Stroma-free, serum-free expansion and differentiation of hematopoietic stem and progenitor cells to the T cell lineage

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Introduction

The use of T cells for cancer immunotherapy and other therapeutic applications relies on the isolation of T cells from peripheral blood and their subsequent activation and expansion in culture. T cells can also be generated from 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 with stromal cell lines that have been engineered to express a Notch-ligand. In such cultures, $CD34^+CD38^{-/lo}$ HSPCs develop into $CD7^+CD5^+$ pro-T cells that further differentiate to T lineage-committed progenitor cells (pre-T cells) characterized by the expression of CD1a. $CD7^+CD1a^+$ pre-T cells can then differentiate to express CD4 and become CD4 immature single-positive (CD4ISP) cells. CD4ISP cells give rise to $CD4^+CD8^+$ double positive (DP) cells. These finally mature into CD4 and CD8 single-positive (SP) $CD3^+TCR\alpha\beta^+$ T cells.

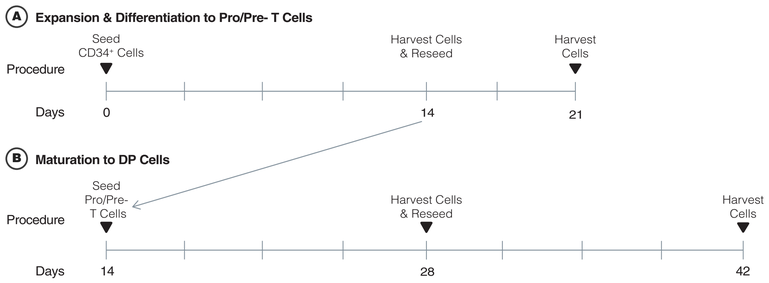
Here we describe a serum-free culture method that recapitulates these differentiation steps in the absence of stromal cells and generates large numbers of functional T lineage cells from a limited number of purified $CD34^+$ CB cells. In this system, $CD7^+CD5^+$ pro-T cells were generated with a frequency of 67% (57 - 77%) and a yield of 144 (99 - 188) cells per initial $CD34^+$ cell at day 14 (mean & 95% CI; n=25). The frequency and yield of these cells increased to 84% (80 - 88%) and 2130 (1500 - 2761), respectively, when cultured under the same conditions for another week. Pro-T cells were able to differentiate into more mature DP and $CD3^+TCR^+$ T cells when cultured for an additional 4 weeks under different stroma-free culture conditions that support T cell maturation.

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 were plated at $1x10^4$ cells/mL in StemSpan™ SFEM II medium supplemented with T Cell Progenitor Expansion Supplement (containing SCF, TPO, Flt3L, and IL-7) onto plates pre-coated with StemSpan T Cell Differentiation Coating Material (StemSpan™ T Cell Progenitor Differentiation Kit). Every 3 - 4 days a half medium exchange was performed. The cells were cultured for 14 days, after which they were harvested, counted and re-plated at $1x10^5$ cells/mL onto freshly coated plates for an additional 7 days of culture. T lineage cells were harvested on day 14 or 21 for analysis and/or further maturation. This protocol is detailed in Figure 1A. For further maturation the pro/pre-T cells harvested on day 14 were seeded at $1x10^5$ cells/mL on freshly coated plates with the same attachment substrate in a modified T Cell Progenitor Maturation medium containing IL-7 and Flt3L. After 14 days, cells were harvested, counted and re-plated at 2×10^5 cells/mL onto freshly coated plates for an additional 14 days of culture. Matured DP T cells were harvested after a total of 6 weeks of culture (Figure 1B).

