T Cell differentiation from induced pluripotent stem cell-derived blood progenitors using DLL4/VCAM beads

Richard Carpenedo, Cole Zmurchok, Alessia Pallaoro, Hiofan Hoi, Elham Afshinmanesh, Marc Ouellette, Avisek Deyati, Dan Kirouac, Steven Woodside, Deepika Rajesh, Chris Bond, Emily Titus | Keystone Emerging Cellular Therapies

OVERVIEW

Adoptive T-cell therapies are effective for some hematological malignancies and could transform the treatment of many other cancers. Despite early clinical success, use of genetically modified patient-derived T cells poses challenges that limit the utility and accessibility of this cellular immunotherapeutic.

T cells derived from genetically modified, clonal, induced pluripotent stem cells (iPSC) can enable a defined, reproducibly manufacturable cell product. However, inducing T-lineage commitment in a scalable and controlled format suitable for clinical manufacturing remains a major obstacle. T-lineage commitment occurs in the thymus through Notch signaling activation in hematopoietic stem/progenitor cells (HSPC) by ligands such as Delta-like 4 (DLL4) expressed on adjacent epithelial cells.

In vitro, Notch signaling in HSPCs can be activated by co-culture with DL4-expressing cell lines, or by culture on DLL4-coated plates. Such systems lack control of the intensity and dynamics of Notch signaling needed for robust T-cell development (especially for iPSC derived HSPC) and are not easily scalable to meet the needs of clinical manufacturing.

To address these limitations, we developed the Engineered Thymic Niche (ETN) platform, consisting of magnetic beads coated with DLL4 and vascular cell adhesion molecule 1 (VCAM-1). ETN enables differentiation of iPSC-derived CD34+ HSPC to progenitor T-cells (ProT).

The ETN can also be controlled to drive differentiation to single positive CD8ab+ cells.

Notch signaling is required for T cell development in the Thymus

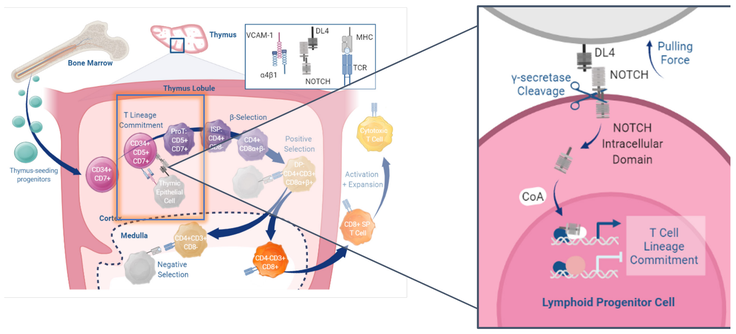


Figure 1. Notch signaling drives T-cell lineage commitment and differentiation from hematopoietic progenitor cells in the thymus. T-cell generation during development and adult homeostasis occur in the thymus. CD34+ progenitors in circulation seed the thymic cortex. Notch signaling, initiated by cell-cell interactions between Notch1+ hematopoietic progenitors and DLL4+ epithelial cells, is required for T-lineage commitment and differentiation. VCAM-Integrin signaling functionally synergizes with Notch signaling to support efficient T-cell lineage commitment, though the exact mechanisms remain unclear. T-cell induction is marked by successive expression of CD5 and CD7 and loss of CD34. CD5+CD7+ Progenitor T-cells (ProTs) then differentiate into CD8+ and CD4+ (single positive) T cells, before exiting the thymus and entering circulation as naive T cells. Generation of functionally mature T cells *in vitro* from stem or progenitors requires synthetically recapitulating these interactions, controlling Notch and VCAM signaling via surface presentation of ligands.

Clinical T-cell manufacturing requires a Xenogeneic-free, scalable culture platform

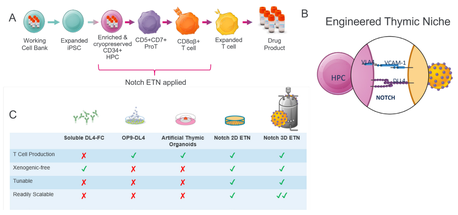
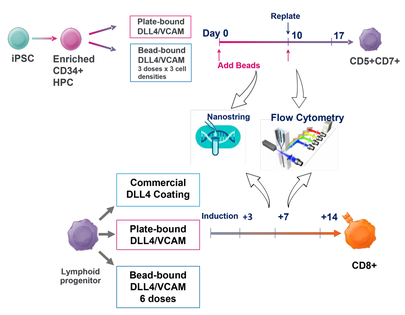


