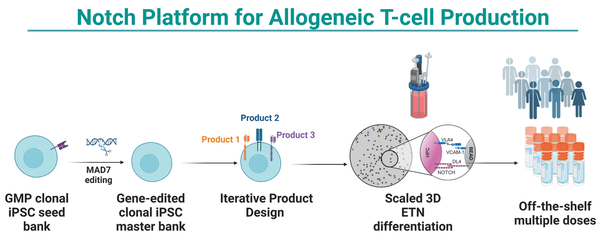
Presentation of DLL4 and VCAM on paramagnetic beads replicates thymic Notch signaling and enables differentiation of functional iPSC derived TCR T-cells in vitro

Siddarth Chandrasekaran, Melanie Kardel, Elisa Martinez, Nabil Zeidan, Avisek Deyati, Valerie Wall, Dylan Lee, Negin Farahani, Amanda Au Yeung, Omar Subedar, Michael Cadell, Sommer Apelu, Laura Prochazka, Zahra Sahaf, Angela Duong, Cole Zmurchok, Boyoung Yoon, Dan Kirouac, Muluken Belew, Jasdeep Mann, Deepika Rajesh, Chris Bond

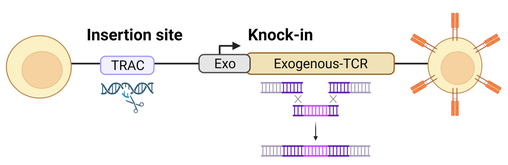
Notch Therapeutics | Society for Immunotherapy of Cancer 2023

INTRODUCTION

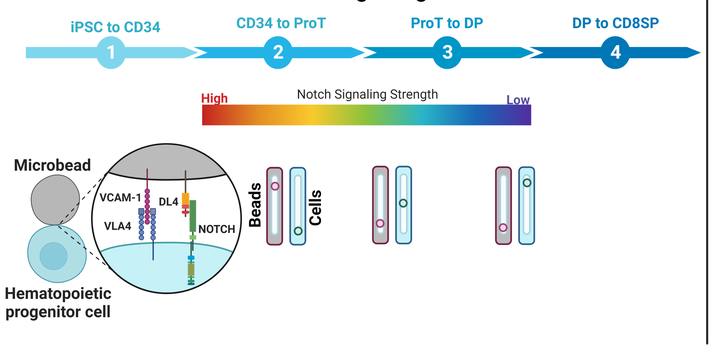
1. Unlimited source for genetically engineered therapeutic T-cells remains a significant roadblock for allogeneic cell therapies.
2. Induced Pluripotent Stem Cells (iPSCs) are emerging as a solution for a reliable starting material for generating unlimited genetically engineered therapeutic T-cells.
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.



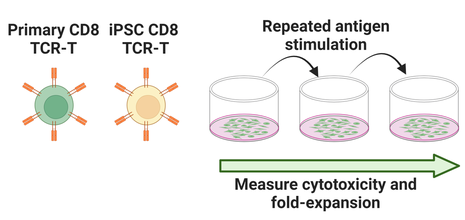
1 Generation of engineered iPSC line with TCR integrated into the TRAC locus

