Generation of Functional CD8αß T-cells from iPSCs via Recapitulation of Thymic Notch Signaling Using DLL4/VCAM1 Microbeads

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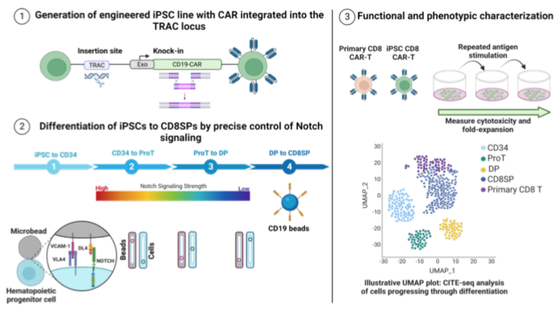
INTRODUCTION

Notch Platform for Allogeneic T-cell Production

1. Unlimited source for genetically engineered therapeutic T-cells remains a significant roadblock for allogeneic cell therapies.
2. Induced Pluripotent Stem Cells (iPSCs) are emerging as a solution for a reliable starting material for generating unlimited genetically engineered therapeutic T-cells.
3. Notch has best-in-class gene editing technology to perform multiple gene edits in our clonal iPSC seed bank to generate a master cell bank that can be used iteratively for multiple product designs.
4. Notch's core 3D Engineered Thymic Niche (ETN) technology can replace the current feeder-layer based approaches to provide Notch signaling to developing cells on suspension beads to enable a precise and scalable process for T-cell differentiation from iPSCs.
5. Notch has end-to-end capabilities to tune T-cell phenotypes and iterate product designs in a commercially scalable format, addressing the limitations of current approaches to T-cell manufacturing to generate multiple doses of off-the-shelf cellular therapeutics.

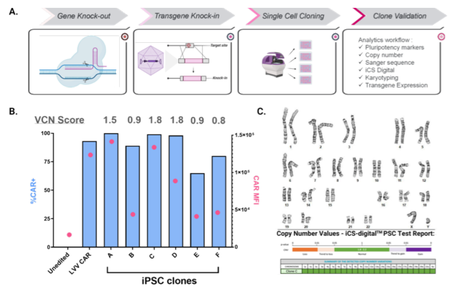
GRAPHICAL ABSTRACT



1. iPSCs were engineered to integrate CD19 CAR into the TRAC locus under the influence of a constitutively active exogenous promoter.
2. iPSCs were differentiated to CD34+ hematopoietic progenitor cells (HPCs) in scalable agitated suspension cultures.
3. T-cell development in thymus is a tightly regulated process that is dependent on sequential activation of Notch and TCR signaling.
4. Notch's proprietary 3D ETN technology allows precise control of Notch signaling to developing HPCs to generate Progenitor T-cells (ProT) and Double Positive (DP) cells by gradually reducing Notch signaling strength by altering bead-to-cell ratio in culture.
5. CAR mediated activation of DP cells using target-antigen functionalized beads mimics TCR signaling to generate CD8 Single Positive (SP) cells.
6. iPSC derived CD8 CAR-T cells were compared to primary CD8 CAR-T cells for target-specific anti-tumor activity in an *in vitro* serial restimulation assay.
7. Cell phenotypes were monitored throughout differentiation by flow cytometry and at select timepoints by single cell RNA sequencing.

RESULTS

Figure 1: Generation and characterization of a clonal iPSC line with CAR integrated at the TRAC locus

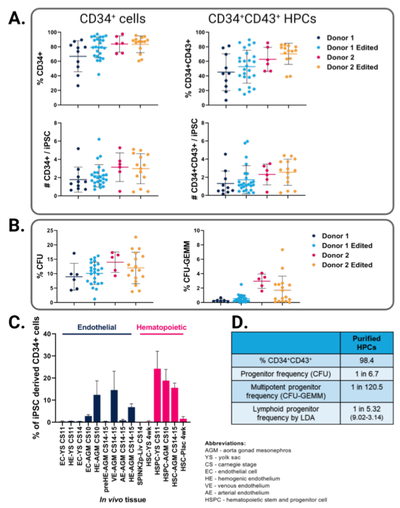


A. Schematic for generation and characterization of clonal iPSC lines with CD19-CAR integrated at TRAC locus. Clones were generated with VIPS single cell deposition and screened for targeted bi-allelic insertion at TRAC locus using standard clone validation techniques.

