TECHNICAL BULLETIN

Generation of T Cells from Human Pluripotent Stem Cells Using STEMdiff™ and StemSpan™ Media and Supplements

Introduction

T cells are lymphocytes that provide defense against pathogens and tumors. As part of the adaptive immune system, T cells recognize a wide range of targets through their antigen-specific T cell receptors (TCRs) and exert effector functions, including cytokine secretion and cell killing. A crucial feature of T cells is the formation of memory cells, which helps establish a much faster response to reinfection than the primary response. While T cells can be isolated from peripheral blood, human pluripotent stem cells (hPSCs) offer a potentially unlimited source of immune cells. The ability to differentiate hPSCs to T cells provides a useful tool for developing adoptive immunotherapy in cancer patients and for research into the basic biology of these cells.

STEMdiff™ T Cell Kit (Catalog #100-0194) facilitates the differentiation of hPSCs to T cells, without the use of stromal cells and in serum-free culture conditions.

Differentiate hPSCs to T Cells

STEMdiff™ T Cell Kit comprises STEMdiff™ Hematopoietic - EB reagents paired with a StemSpan™ T Cell Generation Kit. The STEMdiff™ T Cell Kit is used in a four-stage protocol to differentiate hPSCs to T cells. In the first two stages, STEMdiff™ Hematopoietic - EB reagents are used to differentiate hPSCs to $CD34^+$ hematopoietic progenitor cells (Figure 1). First, hPSCs are cultured for 3 days in EB Formation Medium and EB Medium A to induce mesoderm specification. This is done in AggreWell™, allowing for the formation of uniformly sized embryoid bodies (EBs). In the second stage, the cells are cultured for 9 days in EB Medium B, promoting their specification to hematopoietic progenitor cells. The generated EBs are then dissociated into single cells, and $CD34^+$ cells are enriched using EasySep™ immunomagnetic cell isolation.

In the third stage, $CD34^+$ cells are cultured for 14 days in StemSpan™ Lymphoid Progenitor Expansion Medium on plates coated with StemSpan™ Lymphoid Differentiation Coating Material to stimulate their proliferation and differentiation to lymphoid progenitor (LP) cells. Finally, LP cells are differentiated to $CD4^+CD8^+$ double-positive (DP) T cells (Figure 2).

For DP T cell differentiation, LP cells are cultured for 14 days in StemSpan™ T Cell Progenitor Maturation Medium on plates coated with StemSpan™ Lymphoid Differentiation Coating Material. In this system, approximately 60 $CD4^+CD8^+$ DP T cells can be generated per $CD34^+$ cell (average of results from multiple embryonic stem [ES]/induced pluripotent stem [iPS] cell lines; see Figure 6).

Why Use STEMdiff™ for Generating T Cells?

CONSISTENT. Eliminate variation introduced by serum and stromal cell lines by culturing cells in serum- and feeder-free conditions.

UNIFORM. Reduce variability by producing uniform aggregates for EB formation with AggreWell™.

HIGH YIELD. Produce approximately 60 $CD4^+CD8^+$ DP T cells per input hPSC-derived $CD34^+$ cell.

CONVENIENT. Avoid extra passaging steps required with stromal cell-based cultures.

STEMdiff™ T Cell Kit Components

The following products are components of STEMdiff™ T Cell Kit (Catalog #100-0194) and are also available for individual sale.

Component	Size	Catalog #
STEMdiff™ Hematopoietic - EB Basal Medium	120 mL	100-0171
STEMdiff™ Hematopoietic - EB Supplement A	265 µL	100-0172
STEMdiff™ Hematopoietic - EB Supplement B	7 mL	100-0173
StemSpan™ SFEM II	2 x 100 mL*	09605
StemSpan™ Lymphoid Progenitor Expansion Supplement (10X)	5 mL	09915
StemSpan™ Lymphoid Differentiation Coating Material (100X)	2 x 250 µL	09925
StemSpan™ T Cell Progenitor Maturation Supplement (10X)	12.5 mL	09930

**500 mL format is also available (Catalog #09655).*

Protocol for Differentiation of hPSCs to T Cells

This protocol is designed to promote the differentiation of hPSCs to $CD4^+CD8^+$ DP T cells over 40 days of culture. The first two stages—mesoderm formation and hematopoietic specification—are shown in Figure 1. Stage three—lymphoid progenitor differentiation—and stage four—DP T cell differentiation—are shown in Figure 2. An optional protocol extension for further maturation of DP T cells to $CD8^+$ single-positive (SP) T cells is shown in Figure 3.

