Generation of T Cells from Hematopoietic Stem and Progenitor Cells in a Stroma-Free, Serum-Free Culture System

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Introduction

T-cells isolated from peripheral blood (PB) have been shown to be effective for (development of) adoptive immunotherapy for cancer patients. T cells can also be generated by differentiation of hematopoietic stem and/or progenitor cells (HSPCs) in cord blood (CB) or bone marrow (BM). This approach not only offers a renewable source of T cells, but also provides a model system to study disease mechanisms or validate new drugs that affect T cell development and/or function.

Differentiation of HSPCs to T cells typically requires co-culture of HSPCs with stromal cell lines that have been engineered to express a Notch-ligand. In such cultures, $CD34^+CD38^{-/lo}$ HSPCs develop into $CD7^+CD5^+$ progenitor T (pro-T) cells that further differentiate to CD4 immature single-positive (CD4 ISP) cells. CD4 ISP cells give rise to $CD4^+CD8^+$ double positive (DP) cells. These finally mature into $CD3^+TCR\alpha\beta^+$ CD4 and CD8 single positive (SP) T cells.

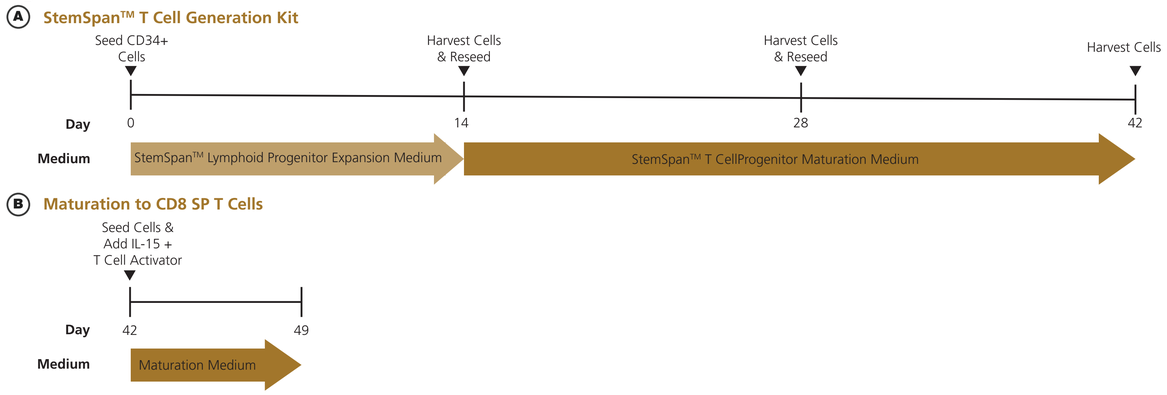
Here we present a serum-free multi-step culture method that recapitulates these differentiation steps in the absence of stromal cells and that generates large numbers of functional T lineage cells from a limited number of purified $CD34^+$ CB cells. In the first step, $CD34^+$ CB cells are differentiated into $CD7^+CD5^+$ pro-T cells: average frequency: 70% (range: 19-96%, n=33), average yield per initial $CD34^+$ cell: 209 (range: 36-697). In the second step, pro-T cells are differentiated into CD4 ISP and more mature DP cells expressing CD3 and TCRαβ. Continued culture of DP cells with additional stimuli, i.e., IL-15 and ImmunoCult CD28/CD3/CD2 T Cell Activator for one week results in the generation of functionally active CD8 SP T cells.

Methods

Culture Protocol

$CD34^+$ cells were enriched from human CB samples by depleting mature cells using RosetteSep™ followed by EasySep™ CD34 positive selection. The isolated $CD34^+$ cells (freshly isolated or frozen) were plated at $1x10^4$ cells/mL in StemSpan™ SFEM II medium supplemented with Lymphoid Progenitor Expansion Supplement (containing SCF, TPO, Flt3L, and IL-7) onto plates pre-coated with StemSpan™ Lymphoid Differentiation Coating Material (**Figure 1A**). Every 3-4 days a half medium exchange was performed. The cells were cultured for 14 days to generate pro-T cells. For further maturation, the pro-T cells were harvested, counted and re-plated at $1x10^5$ cells/mL onto freshly coated plates in StemSpan™ T Cell Progenitor Maturation Medium composed of SFEM II medium and a T Cell Progenitor Maturation Supplement (containing Flt3L and IL-7) for an additional 14 days of culture. Cells were then harvested, counted and re-plated at $5x10^5$ cells/mL onto freshly coated plates for 14 more days of culture. CD4 ISP and $CD4^+CD8^+$ DP T cells were harvested after a total of 6 weeks of culture (**Figure 1A**). A recommended protocol to differentiate DP cells into CD8 SP T cells is shown in **Figure 1B**. Briefly, Day 42 cells were harvested and re-plated at $1x10^6$ cells/mL onto freshly coated plates in T Cell Progenitor Maturation medium with added IL-15 and ImmunoCult Human CD3/CD28/CD2 T Cell Activator. The cells were cultured for one week. ImmunoCult™ Human CD3/CD28 T Cell Activator can also be used (data not shown).