B. CAR expression at the end of clone production from 1 parental iPSC line. Percent of CAR positive cells (bar chart, blue) and median fluorescent intensity (MFI) (pink dots) are depicted. Parent line (unedited) and clonal iPSC line expressing CD19-CAR by random LVV integration (LVV CAR) serve as control. Genetic characterization of copy number integration at TRAC locus by using ddPCR CNV Assay (Biorad) by amplifying genomic TRAC locus - CAR transgene junction.

C. Genomic stability analysis of selected clone C. G-band karyotyping conducted by WiCell showing normal Karyotype and summary of iCS- digital assay showing expected copy number in 24 genomic regions of recurrent iPSC abnormalities.

Figure 2: Generation of multipotent lymphoid-competent CD34+ hematopoietic progenitor cells from engineered iPSCs



A. Purity and yield of CD34+ cells and CD34+CD43+ HPCs obtained from iPSC lines derived from 2 independent donors as well as gene-edited clonal iPSC lines prior to enrichment.

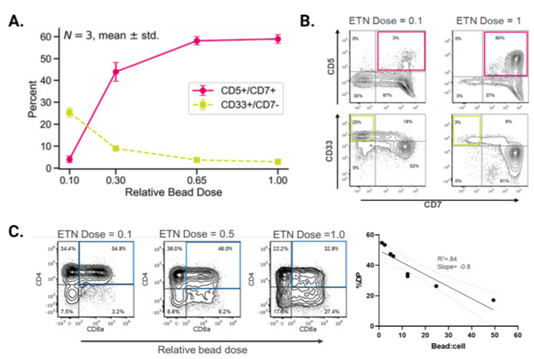
B. HPCs were enriched by CD34 MACS selection or filtration of single cells and assessed for progenitor frequency. Colony-forming units (CFU) and CFU-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) were detected at a high frequency in enriched iPSC derived CD34 cells.

C. iPSC-derived CD34 cells resembled endothelial and hematopoietic progenitors found in Carnegie Stage (CS) 10-15 human embryos when assessed by scRNAseq and compared to in vivo tissue profiles as reported in Calvanese et al. Cell type labels were assigned using ACTINN (Ma and Pellegrini).

D. A clonal iPSC line as described in Figure 1 was differentiated to CD34+ HPCs and purified by filtering for single cells to be used in downstream differentiation. Lymphoid progenitor frequency (confidence limit) was assessed by limiting dilution assay. Wells were scored as positive if they contained >100 viable CD45+CD7+CD56- cells.

RESULTS CONT'D

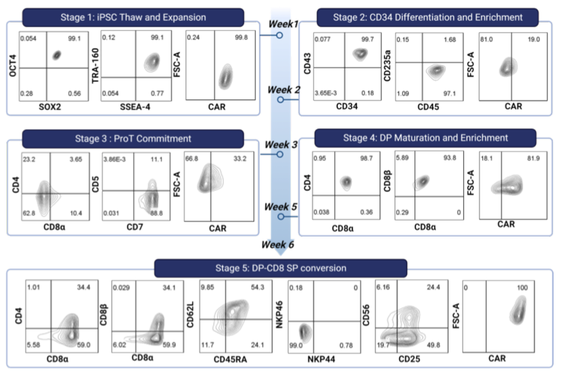
Figure 3: Modulation of ETN-mediated Notch signaling intensity to induce T-cell lineage induction from iPSC-derived CD34 cells



A and B. Proportion of CD5+/CD7+ proT cells (pink) and CD33+/CD7- myeloid cells (yellow) induced from CD34 cells was controlled using ETN bead dose, with higher relative doses (0.65-1.0) resulting in more proT cells after 10 days of differentiation.

C. Subsequent commitment of proT cells to DP cells was also modulated by ETN bead dose, as low relative bead dose (0.1) lead to efficient induction of DP cells, whereas high relative dose (1.0) resulted in a mix of double negative, CD4 immature single positive, DP, and CD8 single positive cells after 11 additional days of differentiation. The proportion of DP cells generated was controlled via bead to cell ratio, with low ratios resulting in the highest DP purity, demonstrating that reduced Notch signaling during this stage is required for T-cell development.

Figure 4: Phenotype of cells at different stages of Notch's T-cell production process



Stage 1: Prior to differentiation, engineered iPSCs are positive for undifferentiated cell makers OCT4, SOX2, SSEA4 and TRA-160. 100% CAR expression detected in iPSCs upon thaw and expansion of a seed bank of an iPSC clone with CAR integrated into TRAC locus.