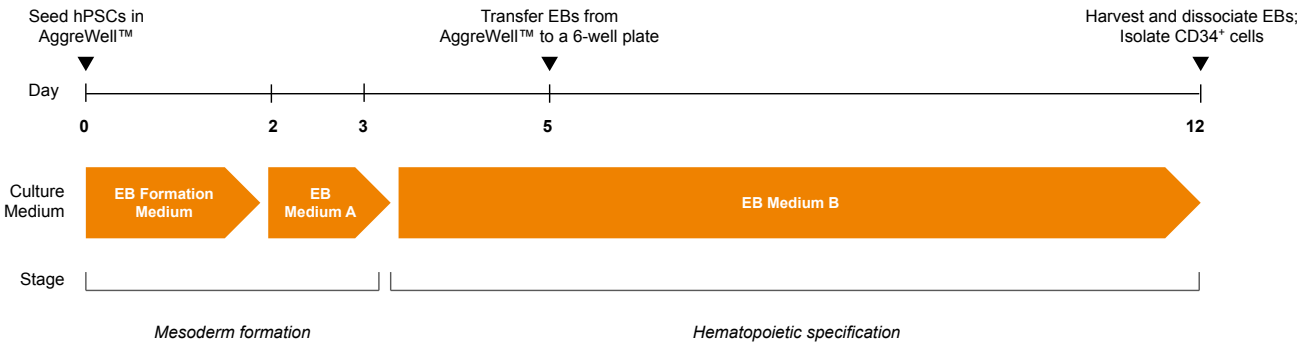

Figure 1. STEMdiff™ Hematopoietic - EB Progenitor Differentiation Protocol

hPSCs are harvested and dissociated into a single-cell suspension prior to seeding into AggreWell™ plates in EB Formation Medium (EB Medium A + 10 µM Y-27632) to form 600-cell aggregates. After 3 days of mesoderm formation, the medium is changed to EB Medium B to induce hematopoietic lineage differentiation. On day 5, EBs are transferred onto non-tissue culture-treated plates. After a total of 12 days, EBs are harvested and dissociated, and $CD34^+$ cells are then enriched by EasySep™ positive selection.

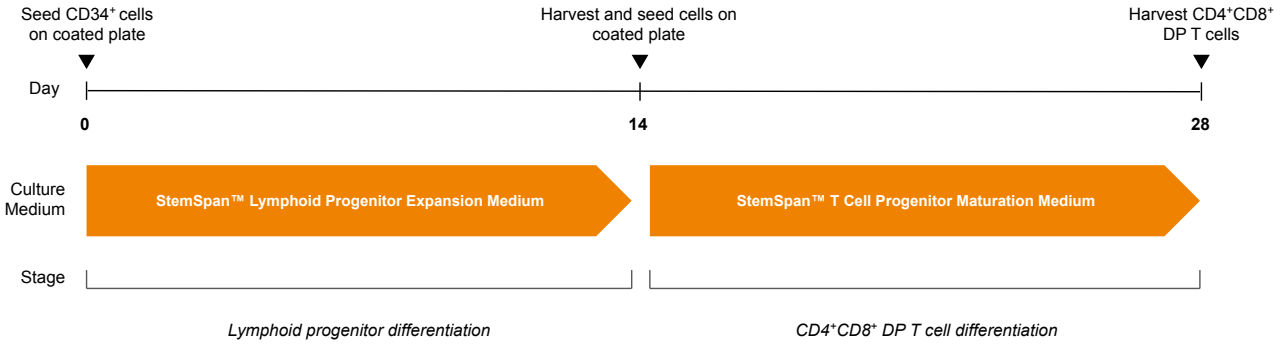

Figure 2. T Cell Generation Protocol

hPSC-derived $CD34^+$ cells are seeded in StemSpan™ Lymphoid Progenitor Expansion Medium on plates coated with StemSpan™ Lymphoid Differentiation Coating Material. On day 14, cells at the lymphoid progenitor stage are harvested and reseeded in StemSpan™ T Cell Progenitor Maturation Medium onto coated plates for further differentiation to $CD4^+CD8^+$ DP T cells. The DP T cells are harvested after 28 days. For further details, see the step-by-step protocol on page 3.

This protocol has been optimized for use with ES and iPS cells; refer to the Technical Manual (Document #10000007541) for complete instructions. Optimal cell yields depend on maintenance of proper cell health, which is achieved by following the recommended schedule of feeding and medium changes.

Differentiation of hPSCs to CD34$^+$ Hematopoeitic Progenitor Cells (Stages 1 and 2)

Day 0

1. Prepare EB Medium A (STEMdiff™ Hematopoietic - EB Basal Medium + STEMdiff™ Hematopoietic - EB Supplement A). Prepare EB Formation Medium by adding Y-27632 at 10 µM to EB Medium A.

2. Prepare an AggreWell™400 plate by rinsing with Anti-Adherence Rinsing Solution, washing with DMEM/F-12 with 15 mM HEPES, and adding 2.5 mL of EB Formation Medium (for one well of a 6-well plate).