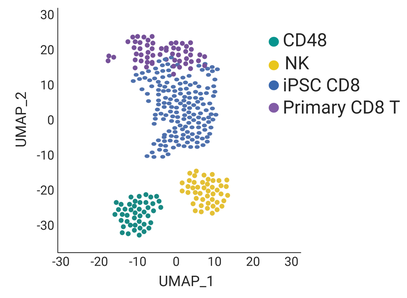


2 Differentiation of iPSCs to CD8SPs by precise control of Notch signaling



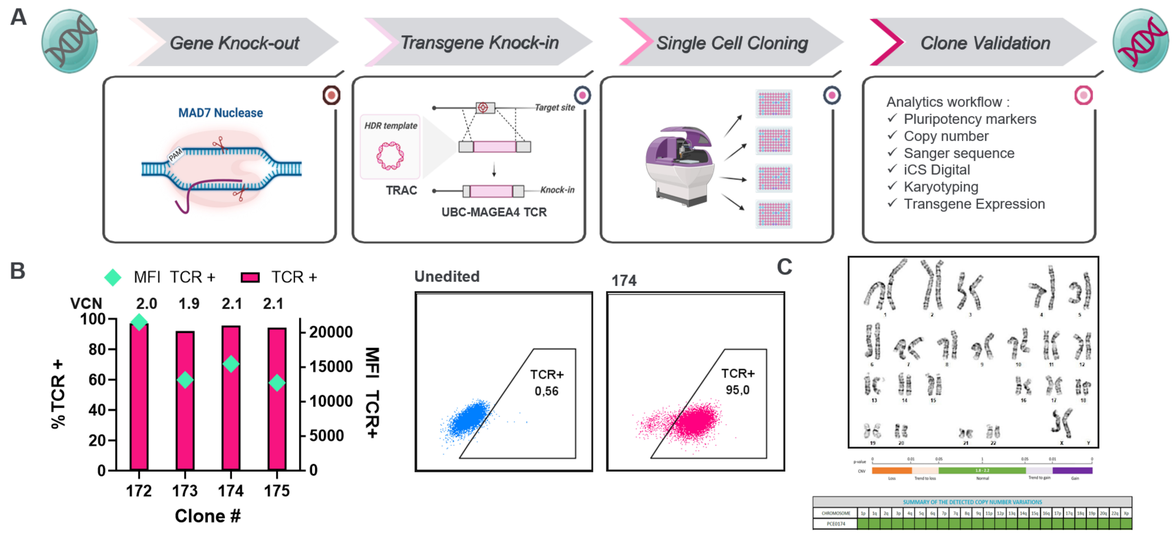
3 Functional and phenotypic characterization





Illustrative UMAP plot: CITE-seq analysis of iPSC derived CD8SPs compared with peripheral blood immune cells

Figure 1: Generation and characterization of a clonal iPSC line with MAGEA4 TCR integrated at the TRAC locus

