Generation of T and NK Cells From Pluripotent Stem Cell-Derived Hematopoietic Progenitors in a Stroma-Free, Serum-Free Culture System

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INTRODUCTION

The use of T and NK cells in the treatment of cancer and other disorders has shown promise, but obtaining sufficient quantities of these cells from patients is hampered by technical challenges and high cost. Human pluripotent stem cells (hPSCs) could represent an unlimited "off-the-shelf" source for generating T and NK cells for clinical applications. Generation of lymphocytes from hPSCs has been difficult because cell cultures often do not support important differentiation processes and rely on undefined factors such as stromal cells and serum. We developed a multi-step culture system that promotes generation of T and NK cells from multiple hPSC lines without the need for stromal cells or serum. hPSCs were first induced to differentiate to mesoderm and hematoendothelial lineages by formation of 3D aggregates. After 10 days of culture, hPSC aggregates were dissociated into a single-cell suspension and CD34$^+$ cells were isolated and cultured for two weeks in stroma-free cultures, promoting differentiation into CD7$^+$CD5$^+$ lymphoid progenitor cells. Finally, lymphoid progenitor cells were differentiated into either T or NK cells using specialized lineage-specific culture conditions. The cells generated in T cell cultures after a total of 38 days of culture were CD4$^+$CD8$^+$ double-positive (DP), and some expressed CD3 and TCRαβ, which is characteristic of mature functional T cells. CD56$^+$ cells arising in the NK cell cultures expressed other characteristic NK cell markers including NKp46, NKG2D, and CD16, and were functional as shown by IFN-γ secretion and cytotoxicity against K562 target cells. These results show that hPSCs can be differentiated under stroma- and serum-free conditions into lymphoid progenitor cells that can generate large numbers of T and NK cells for basic and translational research.

METHODS

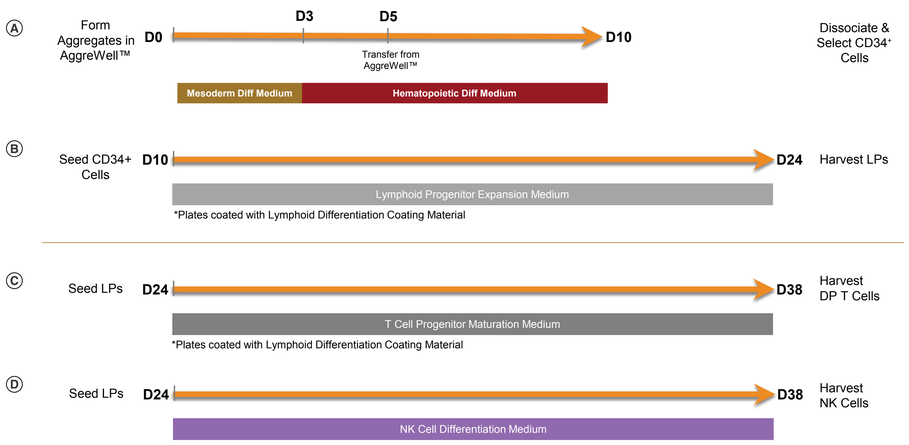

FIGURE 1. Culture Protocol

hPSCs used in this study were from two embryonic stem (ES) cell lines (H1 & H9) and three induced pluripotent stem (iPS) cell lines (WLS-1C [1C], STiPS-M001 [M001], STiPS- F016 [F016]). hPSCs were maintained in mTeSR™1 and harvested and dissociated into single-cell suspensions. The hPSCs were seeded into AggreWell™ plates in a mesoderm differentiation medium to form 500-cell aggregates, with 10 µM Y-27632 added at day 0 (Figure 1A). After 3 days, the medium was changed to induce hematopoietic lineage differentiation. After 2 additional days, aggregates were transferred onto non-tissue culture-treated plates. On day 10, the aggregates were harvested and dissociated using collagenase II & TrypLE™, followed by filtering through a 37 µm cell strainer. CD34$^+$ cells were isolated using EasySep™ Human CD34$^+$ Positive Selection Kit II (Catalog #17856). The isolated CD34$^+$ cells were plated at 5 x 10^4 cells/mL in StemSpan™ SFEM II medium + StemSpan™ Lymphoid Progenitor Expansion Supplement onto plates coated with StemSpan™ Lymphoid Differentiation Coating Material, which provides Notch signaling (Figure 1B). The cells were cultured for 14 days to generate lymphoid progenitor cells (LPs). LPs were harvested, counted, and further differentiated into either DP T cells or NK cells. For T cell differentiation (Figure 1C), LPs were re-plated at 1 x 10^5 cells/mL onto freshly coated plates in StemSpan™ T Cell Progenitor Maturation Medium (composed of StemSpan™ SFEM II and StemSpan™ T Cell Progenitor Maturation Supplement) and cultured for an additional 14 days. CD4$^+$CD8$^+$ DP T cells were harvested after a total of 38 days of culture. For NK cell differentiation (Figure 1D), LPs were re-plated at 1 x 10^5 cells/mL onto uncoated plates in StemSpan™ NK Cell Differentiation Medium (composed of StemSpan™ SFEM II supplemented with StemSpan™ NK Cell Differentiation Supplement and UM729) for an additional 14 days. CD56$^+$ NK cells were harvested after a total of 38 days of culture.

