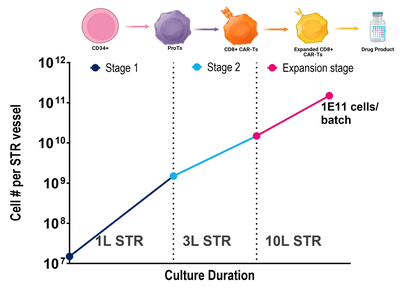


A. Schematic of treatment regimen for NSG mice bearing disseminated A549-CD19 tumor model. A549 CD19+/+ cells co-expressing luciferase were implanted into 6-7 week old NSG mice through lateral tail vein injection, two days before first CAR T injections. A total of 30M iPSC CAR+ CD8+ cells were administered intravenously in 3 doses. A single 3M dose of CD8+ enriched primary CAR-Ts was used as a positive control. Study cohorts received cytokine support intraperitoneally 3 times per week for 4 weeks.

B. Tumor burden was monitored bi-weekly by bioluminescence imaging using IVIS Lumina III through intraperitoneal injection of D-luciferin substrate in sterile Phosphate-buffered saline. Images of representative time points are shown. Quantification of tumor burden as region of interest (ROI) flux values are shown in the right panel with each dot representing mean of the treatment cohorts and error bars represent SEM.

C. Percent change in body weight was monitored throughout study for assessment of acute toxicity.

Figure 6: Path to produce sufficient cell material for an allogeneic CAR-T trial with a single manufacturing batch



CONCLUSIONS

- High density bioprocessing of iPSC-derived CD8+ CAR-T cells in scalable STR platform can be achieved by merging multiplexed genome engineering with step-by-step process improvement and parameter adaptation to achieve *in vivo* scale batch from a single 250mL bioreactor.
- iPSC-derived CD8+ CAR-T cells produced in scalable STR platform are functionally comparable to primary CAR-T cells in serial killing assays and capable of multiple rounds of *in vitro* tumor cell lysis and sustained tumor growth inhibition *in vivo* without acute toxicity.
- This advancement demonstrates proof-of-concept for generation of highly efficacious, off-the-shelf CAR-T cells in a small-footprint STR platform with a controlled and consistent process which will allow us to address the challenge of scalability in clinical manufacturing for off-the-shelf allogeneic cell therapies.