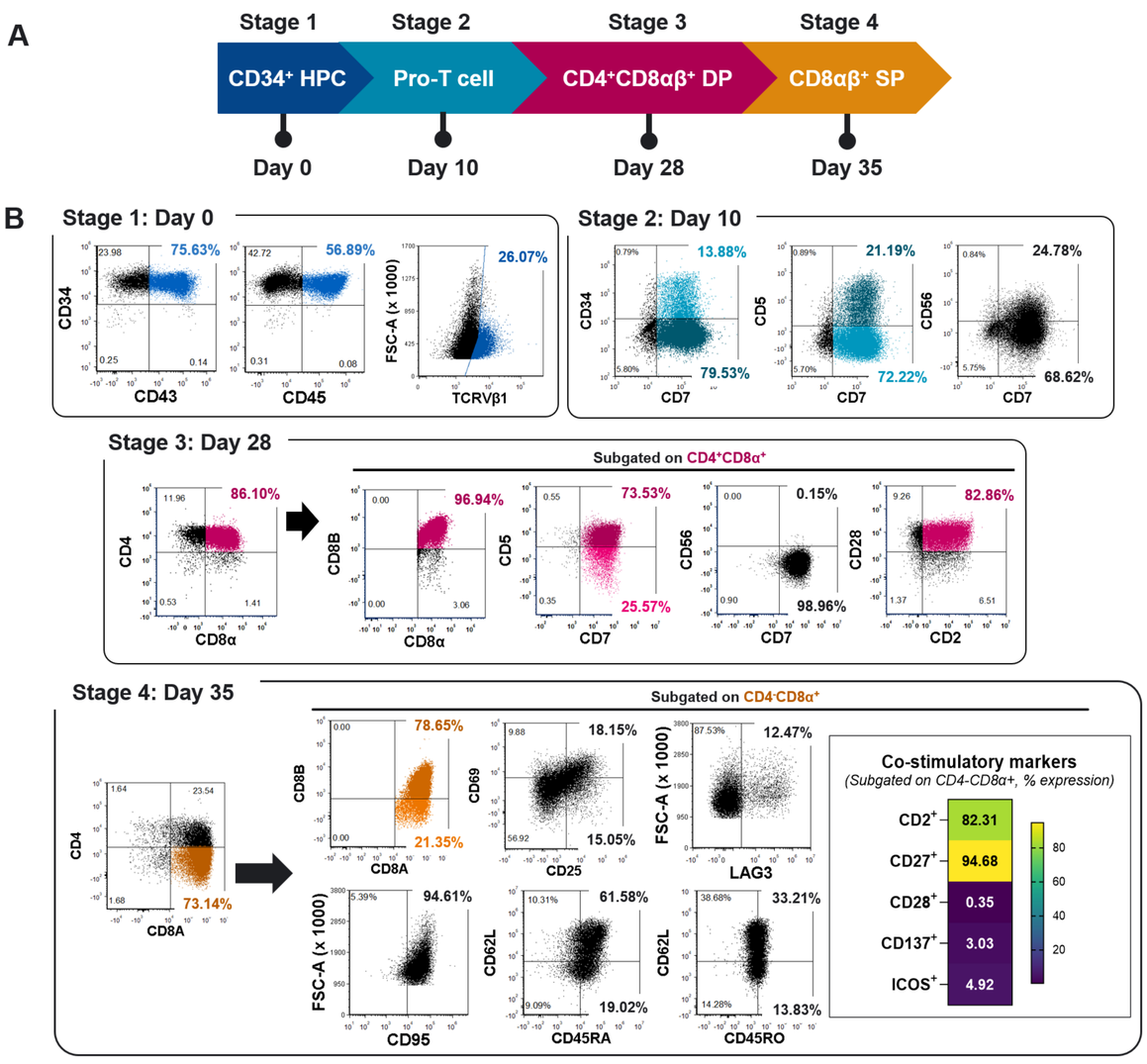


A) Schematic for generation and characterization of clonal iPSC lines with MAGEA4 TCR integrated at TRAC locus. Clones were generated with Namocell single cell deposition and screened for targeted bi-allelic insertion at TRAC locus using ddPCR and standard clone validation techniques.

B) TCR expression at the end of clone production for 4 selected bi-allelic iPSC clones. Representative scatter plot of selected clone (174). VCN values on top of bar chart show genetic characterization of copy number integration at TRAC locus using ddPCR VCN Assay (Biorad) by amplifying genomic TRAC locus - TCR transgene junction.

C) Genomic stability analysis of selected clone 174. G-band karyotyping conducted by WiCell showing normal Karyotype and summary of iCS- digital assay (StemGenomics) showing expected copy number in 24 genomic regions of recurrent iPSC abnormalities.

Figure 2: Differentiation of MAGE-A4 TCR+ CD34 HPCs to CD8SP T cells

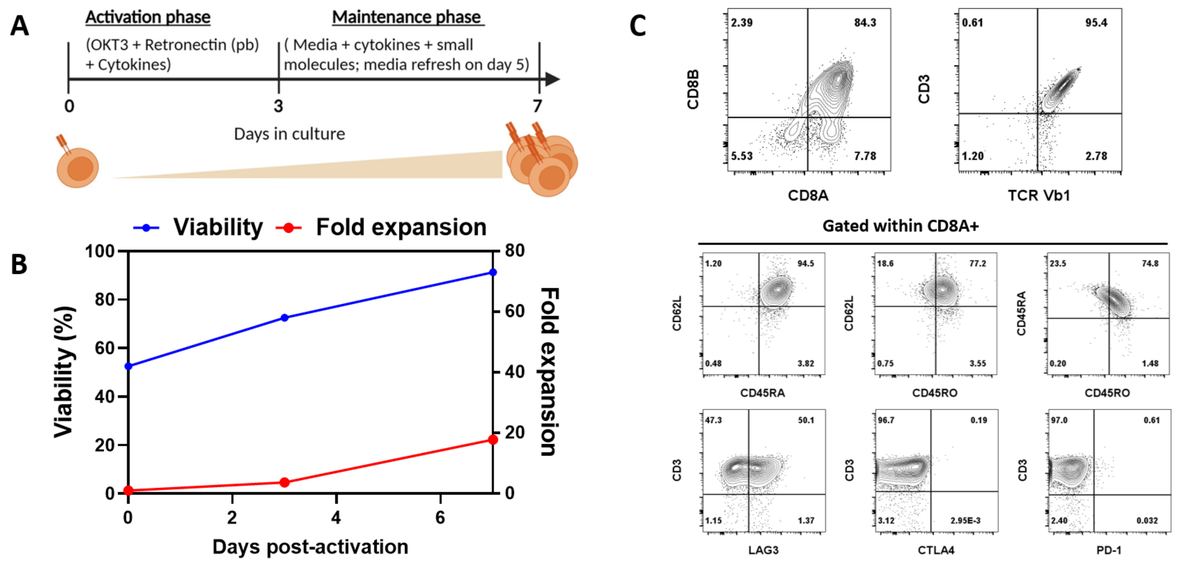


A) T cell differentiation schematic: CD34 HPCs are differentiated into CD8SP T cells using a 4-stage protocol. During Stage # 1, CD34 HPCs are seeded onto 2D ETN coated vessels in progenitor expansion medium (SFEM II + LEM) and cultured for 10 days to generate CD34-CD7+CD5+ ProT cells (stage 2). At this point, cells move onto Stage # 3, where ProT cells are cultured with DL4 and VCAM paramagnetic beads at various doses for 18 days to allow for the emerge of mature Late DPs (CD4+CD8A+CD8B+CD3+TCRαβ+). Lastly, Stage # 4 starts with the enrichment of Late DPs via CD8 Positive Selection kit (STEMCELL Technologies), and activation using Immunocult activator anti-CD3/CD28 for 7 days to allow for DP to SP conversion to occur.