Figure 2. Notch ETN technology enables tunable control of ligand timing, binding and density. Experimental systems for inducing T-cell differentiation from hematopoietic progenitors rely on co-culture with DLL4-expressing cell lines. While suitable for research purposes, these are insufficient for clinical-scale manufacturing of T-cell based therapeutics. Building on our discovery that immobilized DLL4/VCAM supports T-cell differentiation *in vitro*, we have designed the 3D Engineered Thymic Niche (ETN) platform for scalable T-cell manufacturing (B). The ETN is utilized during differentiation from CD34+ HPCs to CD8 single positive T cells (A). The Notch 3D ETN is uniquely amenable to scalable processes (C).

METHODS



iPSC-derived CD34+ cells were cultured with the Engineered Thymic Niche (ETN), either with DLL4 and VCAM immobilized on tissue culture plates (2D ETN) or on magnetic beads (3D ETN). A matrix of conditions comprising 3 bead doses and 3 cell densities was tested. For both 2D ETN and 3D ETN, cells were passaged and reseeded after 10 days. Cells were harvested for gene expression analysis of the Notch regulatory network using Nanostring technology and flow cytometric phenotype analysis (day 10, 12, and 17).

Maturation of progenitor T cells generated using the 2D ETN was induced using commercially available DLL4 coating material, 2D ETN, or 3D ETN beads. Six bead doses were tested, with relative bead doses covering a 30-fold range. Cells were harvested for analysis by flow cytometry and qPCR after 3 and 7 days of maturation. Dimensionality reduction of flow cytometry data was performed using Uniform Manifold Approximation and Reduction (UMAP), and unsupervised clustering was done using FlowSOM. Additionally, cells were matured for 14 days using 3D ETN and assessed by flow cytometry.

RESULTS

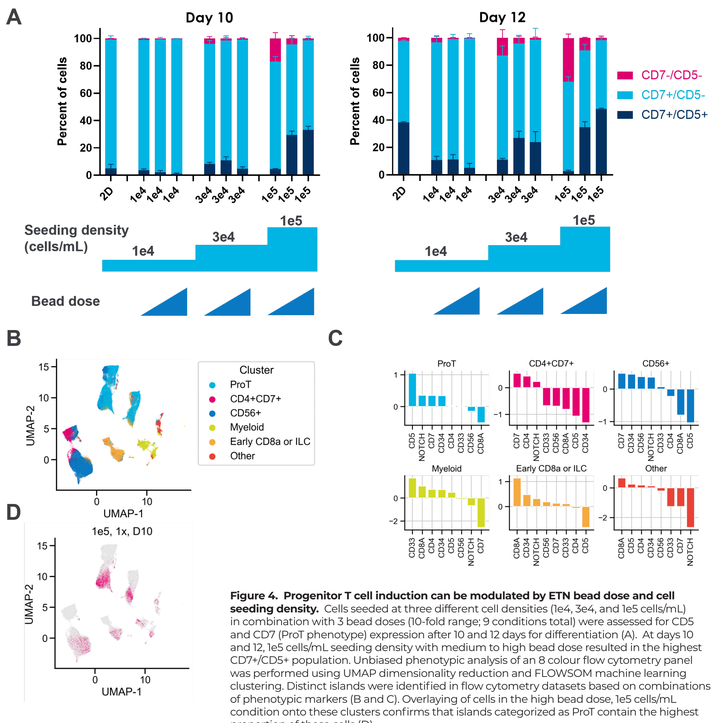


Figure 4. Progenitor T cell induction can be modulated by ETN bead dose and cell seeding density. Cells seeded at three different cell densities (1e4, 3e4, and 1e5 cells/mL) in combination with 3 bead doses (10-fold range; 9 conditions total) were assessed for CD5 and CD7 (ProT phenotype) expression after 10 and 12 days for differentiation (A). At days 10 and 12, 1e5 cells/mL seeding density with medium to high bead dose resulted in the highest CD7+/CD5+ population. Unbiased phenotypic analysis of an 8 colour flow cytometry panel was performed using UMAP dimensionality reduction and FLOWSOM machine learning clustering. Distinct islands were identified in flow cytometry datasets based on combinations of phenotypic markers (B and C). Overlaying of cells in the high bead dose, 1e5 cells/mL condition onto these clusters confirms that islands categorized as ProT contain the highest proportion of these cells (D).