Assessment of Cells

Cultured cells were harvested, counted and analyzed by flow cytometry for expression of markers of hematopoietic development (CD34 and CD144) and lymphoid commitment (CD7 and CD5). T cell maturation cultures were assessed for expression of CD4, CD8α, CD3, and TCRαβ, while NK cell differentiation cultures were assessed for expression of CD56, NKp46, NKG2D, and CD16. Dead cells were excluded by light scatter profile and DRAQ7 staining. The number of hematopoietic progenitor cells (CD34$^+$), lymphoid progenitor cells (CD7$^+$CD5$^+$), DP cells (CD4$^+$CD8$^+$), and NK cells (CD56$^+$) were calculated from the fraction of cells expressing the requisite markers.

RESULTS

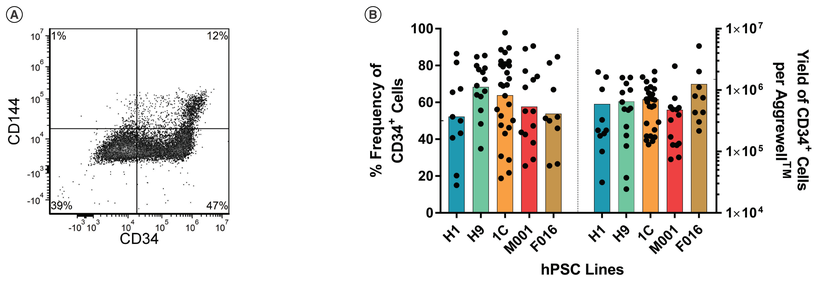

FIGURE 2. hPSCs Differentiate to CD34$^+$ Hematopoietic Progenitor Cells

H1, H9, 1C, M001, and F016 hPSCs were formed into 500-cell aggregates in individual wells of a 6-well AggreWell™ plate and then induced to differentiate to mesoderm and hematopoietic lineages as described in Figure 1A. After 10 days of culture, the aggregates were dissociated and analyzed by flow cytometry for CD34 and CD144 expression; cell yields were also calculated **(A, B)**. **(A)** Some CD34$^+$ cells express the endothelial marker CD144. **(B)** Average frequencies of CD34$^+$ cells were 52%, 68%, 64%, 58%, and 54%, and average yields per well were 5.9 x 10^5, 6.5 x 10^5, 7.1 x 10^5, 4.8 x 10^5, and 1.3 x 10^6 CD34$^+$ cells from H1, H9, 1C, M001, and F016 hPSCs, respectively. Dots show the results of 9 - 27 individual experiments; bars show the mean.