B) Successful in vitro differentiation of iPS-derived MAGE-A4 TCR expressing CD34 HPCs into CD8SP T cells using DL4 and VCAM paramagnetic beads. Representative flow cytometry plots of key subpopulations generated during the 4-stage T cell differentiation process. **Stage 1 CD34 HPCs** express high levels of CD43 and CD45 hematopoietic markers (dark blue subpopulations) and intermediate expression of TCR detected using TCRVβ1 antibody. **Stage 2 ProT cells** are predominantly CD7+ (93.41%), co-expressing CD34 (13.88%), CD5 (21.19%) and CD56 (24.78%). **Stage 3 Late DPs** are primarily CD4+CD8A+CD8B+ (~86%), with high levels of CD5+CD7+ and CD28+CD2+ co-stimulatory molecules (dark pink). **Stage 4 CD8SPs** are 73.14% CD4-CD8A+, co-expressing high levels of CD8B (dark orange). The resulting CD8SPs also express key activation markers (CD25 and CD69), a low exhaustion profile (12.47% LAG3+), a mix of TSCM, TCM and TEM memory phenotypes and are predominantly CD2+ and CD27+.

C) MAGE-A4 TCR expression kinetics during T cell differentiation. Cells were stained with APC-conjugated MAGE-A4 Tetramer reagent on day 10 (ProT), day 28 (Late DP) and day 35 (CD8SP) to detect surface TCR expression levels during T cell differentiation. While MAGE-A4 TCR expression was lower at the CD34 HPC stage (26.07%), as cells become more T-lineage committed, TCR surface expression increases as a result.