Figure 1. Workflow and Culture Protocol



Assessment of T Lineage Cells

Cells harvested from the cultures were counted and analyzed by flow cytometry for the expression of T lineage markers including CD7, CD5, CD4, CD8α, CD8β, CD3, TCRαβ, CD45RA and CD27 as well as γδ T and NK cell markers, TCRγδ and CD56. Dead cells were excluded by light scatter profile and 7-AAD or DRAQ7 staining. The number of pro-T cells ($CD7^+CD5^+$), CD4 ISP ($CD4^+CD8^-TCR^-$), DP ($CD4^+CD8^+$) cells and their $CD3^+TCR\alpha\beta^+$ subset (Day 42), $CD56^+$, $CD3^+TCR\gamma\delta^+$, and $CD3^+TCR\alpha\beta^+$ cell subsets (Day 49) (**Figures 2** and **3**) was calculated based on the fraction of cells expressing the requisite markers.

Quantitative RT-PCR Analysis

Control peripheral blood (PB) CD4 T, CD8 T, γδ T, and CB $CD34^+$ cells were purified using EasySep™ magnetic separation. DP and $CD3^+TCR\alpha\beta^+$ CD8 SP Day 49 cells (**Figure 3D**) were purified by FACS cell sorting. Total RNA was extracted using Qiagen RNeasy® Kit, cDNA was synthesized using High Capacity cDNA Reverse Transcription Kit (ThermoFisher) and qRT-PCR was performed using PrimeTime qPCR Assay primers (IDT) and qPCR Master Mix Kit (STEMCELL Technologies).

Results

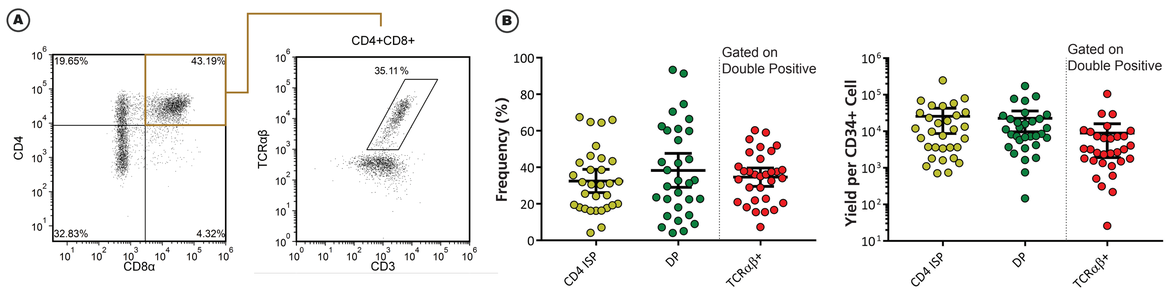


Figure 2. Pro-T Cells Differentiate To CD4 ISP And DP Cells Expressing $CD3^+TCR\alpha\beta^+$ During 4 Weeks Of Maturation Culture. 50,000 pro-T cells generated after 14 days of culture as described in **Figure 1A** were replated in StemSpan™ SFEM II supplemented with the T Cell Progenitor Maturation Supplement in wells of 24-well plates coated with StemSpan™ Lymphoid Differentiation Coating Material, and cultured for another 4 weeks (**Figure 1A**). (**A & B**) Cells were analyzed by flow cytometry for CD3, CD4, CD8, and TCRαβ expression. Cell counts were also obtained (**B**). Dots show the results of 31 individual experiments; lines show the mean and 95% confidence intervals (CI). On average 32% of cells on day 42 were CD4 ISP and 38% were $CD4^+CD8^+$ DP cells with yields of ~26,000 CD4 ISP and ~23,000 DP cells per original $CD34^+$ cell. 35% of DP cells co-expressed CD3 and TCRαβ.