3. Harvest hPSCs and generate a single-cell suspension using ACCUTASE™.

4. Dilute hPSCs to 1.4 x 10^6 cells/mL in 2.5 mL of EB Formation Medium, then seed into the AggreWell™ plate prepared in step 2. Incubate at 37°C for 2 days.

Day 2

5. Perform a half-medium change with EB Medium A. Incubate at 37°C for 24 hours.

Day 3

6. Prepare EB Medium B (STEMdiff™ Hematopoietic - EB Basal Medium + STEMdiff™ Hematopoietic - EB Supplement B). Perform a half-medium change with EB Medium B. Incubate at 37°C for 2 days.

Day 5

7. Harvest EBs, then filter and elute these with 2.5 mL EB Medium B, using a 37 µm reversible strainer.

8. Transfer eluted EBs to a non-tissue culture-treated plate. Incubate at 37°C for 2 days.

Day 7

9. Add 2.5 mL EB Medium B. Incubate at 37°C for 3 days.

Day 10

10. Perform a half-medium change with EB Medium B. Incubate at 37°C for 2 days.

Day 12

11. Harvest EBs and dissociate into a single-cell suspension using Collagenase Type II and TrypLE™ Express. Isolate CD34$^+$ cells using EasySep™ Human CD34 Positive Selection Kit II.

Differentiation of CD34$^+$ Cells to CD4$^+$CD8$^+$ DP T Cells (Stages 3 and 4)

Day 0

12. Coat non-tissue culture-treated plates with StemSpan™ Lymphoid Differentiation Coating Material; refer to the Technical Manual (Document #10000007541) for complete instructions.

13. Prepare StemSpan™ Lymphoid Progenitor Expansion Medium (StemSpan™ SFEM II + StemSpan™ Lymphoid Progenitor Expansion Supplement).

14. Dilute CD34$^+$ cells to 5 x 10^4 cells/mL in StemSpan™ Lymphoid Progenitor Expansion Medium and seed onto the coated plate.

15. Incubate at 37°C for 14 days, following instructions in the Technical Manual (Document #10000007541) for required half-medium changes and plate transfer on day 7.

Day 14

16. Harvest lymphoid progenitor cells (containing CD5$^+$CD7$^+$ cells; see Figure 5) for further differentiation to DP T cells.

17. Prepare StemSpan™ T Cell Progenitor Maturation Medium (StemSpan™ SFEM II + StemSpan™ T Cell Progenitor Maturation Supplement).

18. Dilute lymphoid progenitor cells to 0.5 - 1 x 10^6 cells/mL in StemSpan™ T Cell Progenitor Maturation Medium. Seed onto a freshly coated plate (see step 12), incubate at 37°C, and follow instructions in the manual for required half-medium changes and additions.

    Note: *Removing dead cells using fluorescence-activated cell sorting (FACS) at this stage may improve frequency and yield of DP T cells.*

Day 28

19. Harvest cells containing DP T cells (see Figure 6) for use in downstream assays, or follow the optional protocol extension for further maturation to CD8$^+$ SP T cells (see Figures 3 and 7).

Optional Protocol Extension

An optional protocol to mature CD4$^+$CD8$^+$ DP T cells to CD8$^+$ SP T cells is presented in Figure 3. This extended protocol uses StemSpan™ T Cell Progenitor Maturation Medium prepared with reagents included in STEMdiff™ T Cell Kit and must be combined with additional components, including either ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator or ImmunoCult™ Human CD3/CD28 T Cell Activator, and Human Recombinant IL-15.

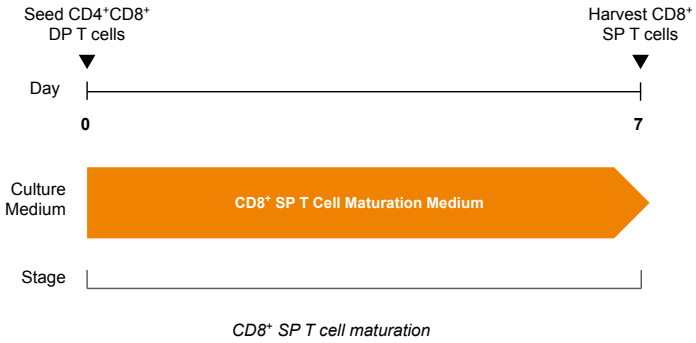

Figure 3. Optional CD8$^+$ SP T Cell Maturation Protocol

DP T cells are seeded in CD8$^+$ SP T Cell Maturation Medium supplemented with ImmunoCult™ T Cell Activator on plates coated with StemSpan™ Lymphoid Differentiation Coating Material. CD8$^+$ SP T cells can be harvested after 7 days.