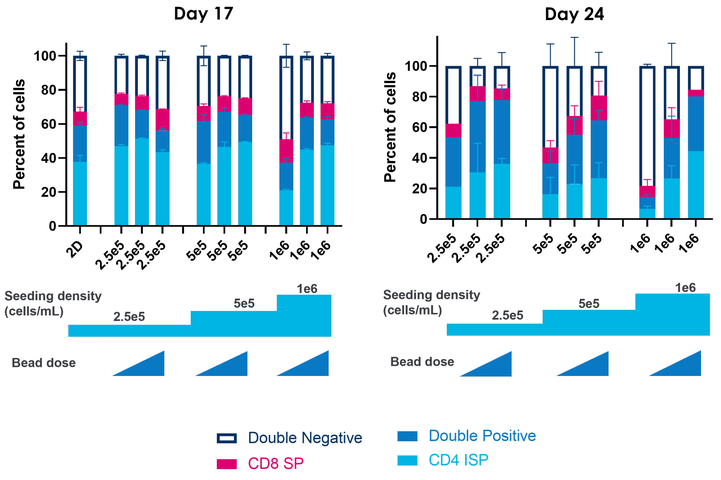


Figure 5. Double positive differentiation can also be modulated through bead dose and cell density. Cells were seeded at D0 using conditions identified in Figure 1 (1e5 cells/mL, high bead dose) and were reseeded at day 10 using a new matrix of cell densities and bead doses (2.5e5, 5e5 and 1e6 cells/mL; 3 bead doses). At day 17, multiple conditions produced similar proportions of CD4 immature single positive (CD4 ISP), double positive (DP), CD8 single positive (CD8 SP) and double negative cells, as determined by flow cytometry. By day 24, high bead dose at all three cell densities produced a high percentage of DPs. The bead dose-response was most apparent at high cell densities, whereas the lowest cell density (2.5e5 cells/mL) was least sensitive to bead dose.

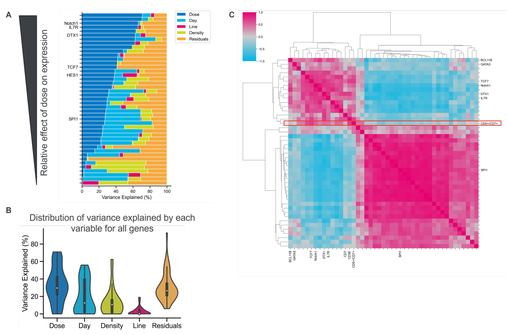


Figure 6. ETN bead dose is the major driver of gene expression changes. Gene expression was assessed at multiple time points (day 10, 12, and 14) during ProT differentiation using a custom 48-gene Nanostring panel. Mixed effects regression modelling was used to identify the fraction of variance explained by each parameter (bead dose, day of differentiation, cell line, and cell seeding density). For the majority of genes, bead dose explains the highest percentage of variance (A and B). Many genes in the panel are strongly correlated (red) or anti-correlated (blue) with other genes or with ProT phenotype at D18 (CD7+CD5+; red box) (C). Many Notch-responsive genes (*Notch1, DTX1, TCF7, IL7R, et al*) correlate well with each other and with ProT phenotype.

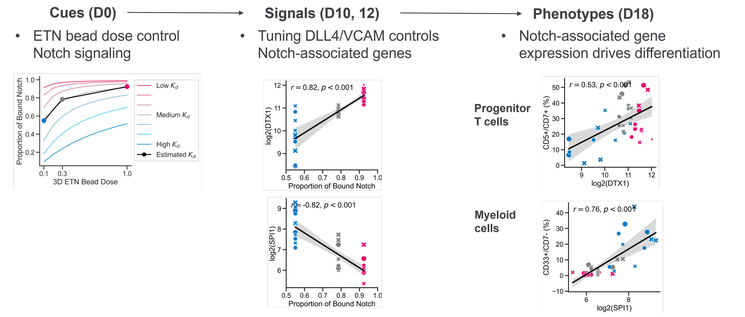


Figure 7. ETN bead dose controls Notch signaling, Notch-responsive gene expression, and cell phenotype. The proportion of bound Notch receptors is a function of ETN bead dose. The expression of Notch-responsive genes (e.g. *DTX1*) can be tuned via ETN bead dose, which in turn drives differentiation to Progenitor T cells. Conversely, Myeloid gene expression (e.g. *SPI1*) is inversely proportional to ETN bead dose, and a higher percentage of Myeloid cells is observed with low bead doses.