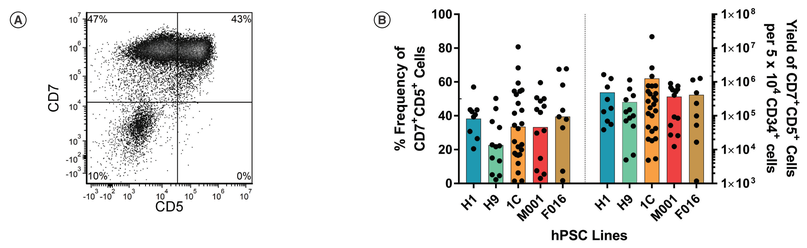

FIGURE 3. CD7$^+$CD5$^+$ Lymphoid Progenitor Cells are Generated From hPSC-Derived CD34$^+$ Cells

hPSCs were differentiated to the hematopoietic lineage as described in Figure 1A and CD34$^+$ cells were then isolated and cultured in StemSpan™ SFEM II + Lymphoid Progenitor Expansion Supplement on plates coated with Lymphoid Differentiation Coating Material. **(A,B)** After 2 weeks, cells were harvested, counted, and analyzed for expression of CD5 and CD7. **(B)** Average frequencies of CD7$^+$CD5$^+$ lymphoid progenitor cells were 38%, 23%, 34%, 33%, and 40%, and average yields per 5 x 10^4 hPSC-derived CD34$^+$ cells were 4.9 x 10^5, 2.6 x 10^5, 1.3 x 10^6, 3.7 x 10^5, and 4.1 x 10^5 CD7$^+$CD5$^+$ lymphoid progenitor cells in cultures of H1, H9, 1C, M001, and F016 hPSCs, respectively.

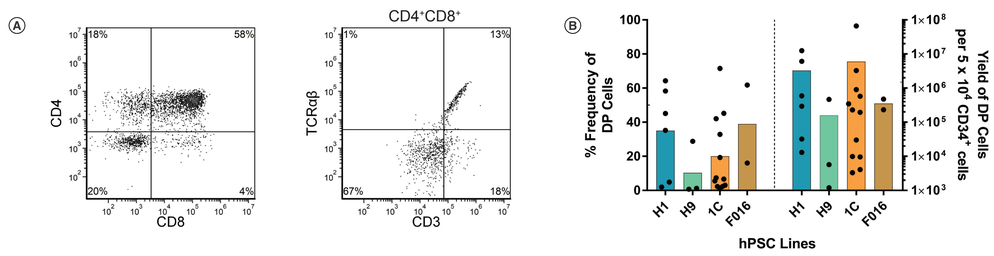

FIGURE 4. hPSC-Derived Lymphoid Progenitor Cells Differentiate to CD4$^+$CD8$^+$ DP Cells Expressing CD3 and TCRαβ Under T Cell Differentiation Conditions

Lymphoid progenitor cells generated from hPSC-derived CD34$^+$ cells were cultured in StemSpan™ SFEM II + T Cell Progenitor Maturation Supplement in plates coated with Lymphoid Differentiation Coating Material for 14 days. **(A,B)** Cells were harvested, counted, and analyzed for expression of CD4, CD8, CD3, and TCRαβ. **(B)** Average frequencies of DP cells derived from hPSC lines H1, H9, 1C, and F016 were 35%, 10%, 20%, and 39%, whereas yields of DP cells per 5 x 10^4 input hPSC-derived CD34$^+$ cells were 3.3 x10^6, 1.6 x 10^5, 6.0 x 10^6, and 3.5 x 10^5 for H1, H9, 1C, and F016 hPSC lines, respectively. An average of 16% (range: 0.4 - 42%, n = 6 experiments with H1 hPSCs) and 13% (0 - 35%, n = 11 experiments with 1C hPSCs) of DP cells co-expressed CD3 and TCRαβ (data not shown).

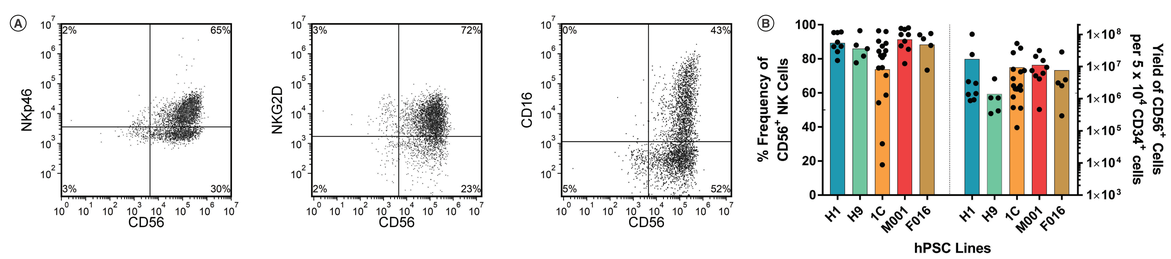

FIGURE 5. hPSC-Derived Lymphoid Progenitor Cells Produce Large Numbers of CD56$^+$ NK Cells in NK Cell Cultures