Applications for STEMdiff™ T Cell Kit

- Research the differentiation of hPSCs to T lymphoid lineage cells
- Assess the efficacy and toxicity of candidate therapeutics on T cell differentiation during drug development
- Research the use of T cells for potential development of cellular therapeutics
- Develop in vitro models to study diseases that involve T cells
- Perform gene editing of progenitors prior to differentiation to T cells

Protocol for Optional CD8$^+$ SP T Cell Maturation

1. Prepare a freshly coated plate (See step 12 of Protocol for Differentiation of hPSCs to T Cells on page 3).
2. Prepare CD8$^+$ SP T Cell Maturation Medium (StemSpan™ SFEM II + StemSpan™ T Cell Progenitor Maturation Supplement + IL-15). See Technical Manual for details.
3. Add ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator or ImmunoCult™ Human CD3/CD28 T Cell Activator at half of the recommended concentration to an aliquot of CD8$^+$ SP T Cell Maturation Medium.
4. Dilute cells to 1 x 10^6 cells/mL in CD8$^+$ SP T Cell Maturation Medium + ImmunoCult™ T Cell Activator, seed onto the coated plate, and incubate at 37°C.
5. After 3 - 4 days of culture, add CD8$^+$ SP T Cell Maturation Medium, without ImmunoCult™ T Cell Activator.
6. Incubate at 37°C and harvest cells after 7 days.

Analysis of Differentiation from hPSCs to T Cells

hPSCs are differentiated to CD34$^+$ hematopoietic progenitor cells over 12 days (Figure 4). CD7$^+$CD5$^+$ lymphoid progenitor cells generated after 14 days of culture of hPSC-derived CD34$^+$ cells (Figure 5), DP T cells generated during a second 14-day culture step (Figure 6), and CD8$^+$ SP T cells generated by following the optional protocol extension (Figure 7) are identified by flow cytometry. Cells may also be stained with antibodies directed against cell surface markers CD3, CD4, CD5, CD7, CD8a, CD8b, CD34, CD144, and TCRαβ for analysis of hematopoietic progenitor cells, lymphoid progenitor cells, and T cell subsets. In the representative flow cytometry plots shown, dead cells were excluded by light scatter profile and DRAQ7™ staining. The average frequency and yield of the indicated hematopoietic progenitor, lymphoid progenitor, and T cell populations are shown in Figures 4C, 5C, 6E, and 7G. CD8$^+$ SP T cells were further stimulated and their ability to degranulate and produce cytokines was assessed in Figure 8.

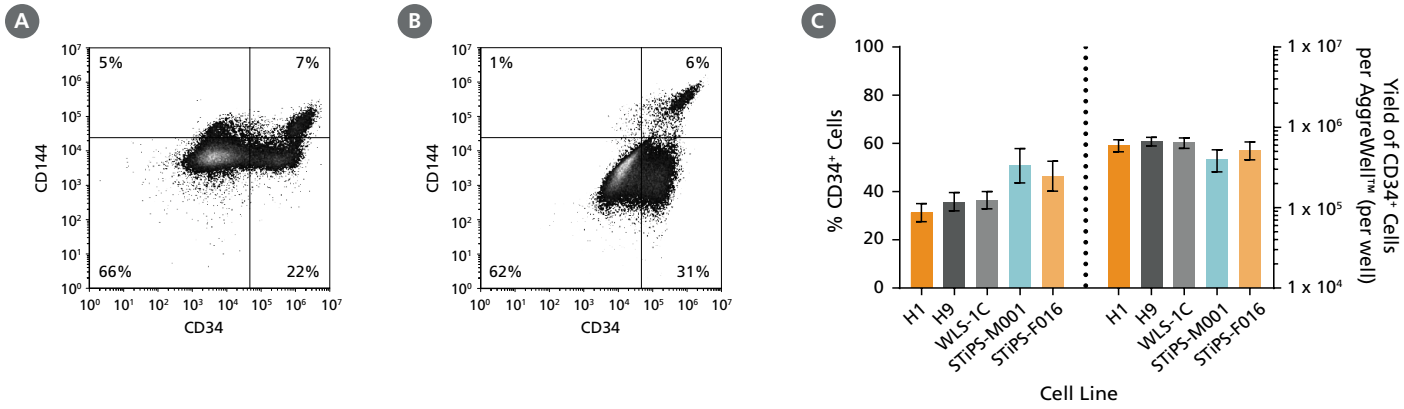

Figure 4. hPSCs Differentiate to CD34$^+$ Hematopoietic Progenitor Cells After 12 Days of Culture