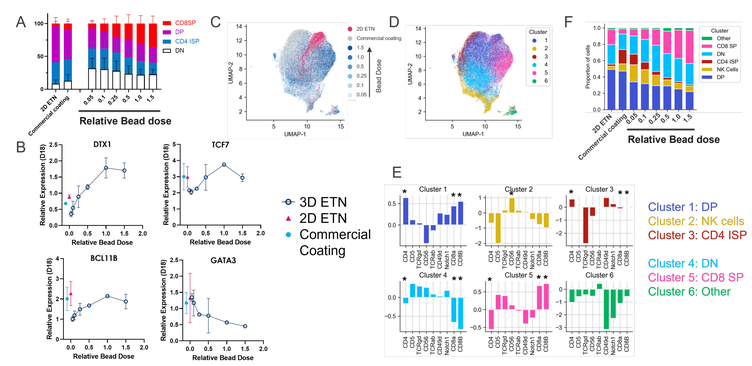


Figure 8. T cell phenotype is modulated by ETN bead dose. iPSC-derived CD34+ cells were differentiated into ProT cells for 18 days using 2D ETN. Subsequently, T cell maturation was induced using 3D ETN beads, with a 30-fold range of bead doses tested. (A) After 7 days of maturation, cell phenotype was assessed by flow cytometry. High bead doses produced more CD8 SP cells than lower bead doses, while low bead doses produced more CD4 ISPs and double negative cells (DN). (B) After 3 days of maturation, expression of *DTX1, TCF7 and BCL11B* was dose-dependent, with saturation occurring around the 1x bead dose. GATA3 displayed an inverse dose-response, with expression highest at low bead doses. (C) After 7 days of maturation, a 13-color flow cytometry panel was used to assess cell phenotype, and UMAP was used to perform dimensionality reduction of this multi-dimensional dataset. Cells were observed to cluster based on dose of DLL4. (D&E) Unsupervised machine learning was used to identify expression patterns within the multi-color flow cytometry dataset. (E) Six clusters were identified, with expression of CD8a, CD8B and CD4 used to describe the clusters as double positive, CD4 ISP, CD8 SP, and DN. (D) The DP cluster with 2D ETN, and CD4 iSP with commercial coating material. (F) The proportion of cells in each cluster was dependent on DLL4 dose, with high ETN bead doses containing more of the CD8 SP cluster, while lower bead doses contained a higher proportion of CD4 iSP cluster, similar to curated data in panel (A).

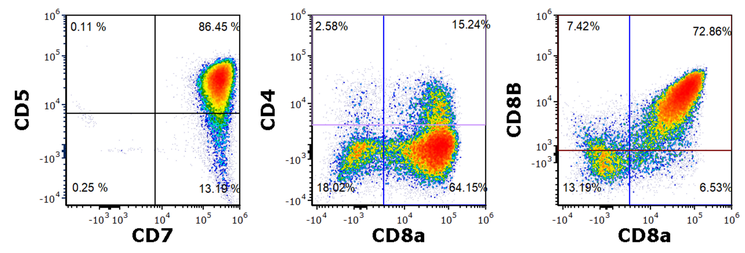


Figure 9. High levels of Notch signaling induced by high ETN bead dose induces differentiation to CD8αβ Single Positive cells. Based on finding in Figure 7, differentiation with high ETN bead dose was extended for an additional 7 days. After 14 days of differentiation using a high ETN bead dose, over 86% of cells were CD7+CD5+, over 64% were CD8α SP, and 73% were CD8αβ.

CONCLUSIONS

3D Engineered Thymic Niche (ETN) serves as surrogate thymic stromal tissue, inducing Notch signaling in iPSC-derived CD34+ cells and resultant T-cell lineage commitment to CD5+CD7+ progenitor T cells

Notch signaling and subsequent T-lineage commitment can be tightly controlled by the dose of ETN beads

3D ETN enables robust and controllable induction of T-cell lineage commitment and subsequent maturation from iPSC cells

High levels of Notch signaling induced by high ETN bead dose was observed to drive robust differentiation to CD8αβ single positive cells

The ETN technology provides a foundation for time and dose-dependent Notch signaling in a manner that enables repeatable manufacturing of iPSC-derived T cells