Figure 3: Expansion of iPSC derived TCR+ T-cells

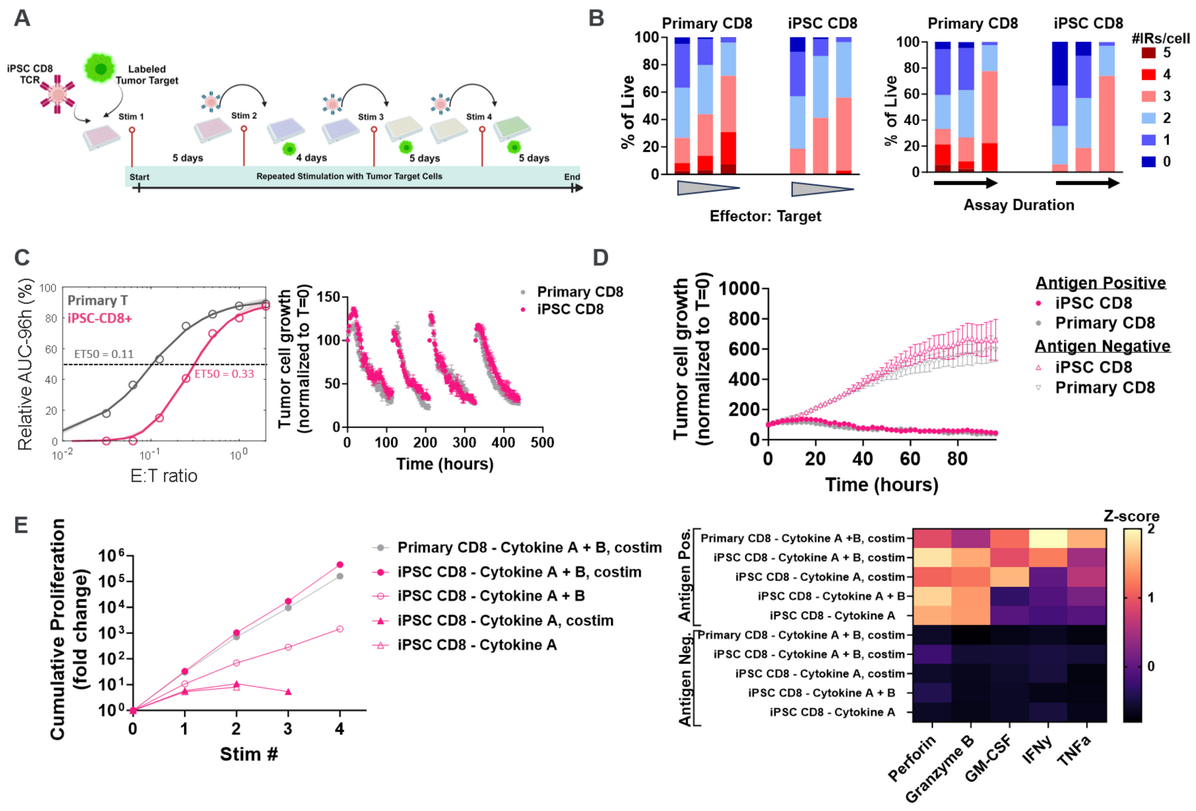


A) A bi-phasic iPSC-T expansion culture protocol comprised of activation and maintenance phases. Cells were activated by engaging CD3 using plate-bound OKT3 and retronectin in a define activation phase media and cells were seeded into expansion media to support proliferation of CD8 T-cells

B) Viability over a 7 day culture period improves with expansion condition from 42% to 75% and we observed over 15-fold expansion during this process.

C) Representative flow plots demonstrating maintenance of key T cell phenotypic markers by end of the expansion protocol. CD8SP cells still retain predominantly stem cell memory phenotype while largely lacking common exhaustion markers.

Figure 4: *In vitro* functional characteristics of iPSC derived TCR+ T-cells



A) Serial restimulation assay schematic. iPSC CD8 TCR or Primary CD8 SSI AAV TCR (effector cells) are seeded with Nuclight™ green-labeled 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 (19 days total).

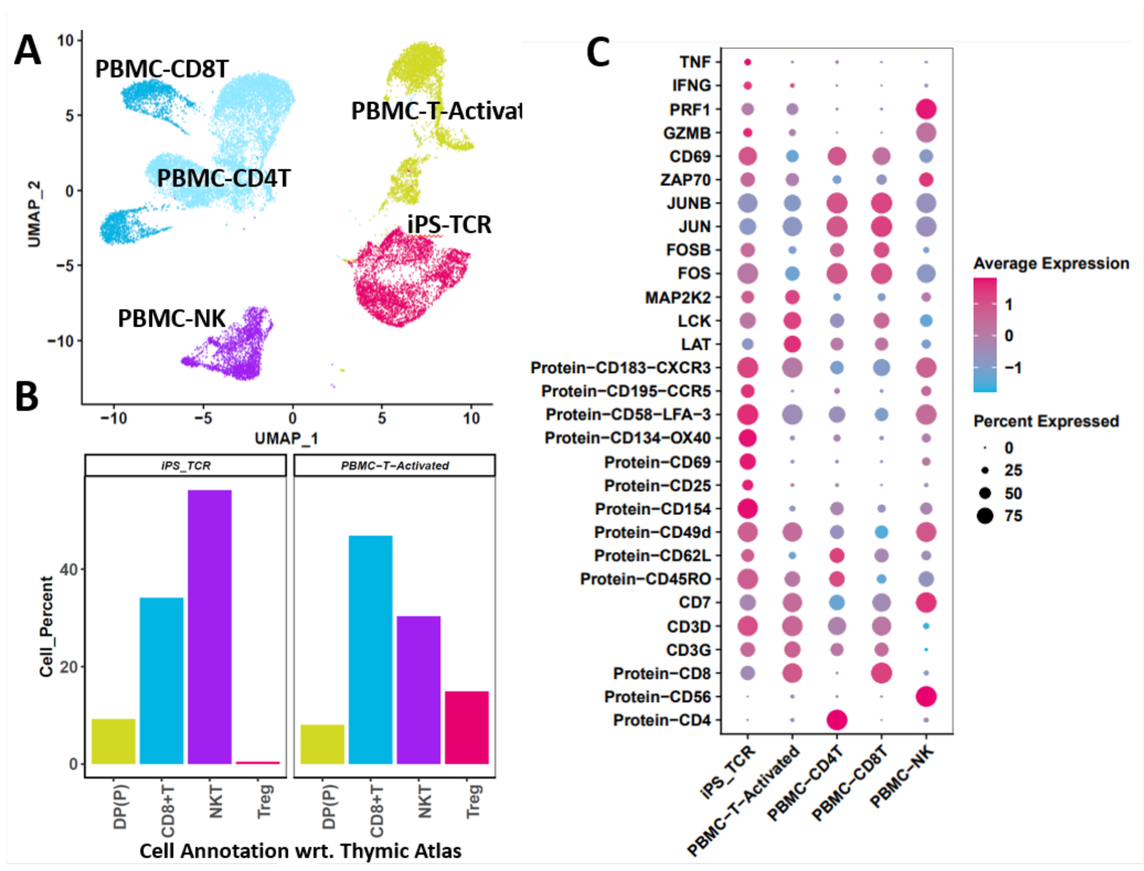
B) T cell phenotype was monitored by flow cytometry throughout the restimulation assay. E:T ratio and assay duration (rounds of activation) influence co-expression of inhibitory receptors (PD-1, TIGIT, LAG3, TIM3 and CD39) on effector cells (a measure of CD8 T cell exhaustion).