Human ES and iPS cells were induced to differentiate to CD34$^+$ cells using the 12-day protocol shown in Figure 1. At the end of the culture period, cells were harvested, dissociated into a single-cell suspension, and analyzed by flow cytometry for CD34 and CD144 expression. Dead cells were excluded by light-scatter profile and DRAQ7™ staining. Representative flow cytometry plot for (A) ES (H1) cell-derived and (B) iPS (STiPS-F016) cell-derived cells analyzed on day 12 are shown. (C) The average frequency of viable CD34$^+$ cells on day 12 (before CD34$^+$ cell isolation) for two ES cell lines (H1 and H9) and three iPS cell lines (WLS-1C, STiPS-M001, and STiPS-F016) ranged between 31% and 51%. The average yield of CD34$^+$ cells produced per well of a 6-well AggreWell™400 plate ranged between 4.0 x 10^5 and 6.7 x 10^5. Data are shown as mean ± SEM (n = 9 - 35). Results are representative of populations prior to magnetic cell separation.

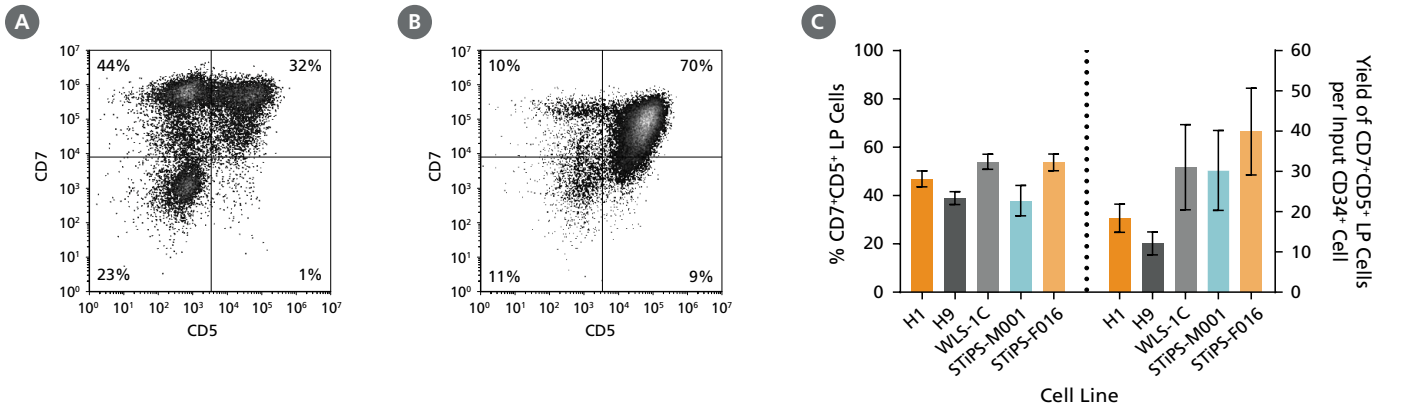

Figure 5. hPSC-Derived CD34$^+$ Cells Differentiate to CD5$^+$CD7$^+$ Lymphoid Progenitor Cells Over 14 Days of Culture

hPSC-derived CD34$^+$ cells were cultured for 14 days in StemSpan™ SFEM II + StemSpan™ Lymphoid Progenitor Expansion Supplement on plates coated with StemSpan™ Lymphoid Differentiation Coating Material (Figure 2). Cells were harvested and analyzed for CD7 and CD5 expression by flow cytometry. Representative flow cytometry plots for (A) ES (H1) cell-derived and (B) iPS (STiPS-F016) cell-derived cells are shown. (C) The average frequency of viable CD7$^+$CD5$^+$ lymphoid progenitor cells on day 14 ranged between 38% and 54%, and the average yield of lymphoid progenitor cells produced per input hPSC-derived CD34$^+$ cell was between 12 and 40. Data are shown as mean ± SEM (n = 8 - 32).