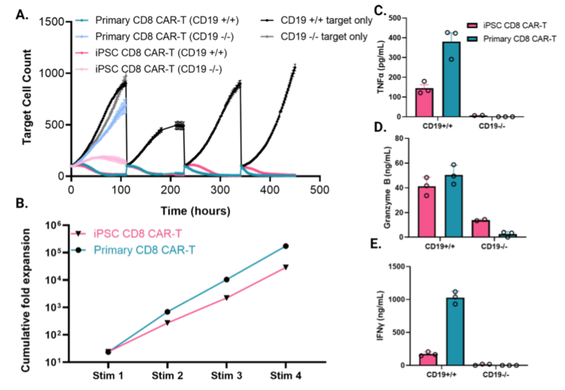
Stage 2: iPSCs were differentiated to CD34+ cells in scalable agitated suspension cultures and enriched HPCs were also positive for CD43 and CD45. Expression of erythroid progenitor marker CD235a is negative showing lack of early lineage commitment. CAR expression is reduced upon CD34 differentiation.

Stage 3: CD34+ HPCs were cultured with ETN beads presenting DLL4 and VCAM-1 with conditions identified to provide high level of Notch signaling and ProT phenotype was assessed by measuring CD5 and CD7. T-cell markers (CD4 and CD8) are low at this stage typical for ProT cells.

Stage 4: Gradual reduction in Notch signaling after ProT stage results in the gain of T lineage fate during week 4 and 5 producing primarily CD4+CD8+ DP cells. CAR expression gradually increases as cells commit to T-cell lineage fate.

Stage 5: CAR engagement facilitates maturation of DP cells to CD8 SP T-cells. iPSC-derived CD8 SP cells are a mix of CD8**αß** and CD8**αα** T-cells. CD8SPs have high levels of expression of stem cell memory markers CD45RA, CD62L and CD95 (data not shown). CAR-stimulated cells expressed the T-cell activation/NK-cell marker CD56 but lacked canonical NK markers such as NKP44 and NKP46. CAR expression is 100% after SP transition.

Figure 5: Functional characterization of iPSC-derived CD8 CAR-T cells in *in vitro* serial restimulation assay

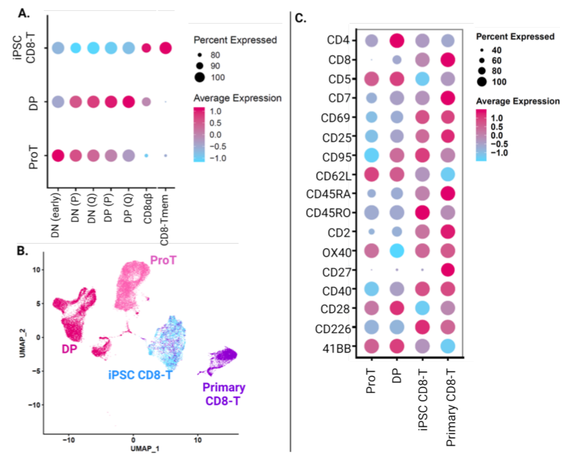


A. Serial restimulation assay to measure cytotoxic activity using an Incucyte assay, with GFP-expressing CD19+ cells as target cells (n= 3 technical replicates). T-cells were co-cultured at a 2:1 E:T every 5 days with target cells and exogenous cytokine support. Target clearance measured by GFP surface area reduction shows comparable activity between iPSC and primary CD8 CAR-T cells over 4 rounds of target exposure.

B. Fold-expansion of cells was calculated by performing a count at the end of each round of target exposure. iPSC-derived CAR-T cells proliferate 28,000-fold over 4 rounds of antigen exposure.

C, D and E. Comparable effector cytokine production 24 hours after stim 1 measured by MSD in iPSC and primary CD8 CAR-Ts co-cultured with CD19+/+ and CD19-/- target cells.

Figure 6: Deep molecular (CITE-Seq) characterization of iPSC to CD8SP differentiation and comparison to primary CD8αß T-cells



A. Single cell GSEA based enrichment of Thymic Atlas Signatures (20 genes within each cell types published by Sarah Teichmann et al.) shows stage- specific differentiation of iPSCs with our 3D ETN platform

B. Unsupervised clustering of the CITE-Seq data at different stages of differentiation along with primary CD8-Ts shows iPSC CD8 T-cells are more similar in molecular profile to primary CD8 T-cells.

C. Protein expression of CITE-Seq data shows the upregulation of T-cell specific markers, such as, CD8, CD45RA, CD45RO, CD2, OX40, CD226 and 41BB as the cells differentiate into T-cells.

CONCLUSIONS

- Notch's proprietary ETN platform enables precision control of notch signaling, which is required for T-cell development.
- The ability to precisely control notch signaling delivers the ability to design and manufacture a uniform and unlimited supply of functional therapeutic T-cells that resemble peripheral CD8**αß** T-cells