FIGURE 1: Workflow and Culture Protocol

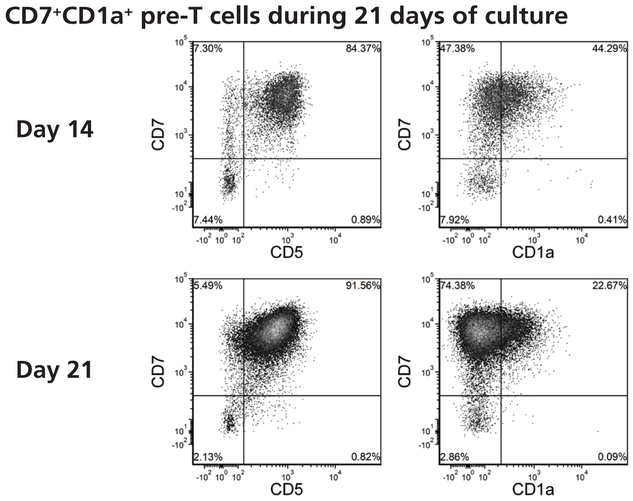


Assessment of T Lineage Cells

Harvested cells were counted and analyzed by flow cytometry for the expression of T lineage markers including CD7, CD5, CD1a, CD4, CD8, CD3, TCRαβ and TCRγδ. Dead cells were excluded by light scatter profile and 7-AAD staining. The number of pro-T ($CD5^+CD7^+$), pre-T ($CD7^+CD1a^+$), CD4ISP ($CD4^+CD3^-TCR^-$), DP ($CD4^+CD8^+$) and $CD3^+TCR\alpha\beta^+$ T cells (Figure 3 and 4) was calculated based on the fraction of cells counted expressing the specified markers.

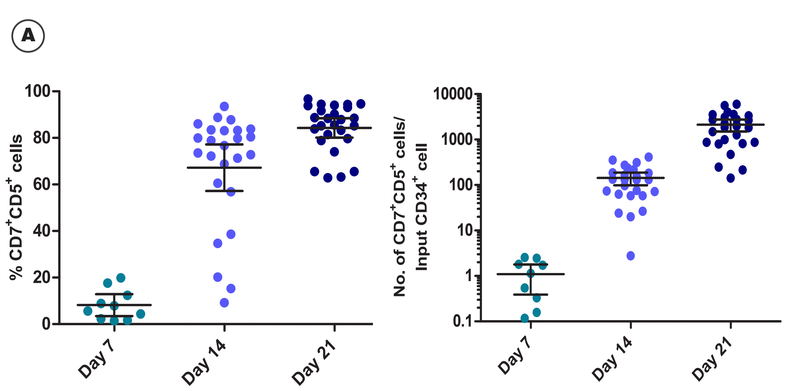
Results

FIGURE 2: $CD34^+$ CB cells differentiate into $CD7^+CD5^+$ pro-T and $CD7^+CD1a^+$ pre-T cells during 21 days of culture



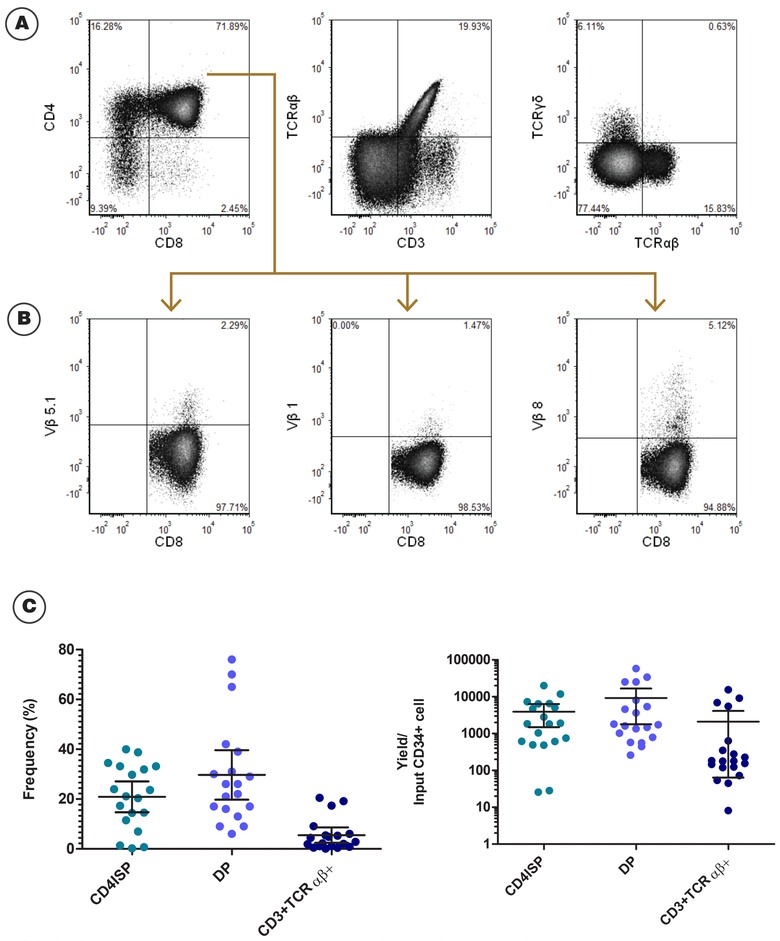
$CD34^+$ CB cells (1,000 cells/well) were cultured for 3 weeks. Cells were analyzed by flow cytometry for the expression of CD7, CD5 and CD1a on days 14 and 21.