C) Using an Incucyte to monitor target cell killing, cells were challenged for a single round of activation at progressively lower E:T ratios. The difference in cytotoxicity between iPSC CD8 TCR and Primary CD8 TCR is most pronounced at lower E:T ratios, showing a 3-fold difference in activity (left). Using an optimal E:T ratio for iPSC CD8 (2:1), iPSC CD8 TCR demonstrate repeated killing of tumor targets comparable to Primary CD8 TCR (right).

D) To assess specificity in an Incucyte cytotoxicity assay, iPSC CD8 TCR or Primary CD8 TCR were seeded with tumor target cells with (antigen positive) or without (antigen negative) β2M expression. IPSC CD8 TCR and Primary CD8 TCR both demonstrate similar specificity for antigen positive cells.

E) Proliferation was assessed by quantification of T cells/per well before and after each round of activation allowing for calculation of fold change. Optimal proliferation of iPSC CD8 TCR requires receiving a combination of Cytokine(s) A, Cytokine B and costimulatory signals. Media components were modified to include combinations of factors which drive each of these signals. Measurement of T cell proliferation shows a profound loss of function when Cytokine B is not provided. Removal of costimulatory signals also reduces the overall magnitude of iPSC CD8 TCR proliferation (left). Optimal cytokine secretion from iPSC CD8 TCR also requires receiving a combination of Cytokines A and B and costimulatory signals (right). Removal of costimulatory signals has the most profound effect on GM-CSF, IFNγ and TNFα. IPSC CD8 TCR demonstrate antigen-specific cytokine release.

Figure 5: Deep molecular (CITE-Seq) characterization of iPSC derived TCR+ T-cells and comparison to primary immune cell subsets



A) Unsupervised clustering with Uniform Manifold Approximation and projection (UMAP) based on CITEseq data (mRNA + surface protein at single cell resolution) with PBMC-CD8T, PBMC-CD4T, PBMC-NK, T-cells from PBMCs activated with CD3/CD28 Dynabeads (PBMC-T-Activated) and IPSC derived TCR cells (iPS-TCR). Spatial proximity between iPS-TCR and PBMC-T-Activated clearly demonstrate that those cells are similar with respect to transcriptome and proteome expression at single cell resolution.

B) Cells from "iPS_TCR" and "PBMC-T-Activated" are annotated with respect to Thymic Atlas reference with SingleR algorithm. 90% of the cells from iPS-TCR and activated T-cells from PBMCs are mature CD8+T/NKT cells when transcriptome of these cells were compared with transcriptome of cells from developing thymus.

C) mRNA and protein markers were screened based on statistical threshold and presented as bubble plot with Seurat. iPS-TCR cells are positive for Protein-CD8, CD45RO and negative for Protein-CD4 and Protein-CD56. These cells are high in CD3 expression and TCR signaling (LAT, LCK, MAP2K2, FOS, FOSB, JUN, JUNB, ZAP70). iPS-TCR cells express GZMB, PRF1 similar to activated T-cells from PBMCs.

CONCLUSIONS

1. Notch's proprietary ETN platform enables precision control of notch signaling, which is required for T-cell development. We have demonstrated control T lineage commitment in TCR engineered iPSC lines and iPSC derived TCR-Ts are phenotypically and functionally similar to primary T cells.
2. The ability to precisely control notch signaling delivers the ability to design and manufacture a uniform and unlimited supply of functional therapeutic T-cells