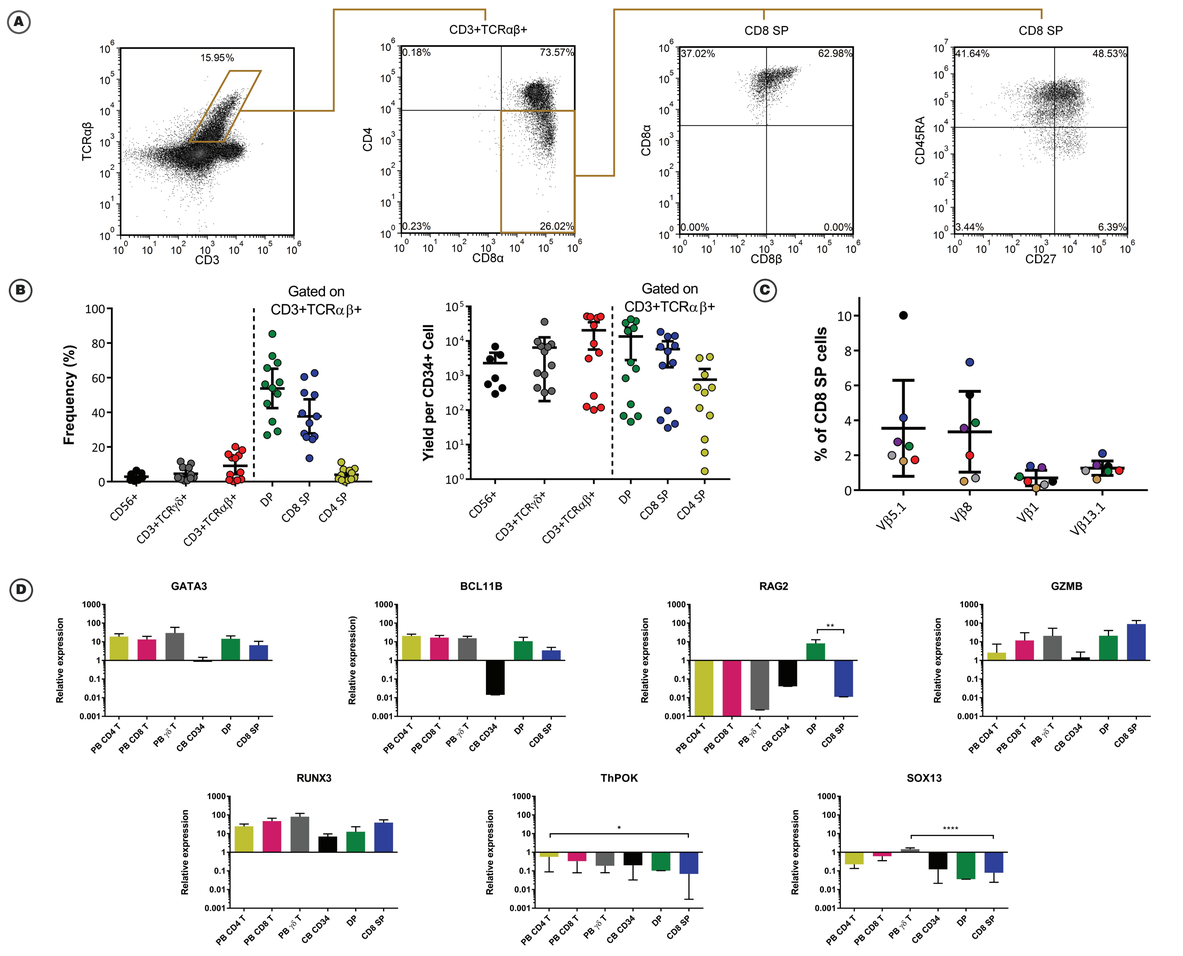


Figure 3. CD8 SP T Cells Derived from DP Cells Resemble Mature Naïve CD8 SP T Cells. To promote further T cell maturation, 500,000 Day 42 cells were cultured for one more week in T Cell Progenitor Maturation Medium further supplemented with IL-15 and CD3/CD28/CD2 T Cell activator (**Figure 1B**). **A)** The cultured Day 49 cells were stained for CD3, TCRαβ, CD4, CD8α, CD8β, CD45RA and CD27 and analyzed by flow cytometry. CD8 SP T cells co-expressed CD8 α and CD8β, and naïve T cell markers CD27 and CD45RA. **B)** Frequency and yield of $CD3^+TCR\alpha\beta^+$ cells and subpopulations, and of $CD56^+$ and $CD3^+TCR\gamma\delta^+$ cells, for Day 49 cells (n=12, mean and CI). On average, 9% of Day 49 cells were $CD3^+TCR\alpha\beta^+$; 38% of $CD3^+TCR\alpha\beta^+$ cells were CD8 SP and 54% were DP. The yield of CD8 SP T cells per initial $CD34^+$ cell was on average ~6000, but was highly variable between experiments (range: 31-18,000). $CD56^+$ and $CD3^+TCR\gamma\delta^+$ cells were also detected, but at lower frequencies and yields. **C)** TCR polyclonality of CD8 SP cells was examined by flow cytometry analysis of the frequency of four typical TCR Vβ clones. **D)** Expression of GATA3 and BCL11B, transcription factors involved in T cell lineage specification and commitment, RAG2, protein responsible for rearranging TCR genes, GZMB, intracellular molecule inducing target cell lysis, and RUNX3, ThPOK, and SOX13, the signature transcription factors for CD8, CD4 and γδ T cell lineages, respectively in Day 49 T cell subsets. Data shown are expressed relative to expression of TATA Binding Protein (TBP) housekeeping gene and represent the average and standard deviation (SD) of 4-6 independent donors. Purified PB CD4 T, CD8 T, γδ T, and CB $CD34^+$ cells were used as controls. Expression levels of GATA3, BCL11B, RUNX3 and GZMB transcripts in cultured CD8 SP cells were similar to the levels in PB CD8 T cells. RAG2 was expressed in DP cells but downregulated in CD8 SP cells (paired t-test, p value=0.0089). ThPOK and SOX13 expression was lower in CD8 SP cells compared to control PB CD4 T and γδ T cells, respectively (non-paired t-test, p value=0.03 and <0.0001).