FIGURE 3: StemSpan™ T Cell Progenitor Differentiation Kit promotes expansion of $CD34^+$ CB cells and their differentiation into $CD7^+CD5^+$ pro-T and $CD7^+CD1a^+$ pre-T cells



$CD34^+$ CB cells were cultured for the indicated times as shown in Figure 1A, and then counted and analysed by flow cytometry as described in Methods. The percentage and number of **(A)** $CD7^+CD5^+$ cells and **(B)** $CD7^+CD1a^+$ cells generated are shown for 10 - 26 independent experiments. Horizontal lines indicate the mean. Vertical lines indicate 95% confidence interval. **(A)** The frequency of $CD7^+CD5^+$ pro-T cells increased each week up to, on average, 84% on day 21. The number of pro-T cells also increased ~10 - 100 fold every week, resulting in an average of ~2000 pro-T cells per initial $CD34^+$ cell on day 21. **(B)** On day 21 on average 28% of the cells express CD7 and CD1a, indicating further differentiation into pre-T cells. The yield of $CD7^+CD1a^+$ cells on day 21 was ~800 per initial $CD34^+$ cell.

FIGURE 4: Pro-T cells differentiate into CD4ISP, DP and $CD3^+TCR^+$ T cells during continued stroma-free culture



50,000 pro/pre-T cells generated after 14 days of culture as described in Figure 1A were replated in StemSpan™ SFEM II medium supplemented with a T Cell Progenitor Maturation Supplement (containing Flt3L and IL-7) in 24-well plates coated with StemSpan™ T Cell Differentiation Coating Material and cultured for another 4 weeks (Figure 1B). **(A & B)** Cells were then analyzed by flow cytometry for the expression of CD3, CD4, CD8, TCRαβ and TCRγδ. **(B)** Cells were also stained with antibodies that recognize variants of TCR Vβ chain to assess polyclonality of TCR. These cells were first gated on DP cells. **(C)** Cell counts were also obtained. Data shows the mean with 95% CI of 19 experiments. On average 21% $CD4^+CD8^-$ CD4ISP, 30% $CD4^+CD8^+$ DP and 6% $CD3^+TCR\alpha\beta^+$ cells arise in these stroma-free maturation cultures with a yield of ~4000 CD4ISP, 9000 DP and 2000 $CD3^+TCR\alpha\beta^+$ cells, respectively, per original $CD34^+$ cell. The majority of $CD3^+TCR\alpha\beta^+$ cells are DP.

Conclusions

- $CD34^+$ HSPCs from CB proliferate and differentiate efficiently into T cell progenitors in the absence of serum and stromal cells when cultured for 2 - 3 weeks using serum-free StemSpan medium and T Cell Progenitor Differentiation Supplements.
- This culture system can generate >2000 $CD7^+CD5^+$ pro-T cells with ~80% frequency (and ~800 pre-T cells) per original $CD34^+$ CB cell during 3 weeks of culture.
- Pro- and pre-T cells can differentiate further into DP and $CD3^+TCR^+$ T cells during 4 weeks of continued stroma-free culture using a T Cell Progenitor Maturation Supplement.
- The overall yield of $CD3^+TCR\alpha\beta^+$ T cells is ~2000 per initial $CD34^+$ CB cell (with average frequency of 6%) after a total of 42 days of culture.