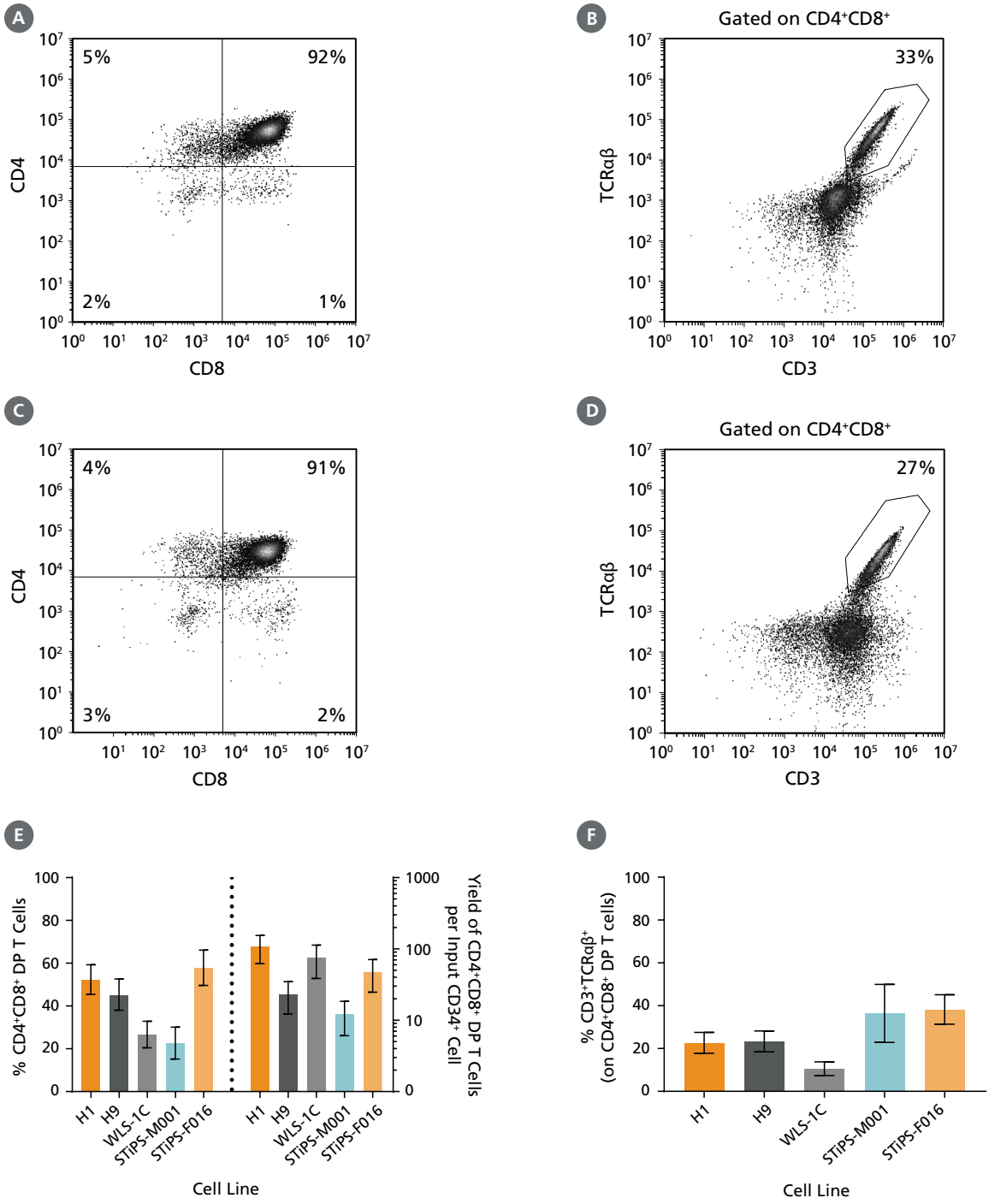

Figure 6. CD4⁺CD8⁺ DP T Cells Can Be Generated from Human hPSC-Derived CD34⁺ Cells After 28 Days of Culture

DP T cells were differentiated from hPSC-derived CD34⁺ cells as described (Figure 2). Cells were harvested and analyzed for expression of CD3, CD4, CD8, and TCRαβ by flow cytometry. Representative flow cytometry plots are shown for (A,B) ES (H1) cell-derived and (C,D) iPS (STiPS-F016) cell-derived cells. (E) The average frequency of viable CD4⁺CD8⁺ DP T cells on day 28 ranged between 23% and 58%, and the average yield of DP T cells produced per input hPSC-derived CD34⁺ cell was between 12 and 108. (F) The average frequency of CD3⁺TCRαβ⁺ expressed on DP T cells ranged between 11% and 38%. Data are shown as mean ± SEM (n = 6 - 17).