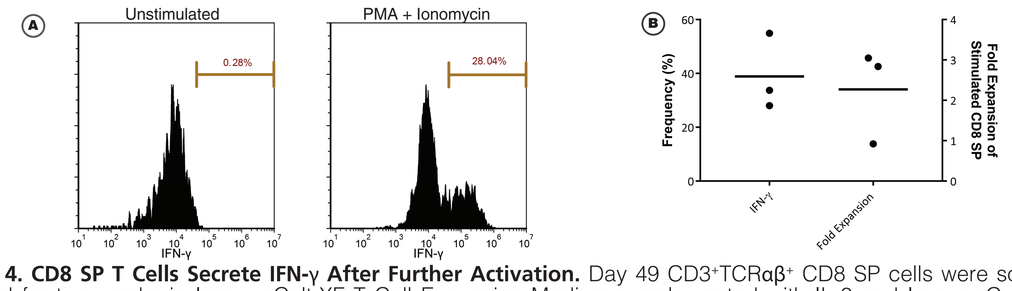


Figure 4. CD8 SP T Cells Secrete IFN-γ After Further Activation. Day 49 $CD3^+TCR\alpha\beta^+$ CD8 SP cells were sorted and cultured for two weeks in ImmunoCult-XF T Cell Expansion Medium supplemented with IL-2 and ImmunoCult Human CD3/CD28/CD2 T Cell Activator. The culture was fed every 3-4 days with ImmunoCult-XF T Cell Expansion Medium supplemented with IL-2. The cells were then stimulated with PMA+Ionomycin for 6 hours with Brefeldin A added in the last 4 hours. Cells were harvested and stained for viability using Zombie NIR fixable viability dye and CD3, CD8, CD4 and TCR αβ surface markers. Cells were fixed and stained for intracellular IFN-γ and analyzed by flow cytometry. **A)** Example flow cytometry plot showing that 28% of stimulated CD8 SP cells were IFN-γ^+ as compared to <1% of non-stimulated cells. **B)** On average, 39% (range 28-55%, n=3) of PMA/Ionomycin stimulated CD8 SP cells produced IFN-γ. CD8 SP expanded on average 2.3 fold (range: 1-3) during two weeks of activation with the T Cell activator.

Summary

- $CD34^+$ HSPCs can proliferate and differentiate to $CD4^+CD8^+$ DP cells during 6 weeks of culture under serum-free and stroma-free conditions, resulting in the generation of thousands of DP cells per original $CD34^+$ HSPC
- DP cells can differentiate further to CD8 SP T cells when stimulated with CD3/CD28/CD2 (or CD3/CD28) T Cell Activator and IL-15
- HSPC-derived CD8 SP T cells show hallmarks of naive $CD8^+$ T cells as indicated by:
  - Expression of CD8α, CD8β, CD45RA & CD27
  - Polyclonal TCR Vβ profile
  - Gene expression profile: expression of GATA3, BCL11B, RUNX3 and GZMB and lack of expression of RAG2, ThPOK & SOX13
  - Ability to proliferate and secrete IFN-γ after further stimulation