Lymphoid progenitor cells generated from hPSC-derived CD34$^+$ cells were cultured in StemSpan™ SFEM II + NK Cell Differentiation Supplement and UM729 in uncoated plates for 14 days. Cells were harvested, counted, and analyzed for the expression of NK cell markers CD56, NKp46, NKgGD, and CD16. **(A)** Representative flow cytometry plots show co-expression of CD56 with indicated NK cell markers. **(B)** CD56$^+$ cells were generated from hPSC lines H1, H9, 1C, M001, and F016 at average frequencies of 89%, 86%, 74%, 91%, and 88%, and with yields of 1.7 x 10^7, 1.4 x 10^6, 9.4 x 10^6, 1.1 x 10^7, and 7.7 x 10^6 CD56$^+$ cells per 5 x 10^4 input hPSC-derived CD34$^+$ cells, respectively.

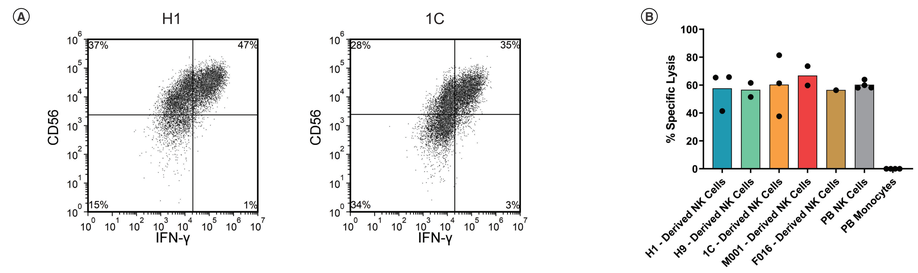

FIGURE 6. hPSC-Derived NK Cells Secrete IFN-γ and Kill Target Cells

(A) NK cells were generated from H1 and 1C hPSC lines after 38 days of culture. The NK cells were stimulated with PMA/ionomycin for 2 hours followed by Brefeldin A for an additional 2 hours. Cells were harvested and stained for CD56 and intracellular IFN-γ. The flow cytometry plots show that 56% of CD56$^+$ cells generated from both hPSC lines expressed intracellular IFN-γ upon stimulation. **(B)** CD56$^+$ NK cells were generated from ES cell lines H1 and H9, and iPS cell lines 1C, M001, and F016. NK cells were co-cultured with calcein AM-labeled K562 cells for 2 hours at an effector (NK cells) to target (labeled K562 cells) ratio of 2:1. Peripheral blood (PB) NK cells and PB monocytes were also co-cultured with labeled K562 cells as positive and negative controls, respectively. PB NK cells were cultured overnight with NK Cell Differentiation Supplement and SFEM II, while PB monocytes were cultured overnight in SFEM II only. To detect spontaneous label release, control wells containing only calcein AM-labeled K562 target cells were set up. The labeled K562 cells were treated with 1% Triton™ X-100 to measure maximum release. After incubation, plates were centrifuged at 500 x *g* for 5 minutes and 100 µL of supernatant was transferred to black plates for analysis using a SpectraMax® microplate reader (excitation 485 nm/emission 530 nm). Results are expressed as % specific lysis: [(test release - spontaneous release) x 100] / (maximum release - spontaneous release). hPSC-derived NK cells showed similar killing activity toward K562 target cells as PB NK cells. Shown are means.

Summary

- hPSCs can be differentiated to T and NK cells in a serum-free, stroma-free culture system.
- High frequency and yield of CD4$^+$CD8$^+$ DP cells and CD56$^+$ NK cells were obtained from CD34$^+$ cells from multiple ES and iPS cell lines.
- The hPSC-derived DP T cells express T cell markers CD3 and TCRαβ, indicating further maturation. Future work will aim to further promote maturation into functional T cells.
- The hPSC-derived CD56$^+$ NK cells co-express CD16, NKG2D, and NKp46, secrete IFN-γ upon stimulation, and exhibit functional killing activity comparable to that of PB NK cells.