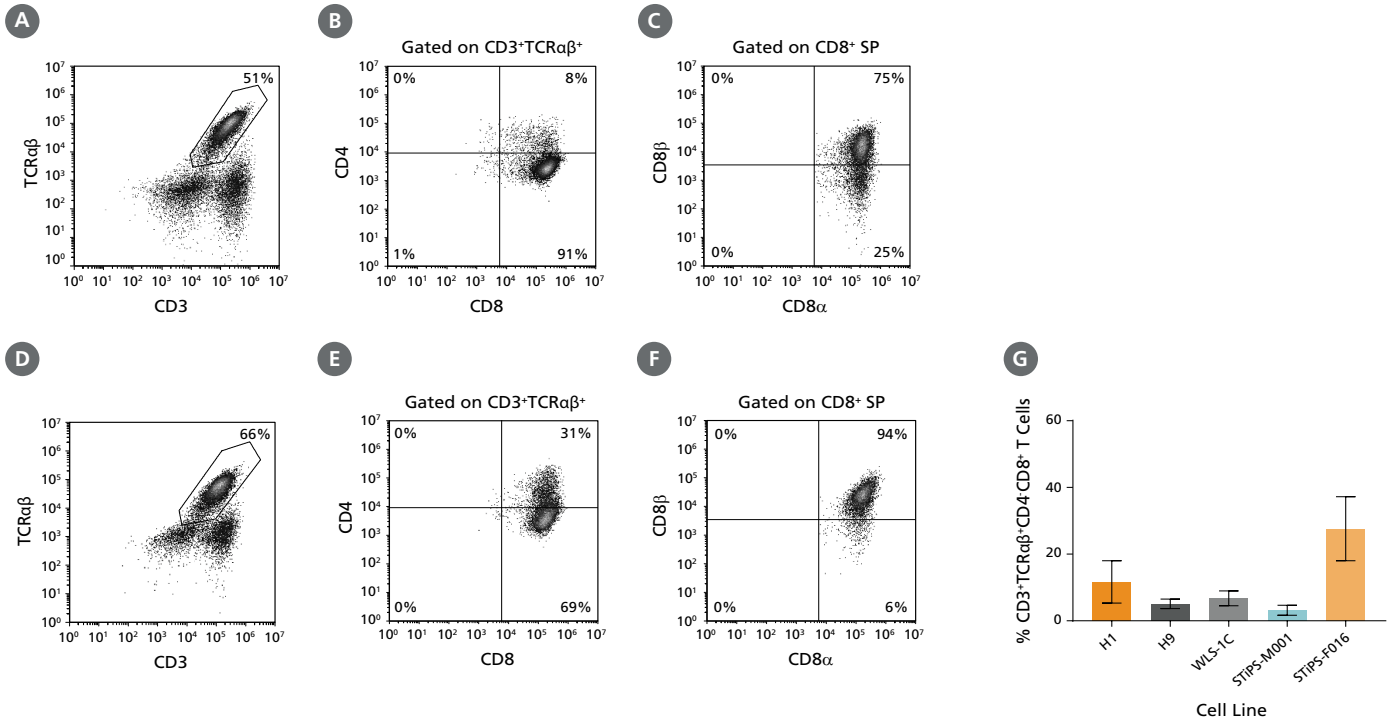

Figure 7. hPSC-Derived CD4$^+$CD8$^+$ DP T Cells Are Able to Mature to CD8$^+$ SP T Cells

hPSC-derived CD34$^+$ cells were first differentiated to DP T cells during 28 days of culture (Figure 2), and were then matured to CD8$^+$ SP T cells using an additional 7-day maturation protocol (Figure 3). Cells were harvested and analyzed by flow cytometry for expression of (A,D) CD3 and TCRαβ, (B,E) CD4 and CD8 (gated on CD3$^+$TCRαβ$^+$), and (C,F) CD8α and CD8β (gated on CD8$^+$ SP). Representative results for (A,B,C) ES (H1) cell-derived and (D,E,F) iPS (STiPS-F016) cell-derived cells are shown. (G) The average frequency of CD3$^+$TCRαβ$^+$CD4$^-$CD8$^+$ (CD8$^+$ SP) T cells was between 3% and 28%. Data are shown as mean ± SEM (n = 2 - 9).

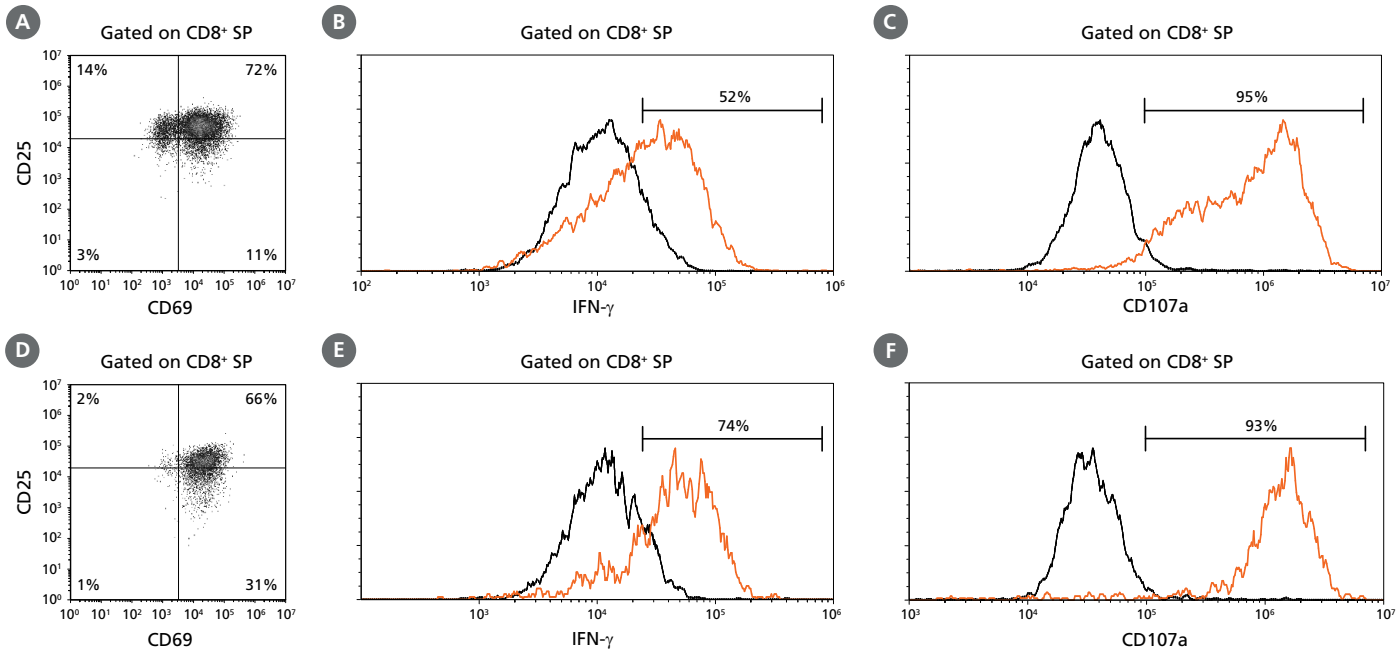

Figure 8. hPSC-Derived CD8$^+$ SP T Cells Can Be Induced to Express Activation Markers CD25 and CD69, Degranulate, and Produce IFN-γ

hPSC-derived CD34$^+$ cells were first differentiated to DP T cells during 28 days of culture and then matured to CD8$^+$ SP T cells using an additional 7-day maturation protocol (Figures 2 and 3). CD8$^+$ SP T cells were sorted using fluorescence-activated cell sorting and cultured for 7 days in ImmunoCult™-XF T Cell Expansion Medium supplemented with IL-2 and stimulated with ImmunoCult™ Human CD3/CD28 T Cell Activator. Cells were harvested and analyzed by flow cytometry for expression of (A,D) CD25 and CD69. For assessment of degranulation and cytokine production, at 4 hours prior to harvest, some cells were also stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin. After one hour, monensin and brefeldin A were added to each well to inhibit protein transport. Control (unstimulated; black histogram) and PMA + ionomycin-stimulated cells (orange histogram) were additionally analyzed by flow cytometry for surface expression of (C,F) CD107a (lysosomal-associated membrane protein 1 or LAMP-1) indicating degranulation and intracellular expression of (B,E) IFN-γ (gated on CD8$^+$ SP). Representative results for (A,B,C) ES (H1) cell-derived and (D,E,F) iPS (STiPS-F016) cell-derived cells are shown.

Product Information

Recommended Antibodies for Analysis*

Product Name	Catalog #
Anti-Human CD34 Antibody, Clone 581	60013
Anti-Human CD5 Antibody, Clone UCHT2	60082
Anti-Human CD7 Antibody, Clone CD7-6B7	N/A
Anti-Human CD3 Antibody, Clone UCHT1	60011
Anti-Human CD4 Antibody, Clone RPA-T4	N/A
Anti-Human CD8a Antibody, Clone RPA-T8	60022
Anti-Human CD8b Antibody, Clone SIDI8BEE	N/A
Anti-Human TCRαβ Antibody, Clone IP26	N/A
Anti-Human CD25, Clone BC96	60158
Anti-Human CD69, Clone FN50	N/A
Anti-Human IFNγ, Clone 4S.B3	N/A
Anti-Human CD107a, Clone H4A3	N/A

**Not included in the kit.*

Accessory Products*

Product Name	Catalog #
Y-27632	72302
AggreWell™400 6-Well (or 24-Well) Plate	34421 (or 34411)
DMEM/F-12 with 15 mM HEPES	36254
Anti-Adherence Rinsing Solution	07010
ACCUTASE™	07920
TrypLE™ Express	Thermo Fisher 12604013
Collagenase Type II	07418
EasySep™ Human CD34 Positive Selection Kit II	17856
37 µm Reversible Strainer, Large	27250
ImmunoCult™ Human CD3/CD28/CD2 T Cell Activator**/***	10970
ImmunoCult™ Human CD3/CD28 T Cell Activator**/***	10971
Human Recombinant IL-15**	78031
ImmunoCult™ -XF T Cell Expansion Medium***	10981
Human Recombinant IL-2 (CHO-Expressed)***	78036
Phorbol 12-Myristate 13-Acetate***	74042
Ionomycin***	73722
Brefeldin A***	73012
Monensin***	BioLegend 420701

**Not included in the kit.*

***Required for the Protocol for Optional CD8⁺ SP T Cell Maturation.*

****Additional products used to generate functional data for Figure 8.*